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REVIEW

Practical progress towards the development of recombinant antivenoms for snakebite envenoming

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ABSTRACT

Introduction: Snakebite envenoming is a neglected tropical disease that affects millions globally each year. In recent years, research into the potential production of recombinant antivenoms, formulated using mixtures of highly defined anti-toxin monoclonal antibodies, has rapidly moved from a theoretical concept to demonstrations of practical feasibility.

Areas covered: This article examines the significant practical advancements in transitioning recombinant antivenoms from concept to potential clinical translation. The authors have based their review on literature obtained from Google Scholar and PubMed between September and November 2024. Coverage includes the development and validation of recombinant antivenom antibody discovery strategies, the characterization of the first broadly neutralizing toxin class antibodies, and recent translational proof-of-concept experiments.

Expert opinion: The transition of recombinant antivenoms from a 'concept' to the current situation where high-throughput anti-venom mAb discovery is becoming routine, accompanied by increasing evidence of their broad neutralizing capacity *in vivo*, has been extraordinary. It is now important to build on this momentum by expanding the discovery of broadly neutralizing mAbs to encompass as many toxin classes as possible. It is anticipated that key demonstrations of whether recombinant antivenoms can match or surpass existing conventional polyvalent antivenoms in terms of neutralizing scope and capacity will be achieved in the next few years.

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Antibody display; monoclonal antibody; neglected tropical diseases; oligoclonal cocktails; venom

1. Introduction

Snakebite envenoming occurs when a venomous snake defensively bites and injects venom through specialized fangs into a victim. It is thought several million people are bitten by venomous snakes each year [1]. The consequences of a bite can be devastating, with estimates of upwards of 130,000 people dying and a further 400,000 permanently disabled annually due to the pathological effects of snake venom [2]. It is overwhelmingly the poorest in society that suffer the greatest snakebite burden, particularly those residing in the impoverished, rural communities of the tropics [3]. Antivenoms have been the mainstay of envenoming treatment for over a century and are the only treatment for snakebite with proven clinical efficacy. Currently, all antivenoms consist of polyclonal antibodies extracted from the plasma of animals, usually horses and occasionally sheep, which have been hyperimmunised with venoms [4]. When administered to a snakebite victim, the anti-toxin antibodies present within an antivenom bind to circulating toxins and block their function, resulting in the halting or reversal of toxin effects. Despite few clinical trials, it is widely accepted from their prolonged use and ample clinical experience that antivenoms are life-saving, essential medicines [2,5–7].

owever, due to their method of manufacture, antivenoms also suffer from a range of issues which ultimately hamper their effectiveness, accessibility and market attractiveness [8]. In particular, despite broad similarity in toxin families across snake families and genera [9], individual venom variation at the species level [10] leads to antivenoms typically being restricted in utility for the species in which they were developed against, resulting in species constrained markets, and consequently, geographically constrained markets [8]. Further issues directly associated with their method of manufacture include substantial variation between antivenom batches [11], poor safety profiles, with frequent adverse reactions to antivenoms reported [12,13], the routine requirement of cold-chain storage which can prove problematic in remote settings [14], low therapeutic potency due to high proportions (often estimated at 80–90%) of therapeutically redundant antibodies [15] and limited efficacy in treating local tissue damage [16]. Furthermore, the specialist method of manufacture for antivenoms, requiring increasingly scarce high-quality crude venom supply as well as specialist horse husbandry, substantially increases manufacturing costs [17], resulting in antivenoms often being unaffordable for already impoverished populations [3,18]. The

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Article highlights

- Snakebite envenoming causes approx. 130,000 deaths and 400,000 permanent disabilities each year.
- Research into the feasibility of recombinant antivenoms, made using highly defined mixtures of broadly neutralizing anti-toxin monoclonal antibodies (mAbs) and hoped to overcome many of the drawbacks of conventional antivenoms, has substantially increased in recent years.
- Several strategies for discovering broadly neutralizing mAbs have been developed and validated.
- In particular, mAb discovery against long-chain α-neurotoxins has been particularly successful, with three mAbs with broad and potent neutralizing efficacy described.
- Challenges remain in discovery of mAbs