Engineering and design considerations for next-generation snakebite antivenoms

Knudsen, Cecilie; Ledsgaard, Line; Dehli, Rasmus Ibsen; Ahmadi, Shirin; Sørensen, Christoffer Vinther; Laustsen, Andreas Hougaard

Published in:
Toxicon

Link to article, DOI:
[10.1016/j.toxicon.2019.06.005](https://doi.org/10.1016/j.toxicon.2019.06.005)

Publication date:
2019

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Knudsen, C., Ledsgaard, L., Dehli, R. I., Ahmadi, S., Sørensen, C. V., & Laustsen, A. H. (2019). Engineering and design considerations for next-generation snakebite antivenoms. *Toxicon*, 167, 67-75.
<https://doi.org/10.1016/j.toxicon.2019.06.005>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Review

Engineering and design considerations for next-generation snakebite antivenoms

Cecilie Knudsen^{1*}, Line Ledsgaard¹, Rasmus Ibsen Dehli¹, Shirin Ahmadi^{1,2}, Christoffer Vinter Sørensen¹, Andreas H. Laustsen^{1*}

¹ Department of Biotechnology and Biomedicine, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark; Cecilie.Knudsen@outlook.dk, liljen@dtu.dk, rasdeh@dtu.dk, shiahm@dtu.dk, s190279@student.dtu.dk, ahola@bio.dtu.dk

² Biotechnology and Biosafety Institute, Eskisehir Osmangazi University, Eskisehir, Turkey

*Corresponding authors:

Andreas Hougaard Laustsen; ahola@bio.dtu.dk;

Cecilie Knudsen; Cecilie.Knudsen@outlook.dk;

Academic Editor: name

Received: date; Accepted: date; Published: date

Abstract:

Snakebite envenoming is a devastating Neglected Tropical Disease, the treatment of which has seen relatively little innovation since the invention of antivenom serotherapy in 1894. Current antivenoms have been and continue to be invaluable in saving thousands of lives. However, these medicines are associated with a number of drawbacks pertaining to availability, safety, and efficacy. Fortunately, with the advent of novel methodologies, such as antibody discovery technologies, high-throughput drug discovery approaches, and improved methods for protein engineering, we are starting to see scientific advances in the field. This review presents relevant engineering and design considerations for exploiting these methodologies to develop next-generation antivenoms with improved safety, efficacy, and affordability. The pros and cons of different treatment modalities will be discussed with regards to immunogenicity, the suitability of preclinical efficacy assays, availability of discovery methods, economic viability of production schemes, and possible regulatory approval paths.

Keywords: Next-generation antivenoms; recombinant antivenoms; small molecule toxin inhibitors; toxin-neutralization; monoclonal antibodies; snakebite envenoming; antivenom development; antivenom manufacture.

1. Introduction

While the progress of human technology has conquered many diseases, it has left others unaddressed and forgotten. One such neglected disease is snakebite envenoming, which continues to plague vulnerable communities around the world, causing death and disability. Inflicting cytotoxicity, neurotoxicity, hemotoxicity, and other toxicities, snakebite envenoming annually claims somewhere from 81,000 to 138,000 lives, and causes hundreds of thousands of people to live with chronic disablement (Gutiérrez et al., 2017a). Snakebite envenoming disproportionately affects poor, agricultural workers, often the breadwinners of their families, and as such acts to destabilize economically vulnerable population segments (Bawaskar et al., 2017; Chaves et al., 2015; Gutiérrez et al., 2017a; Harrison et al., 2009). Hence, snakebite envenoming is not only a public health issue, but also a socioeconomic challenge in many parts of the world.

Currently, snakebite envenoming is treated with antivenom consisting of polyclonal antibodies or antibody fragments derived from the plasma of hyperimmunized animals (Gutiérrez et al., 2017a). Such antivenoms save countless lives and are therapeutically invaluable. Nonetheless, the administration of heterologous antibodies comes at the inherent risk of immunogenicity, and adverse reactions have been reported both due to this and to inadequate purification procedures for some antivenom products (Burnouf et al., 2004; Chippaux et al., 1998; De Silva et al., 2016; Fry, 2018; León et al., 2013, 2008; LoVecchio et al., 2003; Malasit et al., 1986; Moran et al., 1998; Stone et al., 2013; Sutherland, 1977). Along with the limited availability of antivenoms in certain regions of the world, this has highlighted the need for novel types of antivenom with improved therapeutic properties (Laustsen et al., 2016a). In this review, we seek to discuss engineering and design aspects which should be considered in the pursuit of the next generation of antivenoms. These will be supported by examples of the promising research efforts that have been published in recent years within antibody engineering and drug repurposing.

2. Efficacy

One of the most important factors to assess when developing a new antivenom is efficacy. Several assays exist for determining efficacy in the preclinical setting, with the common standard being administering a preincubated mixture of venom and antivenom in an animal model and monitoring survival (see Figure 1A) (Gutiérrez et al., 2017b; World Health Organization, 2018). This assay is suitable for testing antivenoms against lethal, systemic envenomings (e.g. neurotoxicity and venom-induced consumption coagulopathy). However, many snake venoms initiate complex pathophysiological effects resulting in various clinical manifestations. Thus, depending on the venom in question, it might be necessary to assess additional parameters, such as nephrotoxicity, cytotoxicity, myotoxicity, and hemotoxicity (Gutiérrez et al., 2017b, 2013). Several tests capable of measuring these and other parameters preclinically have been developed, while tests for parameters such as cardiovascular and renal toxicity are still missing (Gutiérrez et al., 2017b; Williams et al., 2018; World Health Organization, 2010).

78 An important issue with the preincubation assay for assessing preclinical efficacy is that it largely
79 negates the influence of pharmacokinetics and toxicokinetics. An (experimental) envenoming therapy
80 could appear to be efficacious when preincubated with venom, but fail to neutralize the venom toxins
81 in a rescue assay, where the antivenom is administered a given period after venom administration and
82 at a different anatomical site (typically intravenously, whereas the venom may be administered
83 subcutaneously, intramuscularly, intraperitoneally, or even intravenously) (see Figure 1B). Lack of
84 efficacy in a rescue assay could thus not only be due to inability of the antivenom antitoxins to
85 strongly bind and neutralize the venom toxins (pharmacodynamics). It could also be due to an
86 inappropriate half-life for the antitoxins, limited volume of distribution of the antitoxins, or because
87 of a rapid onset of irreversible toxic effects caused by venom toxins before the antitoxins are able to
88 intercept and neutralize the venom toxins (pharmacokinetics and toxicokinetics). Additionally, the
89 physiological and immunochemical differences between the model organisms used for the tests and
90 the intended human recipients can render preincubation assays less predictive of clinical efficacy
91 (Rojas et al., 2013; Sanhajariya et al., 2018), although the current assay is accepted as providing good
92 indication for human envenoming. However, the conventional preincubation assay might still be most
93 suitable for molecules with well-characterized pharmacokinetics and for comparing antivenoms
94 based on the same type of scaffold molecules with similar therapeutic properties.

95
96 The difference in pharmacokinetics between antibody formats could be hypothesised to make the
97 formats differentially suited for neutralizing different classes of toxins. For instance, smaller antibody
98 formats, which are often assumed to have larger volumes of distribution, might be more suitable for
99 the neutralization of locally acting toxins in distal tissue, such as toxins causing local necrotic effects
100 (Gutiérrez et al., 2003; Seifert and Boyer, 2001). Conversely, larger formats, such as the
101 immunoglobulin G (IgG) format, might be better suited for neutralizing systemically acting toxins,
102 such as neurotoxins and hemotoxins. The superior half-lives of IgGs might also offer prolonged
103 protection against toxins entering circulation from the bite site at a later time point due to depot effects
104 (Seifert and Boyer, 2001). However, careful consideration should still be given as to which format
105 should be chosen, as studies show that the interplay between pharmacokinetics and
106 pharmacodynamics often makes the situation more complex than what theoretical generalisations
107 might lead one to predict (Gutiérrez et al., 2003).

108

Figure 1. A) Schematic representation of a preincubation assay, where either whole venom or selected venom components are incubated with the experimental antivenom, prior to administration into the model organism. B) Schematic representation of a rescue assay, in which venom or toxins are injected into the model organism, followed by injection of the experimental antivenom after a delay.

Understanding the influence of pharmacodynamics and complementary properties of antibodies in an antivenom is of high importance. However, it is also essential to consider the toxicodynamics and synergistic effects of venom toxins that are to be neutralized with a given novel antivenom. For example, it might be worth considering if it is necessary to neutralize all toxins in a venom to neutralize venom lethality. It has been suggested that by only neutralizing key toxins and/or by disrupting toxin synergism (the phenomenon where a mixture of venom components has a toxicity that is significantly higher than the sum of the toxicities of the components alone), it might be possible to neutralize whole venom lethality (Laustsen, 2016a; Laustsen et al., 2015). This has recently been demonstrated to be the case for *Dendroaspis polylepis* venom (Laustsen et al., 2018b), as well as it has been demonstrated that antibodies raised against the snake venom metalloproteinase Ecarin from *Echis carinatus* venom had partial neutralising potential in mice envenomed with *E. ocellatus* venom (Ainsworth et al., 2018). These findings underline that when employing novel drug development strategies, as well as innovative immunization techniques (Bermúdez-Méndez et al., 2018), it may be

possible to develop efficacious antivenom products that only target a subset of the venom components.

For several decades, the use of recombinant antibodies has been investigated in the field of antivenom research (Laustsen et al., 2018a, 2016a). Many of these attempts to create novel antivenoms have employed various types of antibody formats. As such, different antibody formats, including nanobodies (V_HHs), single-chain antibody fragments (scFvs), antigen binding antibody fragments (Fabs and F(ab')₂s), and whole monoclonal IgG molecules (see Figure 2A) have demonstrated efficacy in neutralizing snake venoms (Boyer et al., 2013; Brvar et al., 2017; Castro et al., 2014; Julve Parreño et al., 2017; Laustsen et al., 2018b; Richard et al., 2013). In another of these studies, Richard et al. showed that V_HHs isolated from an immune phage display nanobody library, as well as V_HH-Fc constructs, effectively neutralized α -cobratoxin from *Naja kaouthia* (Richard et al., 2013). Combined, these studies demonstrate the feasibility of using different types of recombinant antibodies for neutralizing snake venom toxins. This may be unsurprising given that conventional antivenoms based on different antibody formats have been used to successfully treat snakebite victims for more than a century.

A newer approach within development of next-generation envenoming therapeutics focuses on inhibitors of enzymatic toxins, such as phospholipases A₂ and snake venom proteases. The strength of these small molecule inhibitors lies in their ability to neutralize the activities of multiple different toxins across different species by exploiting the similarities of the catalytic sites of toxin subfamilies. One of the most promising examples of this category of antitoxins is varespladib (LY315920) and its orally available prodrug methyl-varespladib (LY333013) (see Figure 2B). These drugs were originally developed to treat acute coronary syndrome and went through human clinical trials, but were later repurposed for inhibition of phospholipase A₂ (PLA₂) toxins by Lewin et al. (Lewin et al., 2016). In this study, it was demonstrated that mice pretreated or co-injected with varespladib had higher rates of survival when administered with lethal doses of *Micrurus fulvius* and *Vipera berus* venom than control mice not treated with varespladib (Lewin et al., 2016). These results were bolstered further, when Bryan-Quirós et al. demonstrated that immediate injection of varespladib at the site of toxin or venom administration could significantly reduce the myonecrotic effects of venom from *Pseudechis colletti* and *Bothrops asper* (Bryan-Quirós et al., 2019). In addition to its anti-myonecrotic effects, the methylated version of varespladib has shown neutralizing abilities towards lethal doses of neurotoxic venoms from *Micrurus fulvius* and the Papuan *Oxyuranus scutellatus*, respectively, in rescue assays in porcine and murine models (Lewin et al., 2018a, 2018b). This could prove especially promising for the treatment of neurotoxic snakebites in Papua New Guinea, where taipans are responsible for a large amount of lethal envenoming cases (Vargas et al., 2011).

Another promising example of the use of small molecule inhibitors for the neutralization of enzymatic toxins is the metalloprotease inhibitor batimastat and its orally available prodrug marimastat (see

Figure 2B). Like varespladib, batimastat was originally developed for a different indication and later repurposed for snakebite envenoming (Rasmussen and McCann, 1997). Both batimastat and marimastat have been shown to effectively abrogate the hemorrhagic and coagulopathic effects induced by *Echis ocellatus* venom if administered early and close to the site of venom injection (Arias et al., 2017). Another group of small molecule enzymatic inhibitors is made up by metal chelators, such as EDTA. Several studies show that chelating agents can abrogate toxic effects of snake venom (Ainsworth et al., 2018; Escalante et al., 2017; Howes et al., 2007). However, a common factor for these small molecule inhibitors is that they target only one family of enzymatic toxins. This makes it unlikely that one of them will become a stand-alone therapeutic that can effectively neutralize all toxic effects of a broad range of snake venoms, which typically comprise a multitude of different toxin families. Instead, it seems likely that it may find its use as an adjunctive treatment, as fortification agents for hybrid antivenoms comprising both antibodies and small molecule inhibitors, or as a first aid that can be administered en route to a healthcare facility (Kini et al., 2018). Despite the promise of these and other small molecule inhibitors, to the best of our knowledge, no small molecule inhibitors have ever been marketed for the treatment of snakebite envenoming with the aim of neutralizing the snake toxins.

Other technical avenues for developing improved therapeutics against snakebite envenoming are currently being pursued, including the use of oligonucleotide aptamers and nanoparticles (see Figure 2C and 2D) (Chen et al., 2016; El-Aziz et al., 2017; Karain et al., 2016). More extensive reviews of these molecules can be found elsewhere (Knudsen and Laustsen, 2018; Laustsen et al., 2016a, 2016b).

Figure 2. A) Schematic representation of various antibody formats used within antivenom research. B) Chemical structures of some small molecule inhibitors discussed in this review. C) Examples of

192 secondary structures of oligonucleotide aptamers (generated using software described in (Kerpedijev
193 et al., 2015) with sequences from (Chen et al., 2016). D) Schematic representations of two
194 nanoparticles. The representations in this figure are not to scale.

195

196 In short, many exciting and promising research results have been reported on the use of various types
197 of molecular compounds for snake toxin neutralization. However, none have progressed to clinical
198 trials. In many cases, it is unknown how the pharmacokinetics and pharmacodynamics of the less
199 commonly studied molecular entities or scaffolds will affect their efficacy in such trials.

200

201 **3. Safety**

202

203 The safety profiles of current antivenoms often constitute one of their greatest drawbacks, as many
204 antivenoms have been associated with severe adverse reactions (Chippaux et al., 2017). Therefore,
205 the propensity for eliciting adverse effects must be addressed when developing novel antivenom
206 products. For conventional antivenoms, these adverse reactions can be both early-onset, occurring up
207 to 24 hours after administration of antivenom, and late-onset, occurring between 5 and 24 days after
208 antivenom administration (León et al., 2013). Around 20% of snakebite victims that are administered
209 antivenom experience early-onset adverse reactions (León et al., 2013). However, the incidence rate
210 is related to the antivenom product and can be as high as 88% and as low as 3% (León et al., 2013).
211 These reactions can be serious and in rare cases be life-threatening. Early-onset adverse reactions can
212 be divided into two categories; pyrogenic or anaphylactic reactions. The pyrogenic reactions are a
213 direct result of contamination of the antivenom and these reactions can therefore be completely
214 eliminated by implementation of proper product design and good manufacturing practices (GMP) in
215 the production (León et al., 2013). Late-onset serum sickness is a result of an immune response
216 against the heterologous proteins in the antivenom. The actual incidence of these late-onset reactions
217 are not well known, as patients rarely return to the hospitals after treatment (León et al., 2013). In
218 healthcare facilities, which are poorly resourced and staffed by personnel with inappropriate training
219 to tackle such situations, adverse reactions further complicate treatment of snakebite victims
220 (Chippaux et al., 2017). As a result, antivenom is often only administered when clear signs of
221 envenoming manifest themselves (World Health Organization, n.d.), which also has the consequence
222 that antivenom administration may be delayed, allowing the venom toxins to exert their toxic effects
223 for a longer time period. However, when properly manufactured, animal-derived antivenoms having
224 gone through rigorous quality control are associated with few adverse reactions when used to treat
225 animal envenomings in humans (León et al., 2018).

226

227 Polyclonal plasma-derived camelid IgGs have been investigated for many years (Harrison et al.,
228 2011), as they have demonstrated a lower propensity towards inducing adverse reactions than
229 polyclonal plasma-derived IgGs of equine and ovine origin (Herrera et al., 2005). It has been
230 suggested that intravenous administration of antivenom based on camelid antibodies might decrease

the risk of inducing serum sickness and anaphylaxis compared to administration of antivenoms based on equine antibodies (Harrison et al., 2011). However, more recent research pursues recombinant production of camelid single-domain V_HH antibody fragments (Prado et al., 2016; Richard et al., 2013). The development of snakebite therapeutics based on murine monoclonal IgG antibodies and antibody fragments has also been attempted (Laustsen et al., 2016a), but is no longer pursued (Laustsen et al., 2018a), as their heterologous nature has proven to cause both early and late adverse reactions, as well as loss of efficacy during repeated use in indications other than snakebite envenoming (Descotes, 2009; Hansel et al., 2010; Laustsen et al., 2018a). To mitigate the risk of immunogenicity, manufacturers of therapeutic antibodies for many other indications increasingly make use of recombinant human antibodies (Grilo and Mantalaris, 2018; Nelson et al., 2010). Human antibodies are compatible with the human immune system, and while they are not entirely devoid of the risk of provoking immunogenicity, they have greatly improved safety profiles compared to humanized, chimeric, and murine antibodies (Hansel et al., 2010; Harding et al., 2010). An additional benefit might be found in their manufacture, as recombinant expression of monoclonal antibodies (mAbs) yields a more defined antibody product (in contrast to isolation of polyclonal antibodies from sera with inherent batch-to-batch variation and undefined composition). This provides the manufacturer with control over which antibodies are produced, unlike when immunization methods are used, and will theoretically enable the antivenom manufacturer to produce antivenoms consisting purely of therapeutically relevant antibodies with proven efficacy. Since some current antivenoms consist of undefined mixtures of antibodies, as few as 5% of which target snake venom components (Rawat et al., 1994), a shift towards a defined mixture of therapeutically relevant mAbs could potentially lead to development of more efficacious antivenom products. In turn, this may lead to a scenario, where lower doses may be required to treat a given envenoming, thereby possibly lowering both cost of treatment and the risk of adverse effects.

To date, antivenoms comprised of monoclonal IgGs or any other types of recombinant proteins are yet to enter the clinical setting (Laustsen et al., 2018a), but for many other indications there is a rapid increase in market-approved mAbs, especially mAbs of human origin (Grilo and Mantalaris, 2018). This trend may eventually benefit antivenom manufacturers as production of comparatively safe, recombinant antibodies becomes increasingly standardized and economically attractive.

A high number of small non-antibody-based protein scaffolds with different safety profiles are being explored for various indications and may be relevant to investigate in relation to snakebite envenoming (Jenkins et al., 2019; Vazquez-Lombardi et al., 2015). In some cases, it is found that improving the safety profiles of these non-antibody-based protein scaffolds is recommendable. Elimination of T and/or B cell epitopes is one such strategy employed to decrease immunogenicity and improve safety and efficacy of the protein (King et al., 2014; Mazor et al., 2017; Nagata and Pastan, 2009). Many of non-antibody-based protein scaffolds targeting various indications outside of snakebite have progressed through preclinical trials, and several, like adnectins, affibodies, affilins,

affitins, anticalins, avimers, DARPin, and Kunitz domains have been tested in Phase I, II, or III clinical trials, respectively (Jenkins et al., 2019; Vazquez-Lombardi et al., 2015). The progression through and completion of clinical trials indicate that some of these scaffolds might be developed and used for therapeutic purposes for a wide range of indications. However, to the extent of our knowledge, the mentioned scaffolds remain clinically untested for the treatment of toxins from snake venoms.

Unlike non-antibody-based protein scaffolds, nanoparticles have been investigated specifically for treating experimental snakebite envenoming. In a recent study, abiotic hydrogel nanoparticles were successfully engineered to randomly associate with and modulate the activity of a diverse array of both PLA₂ and three-finger toxin isoforms, inhibiting dermonecrosis caused by the black-necked spitting cobra (*Naja nigricollis*) (O'Brien et al., 2018). Toxicity studies without venom administration, comparing only the effects of nanoparticle injection to the injection of phosphate buffered saline, provided promising indications of preclinical safety. Mice receiving the nanoparticles intravenously did not exhibit signs of toxicity or behavioral changes. Mice receiving the nanoparticles intramuscularly in the gastrocnemius did not exhibit problems with mobility nor did the mice exhibit a higher plasma creatine kinase activity, which would have indicated myotoxicity. When the nanoparticles were injected intradermally, no macroscopic effects on the skin were observed, and additionally, only normal microscopic appearances were observed in histological assessments subsequent to administration of the nanoparticle via the intravenous, intramuscular, and intradermal routes (O'Brien et al., 2018). In 2016, 51 medicines based on nanoparticles had achieved FDA-approval, and another 77 nanoparticle-based therapeutic candidates were in clinical trials (Bobo et al., 2016). Most of these nanoparticles are of polymeric, liposomal, or nanocrystalline material, but also metallic, micelle-, and protein-based nanomedicines have been approved by regulatory agencies (Bobo et al., 2016). The wide array of nanoparticles which have gained regulatory approval, and the diversity of the indications these nanoparticles target, offer hope that nanoparticles might also find a therapeutic use within snakebite envenoming.

Most producers of antibody-based therapeutics outside of the field of snakebite antivenom are moving away from the use of non-human IgG antibodies. While antivenom researchers are exploring the opportunity to mimic this trend by shifting the focus to nanobodies, human antibodies, and non-antibody-based protein scaffolds, none of this research has yet left the laboratories and entered the clinic. The reports of adverse effects of existing antivenoms highlight one of the greatest drawbacks of the current antivenom generation and thereby one of its greatest and most important opportunities for improvement. Although thorough safety studies of alternative therapeutic modalities for treating envenoming remain scarce, modalities which have proved safe for the treatment of other indications might constitute safer alternatives to the frequently immunogenic antivenoms based on heterologous polyclonal antibodies, which are currently in use.

309 4. Developability and manufacturing

310

311 The process of bringing a pharmaceutical to the market is time-consuming, expensive, and has a
312 success rate of less than 1% (Kannt and Wieland, 2016). This, in combination with the poor economic
313 incentive to develop new antivenoms, necessitates focusing on tried and tested molecular formats
314 with standardized discovery and production methods. Doing so might minimize the risk of prolonging
315 the process of bringing the drug to the market. Such a prolongation would further increase the costs
316 of the drug development program, forcing the drug developer to charge a high price for the drug to
317 recoup expenditure, and thereby possibly rendering the drug economically unavailable to those who
318 need it (Kini et al., 2018; Laustsen and Dorrestijn, 2018).

319

320 Due to their complexity, snake venoms are unlikely to be neutralized by a single molecule, and this
321 makes ‘one hit’ drugs therapeutically unfeasible. Furthermore, as snake venoms are highly diverse
322 and different across continents, developing a ‘universal antivenom’ that theoretically would be able
323 to neutralize all key toxins from all snakes across the world seems highly infeasible and irrelevant.
324 However, future antivenoms will most likely be quite complex therapeutic products with mixed
325 compositions of toxin-neutralizing modalities. For the development of an array of molecules that can
326 neutralize different toxins, it could be beneficial to use molecules for which standardized high-
327 throughput discovery protocols exist and where the development and production process can
328 piggyback on experience from other fields. Examples of molecules for which high-throughput
329 discovery protocols have already been developed include aptamers, peptides, and mAbs that can all
330 be selected against toxins *in vitro* through SELEX (for aptamers) (Tuerk and Gold, 1990) or phage
331 display (for peptides and antibodies) (McCafferty et al., 1990; Smith, 1985). As an example, phage
332 display was employed to select human antibodies against mamba toxins, which were not neutralized
333 by conventional antivenom (Laustsen et al., 2018b). Phage display has additionally been used to
334 discover human scFvs against toxins from *Bothrops jararacussu* and *Crotalus durissus terrificus*
335 venom. These scFvs were *in vitro* reported to inhibit plasma-clotting and prolonged survival of mice
336 injected with lethal doses of the two venoms (Silva et al., 2018).

337

338 Repurposing existing drugs might significantly shorten the process of discovery and lead to
339 development of novel adjunctive treatments targeting specific toxin (sub-)families, with one
340 prominent example being the previously mentioned varespladib (Kini et al., 2018; Lewin et al.,
341 2018b, 2016; Wang et al., 2018). Repurposing of such drugs has the potential to lower the cost of
342 treatment as there are much fewer expenses associated with their development.

343

344 Another important factor for the cost of a novel antivenom is the cost of its manufacture (Laustsen
345 and Dorrestijn, 2018). For antibody-based antivenoms, recombinant production can be undertaken in
346 several ways (see examples in Figure 3), depending on the requirements of glycosylation, yields, ease
347 of purification, and the scale of the production (Laustsen et al., 2018a). Within large scale

pharmaceutical antibody production approximately 95% of therapeutic antibodies are produced in mammalian cell lines such as Chinese Hamster Ovarian (CHO) cells (Jäger et al., 2013; Walsh, 2014). This has prompted antibody researchers to undertake cost-calculations for large scale production of IgG-based antivenoms in mammalian cell lines (see Figure 3C) and lead to the theoretical demonstration that such production methods would likely be cost-competitive with existing manufacturing processes for conventional antivenoms, assuming that oligoclonal expression technologies (such as the Sympress technology) are employed (Laustsen, 2016b; Laustsen et al., 2017; Rasmussen et al., 2012; Wiberg et al., 2006).

Figure 3. Comparison of antibody generation schemes for antivenoms. A) Antibody generation scheme for many antivenoms in clinical use. B) Generation scheme for antibodies via hybridoma technology. C) Generation scheme for antibodies by phage display technology.

For other types of molecules, such as nucleotide-based aptamers, simple chemical production is often used for small scale synthesis. This allows for cheap production of aptamers required for preclinical testing. However, while some studies present a positive outlook on the large scale cost of manufacture of aptamers (Keefe et al., 2010), studies that thoroughly examine this cost of manufacture are hard to find. It therefore remains unclear what the actual cost of aptamer manufacture is in large scale. It therefore remains to be seen whether this cost is prohibitively high for novel aptamer-based antivenom products that are to reach impoverished victims in the tropics.

Generally, in the pharmaceutical industry, many small molecule therapeutics can be manufactured in large volumes at low cost. However, this does not guarantee that the cost of simultaneous production

of multiple small molecules for an antivenom will be equally low, as different production facilities operating in parallel will most likely be needed for each of these molecules.

Another important consideration is the downstream processing needed for purification of the active pharmaceutical ingredients after the upstream process. As an example, IgGs and IgG-like proteins can be produced both in mammalian cell cultures and in plants (Julve Parreño et al., 2017; Laustsen et al., 2018b). Even more important than the upstream process is the complex and expensive downstream process, which often accounts for as much as 80% of the total production cost (Wilken and Nikolov, 2012). Downstream processing of proteins expressed in plants requires harvesting, extraction, clarification of the extract, and purification of the proteins (Lojewska et al., 2016). By comparison, antibodies expressed in cell cultures can be purified using caprylic acid precipitation and/or protein A affinity chromatography (Liu et al., 2010; Morais et al., 2014). This is routinely performed within industrial antibody manufacturing (Hammerschmidt et al., 2014; Liu et al., 2010), with the consequence that these purification methods have been extensively optimized and are highly standardized. Therefore, when choosing a production strategy, it is highly relevant to take the downstream processing, that follows each method of (bio)synthesis, into account.

For a novel antivenom product to be able to replace existing plasma-derived antivenoms, the cost of treatment is of high importance, as these therapies are to be delivered especially to impoverished victims in countries with limited healthcare budgets. To keep costs low, strategies such as choosing a molecule for which standardized discovery and manufacturing protocols exist and considering the cost of both upstream and downstream processing are thus highly relevant. Repurposing of existing drugs could also allow for great reduction of cost of antivenoms' development processes.

5. Regulatory aspects

Antivenoms are unusual medicines and have historically not followed the conventional path for regulatory approval. Antivenoms have been used since 1896 in human patients, with limited attention in many clinical trials towards their safety and effectiveness (World Health Organization, 2010). Even to this day, some manufacturers introduce new antivenoms to the market with no conventional clinical evaluation process, and some antivenoms even lack preclinical data to support effectiveness (Williams et al., 2018).

Bringing a drug candidate to the market through the conventional pathway is notoriously expensive and time-consuming (Kannt and Wieland, 2016): The average time from the conclusion of preclinical testing until FDA-approval is granted is 8-9 years, seven of which are spent on clinical trials and 1-2 of which are for FDA review (Reichert, 2010). All in all, the process from drug development to regulatory approval frequently ends up taking over 15 years and costing between USD 3.7 and 11.8

billion (Kannt and Wieland, 2016); a sum that is prohibitive within antivenom development and marketing (Williams et al., 2018).

Another challenge for current antivenoms is their propensity for inducing adverse effects, which has caused many manufacturers to forfeit phase 1 trials rather than expose healthy individuals to the risk of suffering adverse reactions (Alirol et al., 2017). Fortunately, fundamentally novel types of antivenom (such as recombinant antivenoms based on monoclonal antibodies) are currently being researched and developed (Jenkins et al., 2019; Knudsen and Laustsen, 2018; Laustsen, 2017; Laustsen et al., 2018a, 2016a). These next-generation antivenoms can be designed to have improved safety profiles compared to current antivenoms, as they can be based on less immunogenic modalities. Improved safety could even allow antivenom developers to bring next-generation antivenoms into clinical phase 1 trials. Being able to test novel antivenom products in clinical phase 1 trials has the potential to dramatically improve future envenoming therapies for patients by allowing for early systematic assessment of clinical safety prior to market entry. It can further be speculated, that if the safety of next-generation antivenom products can be demonstrated to be superior compared to existing antivenoms, an opportunity may present itself for earlier antivenom administration, e.g. in an ambulance en route to a treatment facility. Earlier treatment of snakebite envenoming has been correlated with faster recovery and hospital discharge (Anderson et al., 2018; Johnston et al., 2017) and might be especially helpful in the case of rapidly acting toxins, e.g. neurotoxins. Thus, improved safety may very well lead to improved effectiveness for snakebite antivenoms.

Repurposing of approved therapeutics or therapeutic candidates, which have already gone through (some) clinical trials, could be a way to reuse the results from costly clinical safety trials. As an example, varespladib has previously gone through clinical trials, but was arrested in phase 3 due to insufficient efficacy against acute coronary syndrome (Adis, 2011; U.S. National Library of Medicine, n.d.). However, the phase 2 trials of varespladib suggested that six months of daily treatment for acute coronary syndrome with 500 mg active pharmaceutical ingredient generated no adverse effects compared to the placebo treatment. The estimated dose of varespladib required to treat snakebite envenoming in an adult man of 70 kg has been estimated to be 280 mg, which suggests that treatment with varespladib against snakebite would be safe from adverse reactions (Wang et al., 2018). Similarly, the safety of batimastat and marimastat has already been clinically investigated (Parsons et al., 1997; Sparano et al., 2004; Steward, 1999). As such, relying on previous safety assessments of repurposed therapeutics and therapeutic candidates poses another potential path through the regulatory approval system for novel antitoxin molecules.

Finally, it is possible that future antivenoms will combine existing molecular formats with new ones. For example, it could be envisioned that traditional antivenoms could be spiked with some of the novel antitoxins discussed here. Small molecule inhibitors with broad neutralizing capacities and human monoclonal antibodies targeting poorly immunogenic toxins could bolster current antivenoms

(Kini et al., 2018). However, it is difficult to predict how such hybrid products will be viewed by the regulatory authorities, and which regulatory demands hybrid antivenom products may be met with.

In conclusion, next-generation antivenoms might display improved safety and efficacy profiles, which could enable them to go through more conventional clinical trials and regulatory processes. However, funding and practical challenges associated with such trials, as described by Williams et al. (Williams et al., 2018), might still prove problematic or even prohibitive for the completion of clinical trials, and it is difficult to predict how next-generation antivenoms or hybrid antivenoms will be evaluated by the regulatory authorities.

6. Closing remarks

An urgent need for improving prevention and treatment of snakebite victims exists. Such an improvement must occur on multiple levels, from the creation of international guidelines and public health policies, over national strategies for snakebite management, to improved therapies that are affordable and available to snakebite victims (Gutiérrez et al., 2014; Harrison and Gutiérrez, 2016). While some countries are reliably served with high-quality antivenom products by several manufacturers, other countries are struck by an antivenom paucity in part due to the poor economic viability of current antivenoms in these markets. This accentuates the need for considering antivenom development and production from an economic perspective. Based on these considerations, it might be relevant to focus on therapeutic modalities which are either inherently broadly specific against certain toxin subfamilies (e.g. some enzymatic inhibitors) or scaffold molecules which can be easily adapted to neutralize multiple targets (e.g. antibodies or similar scaffold proteins). It would further be advantageous to focus on types of molecules, which can be discovered and developed in a parallelized high-throughput fashion and which are manufacturable using standardized large scale, low-cost production processes. Additionally, it might be relevant for the antivenom researcher to focus on modalities with known pharmacokinetics to minimize the risk of failure in preclinical and clinical studies. By allowing these engineering and design considerations to direct the development process for fundamentally novel types of antivenoms, it may one day be possible to bring about safer, more effective, and economically viable antivenom products to snakebite victims in need.

Acknowledgments: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Adis, 2011. Varespladib. *Am. J. Cardiovasc. Drugs* 11, 137–143. doi:10.2165/11533650-000000000-00000
- Ainsworth, S., Slagboom, J., Alomran, N., Pla, D., Alhamdi, Y., King, S. i., Bolton, F.M.S., Gutiérrez, J.M., Vonk, F.J., Toh, C.-H., Calvete, J.J., Kool, J., Harrison, R.A., Casewell, N.R., 2018. The

489 paraspecific neutralisation of snake venom induced coagulopathy by antivenoms. Commun.
 490 Biol. 1. doi:10.1038/s42003-018-0039-1
 491 Alirol, E., Sharma, S.K., Ghimire, A., Poncet, A., Combescure, C., Thapa, C., Paudel, V.P., Adhikary,
 492 K., Taylor, W.R., Warrell, D.A., Kuch, U., Chappuis, F., 2017. Dose of antivenom for the
 493 treatment of snakebite with neurotoxic envenoming: Evidence from a randomised controlled
 494 trial in Nepal. PLoS Negl. Trop. Dis. 11. doi:10.1371/journal.pntd.0005612
 495 Anderson, V.E., Gerardo, C.J., Rapp-Olsson, M., Bush, S.P., Mullins, M.E., Greene, S., Toschlog,
 496 E.A., Quackenbush, E., Rose, S.R., Schwartz, R.B., Charlton, N.P., Lewis, B., Kleinschmidt,
 497 K.C., Sharma, K., Lavonas, E.J., 2018. Early administration of Fab antivenom resulted in faster
 498 limb recovery in copperhead snake envenomation patients. Clin. Toxicol. 0, 1–6.
 499 doi:10.1080/15563650.2018.1491982
 500 Arias, A.S., Rucavado, A., Gutiérrez, J.M., 2017. Peptidomimetic hydroxamate metalloproteinase
 501 inhibitors abrogate local and systemic toxicity induced by *Echis ocellatus* (saw-scaled) snake
 502 venom. Toxicon 132, 40–49. doi:10.1016/j.toxicon.2017.04.001
 503 Bawaskar, H.S., Bawaskar, P.H., Bawaskar, P.H., 2017. Snake bite in India: a neglected disease of
 504 poverty. Lancet 390, 1947–1948. doi:10.1016/S0140-6736(17)32175-X
 505 Bermúdez-Méndez, E., Fuglsang-madsen, A., Føns, S., Lomonte, B., Gutiérrez, J.M., Laustsen, A.H.,
 506 2018. Innovative Immunization Strategies for Antivenom Development. Toxins (Basel). 10, 1–
 507 37. doi:10.3390/toxins10110452
 508 Bobo, D., Robinson, K.J., Islam, J., Thurecht, K.J., Corrie, S.R., 2016. Nanoparticle-Based
 509 Medicines: A Review of FDA-approved Materials and Clinical Trials to Date. Pharm. Res. 33,
 510 2373–2387. doi:10.1007/s11095-016-1958-5
 511 Boyer, L. V., Chase, P.B., Degan, J.A., Figge, G., Buelna-Romero, A., Luchetti, C., Alagón, A., 2013.
 512 Subacute coagulopathy in a randomized, comparative trial of Fab and F(ab')₂ antivenoms.
 513 Toxicon 74, 101–108. doi:10.1016/j.toxicon.2013.07.018
 514 Brvar, M., Kurtović, T., Grenc, D., Lang Balija, M., Križaj, I., Halassy, B., 2017. *Vipera ammodytes*
 515 bites treated with antivenom ViperaTAB: a case series with pharmacokinetic evaluation. Clin.
 516 Toxicol. 55, 241–248. doi:10.1080/15563650.2016.1277235
 517 Bryan-Quirós, W., Fernández, J., Gutiérrez, J.M., Lewin, M.R., Lomonte, B., 2019. Neutralizing
 518 properties of LY315920 toward snake venom group I and II myotoxic phospholipases A₂.
 519 Toxicon 157, 1–7. doi:10.1016/j.toxicon.2018.11.292
 520 Burnouf, T., Griffiths, E., Padilla, A., Seddik, S., Stephano, M.A., Gutiérrez, J.M., 2004. Assessment
 521 of the viral safety of antivenoms fractionated from equine plasma. Biologicals 32, 115–128.
 522 doi:10.1016/j.biologicals.2004.07.001
 523 Castro, J.M.A., Oliveira, T.S., Silveira, C.R.F., Caporrino, M.C., Rodriguez, D., Moura-da-Silva,
 524 A.M., Ramos, O.H.P., Rucavado, A., Gutiérrez, J.M., Magalhães, G.S., Faquim-Mauro, E.L.,
 525 Fernandes, I., 2014. A neutralizing recombinant single chain antibody, scFv, against BaP1, A P-
 526 I hemorrhagic metalloproteinase from *Bothrops asper* snake venom. Toxicon 87, 81–91.
 527 doi:10.1016/j.toxicon.2014.05.017
 528 Chaves, L.F., Chuang, T.-W., Sasa, M., Gutiérrez, J.M., 2015. Snakebites are associated with poverty,
 529 weather fluctuations, and El Niño. Sci. Adv. 1, e1500249–e1500249.
 530 doi:10.1126/sciadv.1500249
 531 Chen, Y.J., Tsai, C.Y., Hu, W.P., Chang, L. Sen, 2016. DNA aptamers against Taiwan banded krait
 532 α-bungarotoxin recognize Taiwan cobra cardiotoxins. Toxins (Basel). 8.
 533 doi:10.3390/toxins8030066
 534 Chippaux, J.-P., Lang, J., Amadi-Eddines, S., Fagot, I., Rage, V., Le Mener, V., 1998. Clinical safety
 535 of a polyvalent F(ab')₂ equine antivenom in 223 African snake envenomations: a field trial in
 536 Cameroon. Trans. R. Soc. Trop. Med. Hyg. 92, 657–662.

537 Chippaux, J.-P., Stock, R.P., Massougbodji, A., 2017. Antivenom Safety and Tolerance for the
538 Strategy of Snake Envenomation Management, in: Snake Venoms. pp. 475–495.
539 doi:0.1007/978-94-007-6410-1_25

540 De Silva, H.A., Ryan, N.M., De Silva, H.J., 2016. Adverse reactions to snake antivenom, and their
541 prevention and treatment. *Br. J. Clin. Pharmacol.* 81, 446–452. doi:10.1111/bcp.12739

542 Descotes, J., 2009. Immunotoxicity of monoclonal antibodies. *MAbs* 1, 104–111.
543 doi:10.4161/mabs.1.2.7909

544 El-Aziz, T.M.A., Ravelet, C., Molgo, J., Fiore, E., Pale, S., Amar, M., Al-Khoury, S., Dejeu, J., Fadl,
545 M., Ronjat, M., Taiwe, G.S., Servent, D., Peyrin, E., De Waard, M., 2017. Efficient functional
546 neutralization of lethal peptide toxins in vivo by oligonucleotides. *Sci. Rep.* 7, 1–9.
547 doi:10.1038/s41598-017-07554-5

548 Escalante, T., Ovadia, M., Cury, Y., Franceschi, A., León, G., Chaves, F., Gutiérrez, J.M., Rucavado,
549 A., 2017. Inhibition of local hemorrhage and dermonecrosis induced by *Bothrops asper* snake
550 venom: effectiveness of early in situ administration of the peptidomimetic metalloproteinase
551 inhibitor batimastat and the chelating agent CaNa₂EDTA. *Am. J. Trop. Med. Hyg.* 63, 313–319.
552 doi:10.4269/ajtmh.2000.63.313

553 Fry, B.G., 2018. Snakebite: When the Human Touch Becomes a Bad Touch. *Toxins (Basel)*. 10, 1–
554 23. doi:10.3390/toxins10040170

555 Grilo, A.L., Mantalaris, A., 2018. The Increasingly Human and Profitable Monoclonal Antibody
556 Market. *Trends Biotechnol.* doi:10.1016/j.tibtech.2018.05.014

557 Gutiérrez, J.M., Burnouf, T., Harrison, R.A., Calvete, J.J., Kuch, U., Warrell, D.A., 2014. A
558 multicomponent strategy to improve the availability of antivenom for treating snakebite
559 envenoming. *Bull. World Health Organ.* 92, 526–532. doi:10.2471/BLT.13.132431

560 Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017a.
561 Snakebite envenoming. *Nat. Rev. Dis. Prim.* 3, 17063. doi:10.1038/nrdp.2017.63

562 Gutiérrez, J.M., León, G., Lomonte, B., 2003. Pharmacokinetic-pharmacodynamic Relationships of
563 Immunoglobulin Therapy for Envenomation. *Clin. Pharmacokinet.* 42, 721–741.

564 Gutiérrez, J.M., Solano, G., Pla, D., Herrera, M., Segura, Á., Vargas, M., Villalta, M., Sánchez, A.,
565 Sanz, L., Lomonte, B., León, G., Calvete, J.J., 2017b. Preclinical evaluation of the efficacy of
566 antivenoms for snakebite envenoming: State-of-the-art and challenges ahead. *Toxins (Basel)*. 9,
567 1–22. doi:10.3390/toxins9050163

568 Gutiérrez, J.M., Solano, G., Pla, D., Herrera, M., Segura, Á., Villalta, M., Vargas, M., Sanz, L.,
569 Lomonte, B., Calvete, J.J., León, G., 2013. Assessing the preclinical efficacy of antivenoms:
570 From the lethality neutralization assay to antivenomics. *Toxicon* 69, 168–179.
571 doi:10.1016/j.toxicon.2012.11.016

572 Hammerschmidt, N., Tscheliessnig, A., Sommer, R., Helk, B., Jungbauer, A., 2014. Economics of
573 recombinant antibody production processes at various scales: Industry-standard compared to
574 continuous precipitation. *Biotechnol. J.* 9, 766–775. doi:10.1002/biot.201300480

575 Hansel, T.T., Kropshofer, H., Singer, T., Mitchell, J.A., George, A.J.T., 2010. The safety and side
576 effects of monoclonal antibodies. *Nat. Rev. Drug Discov.* 9, 325–338. doi:10.1038/nrd3003

577 Harding, F.A., Stickler, M.M., Razo, J., DuBridge, R.B., 2010. The immunogenicity of humanized
578 and fully human antibodies: Residual immunogenicity resides in the CDR regions. *MAbs* 2,
579 256–265. doi:10.4161/mabs.2.3.11641

580 Harrison, R.A., Cook, D.A., Renjifo, C., Casewell, N.R., Currier, R.B., Wagstaff, S.C., 2011.
581 Research strategies to improve snakebite treatment: Challenges and progress. *J. Proteomics* 74,
582 1768–1780. doi:10.1016/j.jpro.2011.06.019

583 Harrison, R.A., Gutiérrez, J.M., 2016. Priority Actions and Progress to Substantially and Sustainably
584 Reduce the Mortality, Morbidity and Socioeconomic Burden of Tropical Snakebite. *Toxins*

(Basel). 8, 351. doi:10.3390/toxins8120351

Harrison, R.A., Hargreaves, A., Wagstaff, S.C., Faragher, B., Lalloo, D.G., 2009. Snake Envenoming: A disease of poverty. *PLoS Negl. Trop. Dis.* 3. doi:10.1371/journal.pntd.0000569

Herrera, M., León, G., Segura, Á., Meneses, F., Lomonte, B., Chippaux, J.-P., Gutiérrez, J.M., 2005. Factors associated with adverse reactions induced by caprylic acid-fractionated whole IgG preparations: Comparison between horse, sheep and camel IgGs. *Toxicon* 46, 775–781. doi:10.1016/j.toxicon.2005.08.004

Howes, J.M., Theakston, R.D.G., Laing, G.D., 2007. Neutralization of the haemorrhagic activities of viperine snake venoms and venom metalloproteinases using synthetic peptide inhibitors and chelators. *Toxicon* 49, 734–739. doi:10.1016/j.toxicon.2006.11.020

Jäger, V., Bussow, K., Wagner, A., Weber, S., Hust, M., Frenzel, A., Schirrmann, T., 2013. High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells. *BMC Biotechnol.* 13, 52. doi:10.1186/1472-6750-13-52

Jenkins, T.P., Fryer, T., Ibsen Dehli, R., Jürgensen, J.A., Fuglsang-Madsen, A., Føns, S., Laustsen, A.H., 2019. Toxin neutralization using alternative binding proteins. *Toxins (Basel)*. 11. doi:10.3390/toxins11010053

Johnston, C.I., Ryan, N.M., O’Leary, M.A., Brown, S.G.A., Isbister, G.K., 2017. Australian taipan (*Oxyuranus* spp.) envenoming: clinical effects and potential benefits of early antivenom therapy—Australian Snakebite Project (ASP-25). *Clin. Toxicol.* 55, 115–122. doi:10.1080/15563650.2016.1250903

Julve Parreño, J.M., Huet, E., Fernández-del-Carmen, A., Segura, Á., Venturi, M., Gandía, A., Pan, W. song, Albaladejo, I., Forment, J., Pla, D., Wigdorovitz, A., Calvete, J.J., Gutiérrez, C., Gutiérrez, J.M., Granell, A., Orzáez, D., 2017. A synthetic biology approach for consistent production of plant-made recombinant polyclonal antibodies against snake venom toxins. *Plant Biotechnol. J.* 1–10. doi:10.1111/pbi.12823

Kannt, A., Wieland, T., 2016. Managing risks in drug discovery: Reproducibility of published findings. *Naunyn. Schmiedeberg’s. Arch. Pharmacol.* 389, 353–360. doi:10.1007/s00210-016-1216-8

Karain, B.D., Lee, M.K.H., Hayes, W.K., 2016. C60 Fullerenes as a Novel Treatment for Poisoning and Envenomation: A Proof-of-Concept Study for Snakebite. *J. Nanosci. Nanotechnol.* 16, 7764–7771. doi:10.1166/jnn.2016.12851

Keefe, A.D., Pai, S., Ellington, A., 2010. Aptamers as therapeutics. *Nat. Rev. Drug Discov.* 9, 537–550. doi:10.1038/nrd3141

Kerpedijev, P., Hammer, S., Hofacker, I.L., 2015. Forna (force-directed RNA): Simple and effective online RNA secondary structure diagrams. *Bioinformatics* 31, 3377–3379.

King, C., Garza, E.N., Mazor, R., Linehan, J.L., Pastan, I., Pepper, M., Baker, D., 2014. Removing T-cell epitopes with computational protein design. *Proc. Natl. Acad. Sci.* 111, 8577–8582. doi:10.1073/pnas.1321126111

Kini, R.M., Sidhu, S.S., Laustsen, A.H., 2018. Biosynthetic Oligoclonal Antivenom (BOA) for Snakebite and Next-Generation Treatments for Snakebite Victims. *Toxins (Basel)*. 10, 534. doi:10.3390/toxins10120534

Knudsen, C., Laustsen, A.H., 2018. Recent Advances in Next Generation Snakebite Antivenoms. *Trop. Med. Infect. Dis.* 3, 42. doi:10.3390/tropicalmed3020042

Laustsen, A.H., 2017. Guiding recombinant antivenom development by omics technologies. *N. Biotechnol.* doi:10.1016/j.nbt.2017.05.005

Laustsen, A.H., 2016a. Toxin synergism in snake venoms. *Toxin Rev.* 35, 165–170. doi:10.1080/15569543.2016.1220397

Laustsen, A.H., 2016b. Costing recombinant antivenoms. *Nat. Corresp.* 538, 41.

Laustsen, A.H., Dorrestijn, N., 2018. Integrating Engineering, Manufacturing, and Regulatory Considerations in the Development of Novel Antivenoms. *Toxins* (Basel). 10. doi:10.3390/toxins10080309

Laustsen, A.H., Engmark, M., Milbo, C., Johannesen, J., Lomonte, B., Gutiérrez, J.M., Lohse, B., 2016a. From Fangs to Pharmacology: The Future of Antivenoms. *Curr. Pharm. Des.* 9. doi:10.2174/1381612822666160623073438

Laustsen, A.H., Gutiérrez, J.M., Knudsen, C., Johansen, K.H., Méndez, E.B., Cerni, F.A., Jürgensen, J.A., Øhlenschläger, M., Ledsgaard Jensen, L., Esteban, A.M., Pus, U., Andersen, M.R., Lomonte, B., Engmark, M., Pucca, M.B., 2018a. Pros and cons of different therapeutic antibody formats for recombinant antivenom development. *Toxicon*. doi:10.1016/j.toxicon.2018.03.004

Laustsen, A.H., Johansen, K.H., Engmark, M., Andersen, M.R., 2017. Recombinant snakebite antivenoms: A cost-competitive solution to a neglected tropical disease? *PLoS Negl. Trop. Dis.* 11, 1–14. doi:10.1371/journal.pntd.0005361

Laustsen, A.H., Karatt-Vellatt, A., Masters, E.W., Arias, A.S., Pus, U., Knudsen, C., Oscoz, S., Slavny, P., Griffiths, D.T., Luther, A.M., Leah, R.A., Lindholm, M., Lomonte, B., Gutiérrez, J.M., McCafferty, J., 2018b. In vivo neutralization of dendrotoxin-mediated neurotoxicity of black mamba (*Dendroaspis polylepis*) venom by mixtures of human IgG monoclonal antibodies. *Nat. Commun.* 9, 3928. doi:10.1038/s41467-018-06086-4

Laustsen, A.H., Lohse, B., Lomonte, B., Engmark, M., Gutiérrez, J.M., 2015. Selecting key toxins for focused development of elapid snake antivenoms and inhibitors guided by a Toxicity Score. *Toxicon* 104, 43–45. doi:10.1016/j.toxicon.2015.07.334

Laustsen, A.H., Solà, M., Jappe, E.C., Oscoz, S., Lauridsen, L.P., Engmark, M., 2016b. Biotechnological trends in spider and scorpion antivenom development. *Toxins* (Basel). 8. doi:10.3390/toxins8080226

León, G., Herrera, M., Segura, Á., Villalta, M., Vargas, M., Gutiérrez, J.M., 2013. Pathogenic mechanisms underlying adverse reactions induced by intravenous administration of snake antivenoms. *Toxicon* 76, 63–76. doi:10.1016/j.toxicon.2013.09.010

León, G., Segura, Á., Herrera, M., Otero, R., de Siqueira França, F.O., Barbaro, K.C., Cardoso, J.L.C., Wen, F.H., de Medeiros, C.R., Prado, J.C.L., Malaque, C.M.S.A., Lomonte, B., Gutiérrez, J.M., 2008. Human heterophilic antibodies against equine immunoglobulins: assessment of their role in the early adverse reactions to antivenom administration. *Trans. R. Soc. Trop. Med. Hyg.* 102, 1115–1119. doi:10.1016/j.trstmh.2008.04.038

León, G., Vargas, M., Segura, Á., Herrera, M., Villalta, M., Sánchez, A., Solano, G., Gómez, A., Sánchez, M., Estrada, R., 2018. Current technology for the industrial manufacture of snake antivenoms. *Toxicon* 151, 63–73. doi:10.1016/j.toxicon.2018.06.084

Lewin, M.R., Gilliam, L., Gilliam, J., Samuel, S., Bulfone, T., Bickler, P., Gutiérrez, J.M., 2018a. Delayed LY333013 (Oral) and LY315920 (Intravenous) Reverse Severe Neurotoxicity and Rescue Juvenile Pigs from Lethal Doses of *Micrurus fulvius* (Eastern Coral Snake) Venom. *Toxins* (Basel). 10, 479. doi:10.3390/toxins10110479

Lewin, M.R., Gutiérrez, J.M., Samuel, S.P., Herrera, M., Bryan-Quirós, W., Lomonte, B., Bickler, P.E., Bulfone, T.C., Williams, D.J., 2018b. Delayed oral LY333013 rescues mice from highly neurotoxic, lethal doses of papuan taipan (*Oxyuranus scutellatus*) venom. *Toxins* (Basel). 10, 1–7. doi:10.3390/toxins10100380

Lewin, M.R., Samuel, S., Merkel, J., Bickler, P., 2016. Varespladib (LY315920) appears to be a potent, broad-spectrum, inhibitor of snake venom phospholipase A2 and a possible pre-referral treatment for envenomation. *Toxins* (Basel). 8. doi:10.3390/toxins8090248

Liu, H.F., Ma, J., Winter, C., Bayer, R., 2010. Recovery and purification process development for monoclonal antibody production. *MAbs* 2, 480–499. doi:10.4161/mabs.2.5.12645

Lojewska, E., Kowalczyk, T., Olejniczak, S., Sakowicz, T., 2016. Extraction and purification methods in downstream processing of plant-based recombinant proteins. *Protein Expr. Purif.* 120, 110–117. doi:10.1016/j.pep.2015.12.018

LoVecchio, F., Klemens, J., Roundy, E.B., Klemens, A., 2003. Serum Sickness Following Administration of Antivenin (Crotalidae) Polyvalent in 181 cases of presumed Rattlesnake Envenomation. *Wilderness Environ. Med.* 14, 220–221. doi:10.1580/1080-6032(2003)14

Malasit, P., Warrell, D.A., Chanthavanich, P., Viravan, C., Mongkolsapaya, J., Singhthong, B., Supich, C., 1986. Prediction, prevention, and mechanism of early (anaphylactic) antivenom reactions in victims of snake bites. *Br. Med. J. (Clin. Res. Ed.)* 292, 17–20. doi:10.1136/bmj.292.6512.17

Mazor, R., Crown, D., Addissie, S., Jang, Y., Kaplan, G., Pastan, I., 2017. Elimination of murine and human T-cell epitopes in recombinant immunotoxin eliminates neutralizing and anti-drug antibodies in vivo. *Cell. Mol. Immunol.* 14, 432–442. doi:10.1038/cmi.2015.91

McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J., 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*. doi:10.1038/348552a0

Morais, V., Berasain, P., Massaldi, H., 2014. Immunoglobulin Purification by Caprylic Acid. *Methods Mol. Biol.* 1129, 137–143. doi:10.1007/978-1-62703-977-2

Moran, N.F., Newman, W.J., Theakston, R.D.G., Warrell, D.A., Wilkinson, D., 1998. High incidence of early anaphylactoid reaction to SAIMR polyvalent snake antivenom. *Trans. R. Soc. Trop. Med. Hyg.* 92, 69–70. doi:10.1016/S0035-9203(98)90959-2

Nagata, S., Pastan, I., 2009. Removal of B cell epitopes as a practical approach for reducing the immunogenicity of foreign protein-based therapeutics. *Adv. Drug Deliv. Rev.* 61, 977–985. doi:10.1016/j.addr.2009.07.014

Nelson, A.L., Dhimolea, E., Reichert, J.M., 2010. Development trends for human monoclonal antibody therapeutics. *Nat. Rev. Drug Discov.* 9, 767–774. doi:10.1038/nrd3229

O’Brien, J., Lee, S.-H., Gutiérrez, J.M., Shea, K.J., 2018. Engineered nanoparticles bind elapid snake venom toxins and inhibit venom-induced dermonecrosis. *PLoS Negl. Trop. Dis.* 12, e0006736. doi:10.1371/journal.pntd.0006736

Parsons, S.L., Watson, S.A., Steele, R.J.C., 1997. Phase I/II trial of batimastat, a matrix metalloproteinase inhibitor, in patients with malignant ascites. *Eur. J. Surg. Oncol.* 23, 526–531. doi:10.1016/S0748-7983(97)93077-8

Prado, N.D.R., Pereira, S.S., da Silva, M.P., Morais, M.S.S., Kayano, A.M., Moreira-Dill, L.S., Luiz, M.B., Zanchi, F.B., Fuly, A.L., Huacca, M.E.F., Fernandes, C.F., Calderon, L.A., Zuliani, J.P., Pereira da Silva, L.H., Soares, A.M., Stabeli, R.G., Fernandes, C.F.C., 2016. Inhibition of the Myotoxicity Induced by *Bothrops jararacussu* Venom and Isolated Phospholipases A2 by Specific Camelid Single-Domain Antibody Fragments. *PLoS One* 11. doi:10.1371/journal.pone.0151363

Rasmussen, H.S., McCann, P.P., 1997. Matrix Metalloproteinase Inhibition as a Novel Anticancer Strategy: A Review with Special Focus on Batimastat and Marimastat. *Pharmacol. Ther.* 75, 69–75. doi:10.1016/S0163-7258(97)00023-5

Rasmussen, S.K., Næsted, H., Müller, C., Tolstrup, A.B., Frandsen, T.P., 2012. Recombinant antibody mixtures: Production strategies and cost considerations. *Arch. Biochem. Biophys.* 526, 139–145. doi:10.1016/j.abb.2012.07.001

Rawat, S., Laing, G.D., Smith, D.C., Theakston, R.D.G., Landon, J., 1994. A new antivenom to treat eastern coral snake (*Micrurus fulvius fulvius*) envenoming. *Toxicon* 32, 185–190. doi:10.1016/0041-0101(94)90107-4

Reichert, J.M., 2010. Metrics for antibody therapeutics development. *MAbs* 2, 695–700. doi:10.4161/mabs.2.6.13603

Richard, G., Meyers, A.J., McLean, M.D., Arbabi-Ghahroudi, M., MacKenzie, R., Hall, J.C., 2013. In Vivo Neutralization of alpha-Cobratxin with High-Affinity Llama Single-Domain Antibodies (VHHs) and a VHH-Fc Antibody. *PLoS One* 8. doi:10.1371/journal.pone.0069495
 Rojas, A., Vargas, M., Ramírez, N., Estrada, R., Segura, Á., Herrera, M., Villalta, M., Gómez, A., Gutiérrez, J.M., León, G., 2013. Role of the animal model on the pharmacokinetics of equine-derived antivenoms. *Toxicon* 70, 9–14. doi:10.1016/j.toxicon.2013.03.013
 Sanhajariya, S., Duffull, S.B., Isbister, G.K., 2018. Pharmacokinetics of snake venom. *Toxins* (Basel). 10. doi:10.3390/toxins10020073
 Seifert, S.A., Boyer, L. V., 2001. Recurrence phenomena after immunoglobulin therapy for snake envenomations: Part 1. Pharmacokinetics and pharmacodynamics of immunoglobulin antivenoms and related antibodies. *Ann. Emerg. Med.* 37, 189–195. doi:10.1067/mem.2001.113135
 Silva, L.C., Pucca, M.B., Pessenda, G., Campos, L.B., Martinez, E.Z., Cerni, F.A., Barbosa, J.E., 2018. Discovery of human scFvs that cross-neutralize the toxic effects of *B. jararacussu* and *C. d. terrificus* venoms. *Acta Trop.* doi:10.1016/j.actatropica.2017.09.001
 Smith, G.P., 1985. Filamentous Fusion Phage: Novel Expression Vectors that Display Cloned Antigens on the Virion Surface. *Science* (80-.). 228, 1315–1317.
 Sparano, J.A., Bernardo, P., Stephenson, P., Gradishar, W.J., Ingle, J.N., Zacker, S., Davidson, N.E., 2004. Randomized phase III trial of marimastat versus placebo in patients with metastatic breast cancer who have responding or stable disease after first-line chemotherapy: Eastern Cooperative Oncology Group Trial E2196. *J. Clin. Oncol.* 22, 4683–4690. doi:10.1200/JCO.2004.08.054
 Steward, W.P., 1999. Marimastat (BB2516): Current status of development. *Cancer Chemother. Pharmacol.* 43 (Suppl., 56–60. doi:10.1007/s002800051099
 Stone, S.F., Isbister, G.K., Shahmy, S., Mohamed, F., Abeysinghe, C., Karunathilake, H., Ariaratnam, C.A., Jacoby-Alner, T.E., Cotterell, C.L., Brown, S.G.A., 2013. Immune Response to Snake Envenoming and Treatment with Antivenom; Complement Activation, Cytokine Production and Mast Cell Degranulation. *PLoS Negl. Trop. Dis.* 7. doi:10.1371/journal.pntd.0002326
 Sutherland, S.K., 1977. Serum Reactions: An Analysis of commercial antivenoms and the possible role of anticomplementary activity in *de novo* reactions to antivenoms and antitoxins. *Med. J. Aust.* 2, 841–842.
 Tuerk, C., Gold, L., 1990. Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase. *Science* (80-.). 249, 505–510.
 U.S. National Library of Medicine, n.d. Vista-16 trial: Evaluation of Safety and Efficacy of Short-term A-002 Treatment in Subjects With Acute Coronary Syndrome [WWW Document]. URL <https://clinicaltrials.gov/ct2/show/NCT01130246>
 Vargas, M., Segura, Á., Herrera, M., Villalta, M., Estrada, R., Cerdas, M., Paiva, O.K., Matainaho, T., Jensen, S.D., Winkel, K.D., León, G., Gutiérrez, J.M., Williams, D.J., 2011. Preclinical Evaluation of Caprylic Acid-Fractionated IgG Antivenom for the Treatment of Taipan (*Oxyuranus scutellatus*) Envenoming in Papua New Guinea. *PLoS Negl. Trop. Dis.* 5. doi:10.1371/journal.pntd.0001144
 Vazquez-Lombardi, R., Phan, T.G., Zimmermann, C., Lowe, D., Jermutus, L., Christ, D., 2015. Challenges and opportunities for non-antibody scaffold drugs. *Drug Discov. Today* 20, 1271–1283. doi:10.1016/j.drudis.2015.09.004
 Walsh, G., 2014. Biopharmaceutical benchmarks 2014. *Nat. Biotechnol.* 32. doi:10.1038/nbt.3040
 Wang, Y., Zhang, J., Zhang, D., Xiao, H., Xiong, S., Huang, C., 2018. Exploration of the Inhibitory Potential of Varespladib for Snakebite Envenomation. *Molecules* 23, 391. doi:10.3390/molecules23020391
 Wiberg, F.C., Rasmussen, S.K., Frandsen, T.P., Rasmussen, L.K., Tengbjerg, K., Coljee, V.W.,

777 Sharon, J., Yang, C.-Y., Bregenholt, S., Nielsen, L.S., Haurum, J.S., Tolstrup, A.B., 2006.
 778 Production of target-specific recombinant human polyclonal antibodies in mammalian cells.
 779 Biotechnol. Bioeng. 94, 396–405. doi:10.1002/bit.20865
 780 Wilken, L.R., Nikolov, Z.L., 2012. Recovery and purification of plant-made recombinant proteins.
 781 Biotechnol. Adv. 30, 419–433. doi:10.1016/j.biotechadv.2011.07.020
 782 Williams, D.J., Habib, A.G., Warrell, D.A., 2018. Clinical studies of the effectiveness and safety of
 783 antivenoms. Toxicon 150, 1–10. doi:10.1016/j.toxicon.2018.05.001
 784 World Health Organization, 2018. Guidelines for the production, control and regulation of snake
 785 antivenom immunoglobulins.
 786 World Health Organization, 2010. WHO guidelines for the production, control and regulation of
 787 snake antivenom immunoglobulins. doi:10.1051/jbio/2009043
 788 World Health Organization, n.d. WHO guidelines for the management of snakebite.
 789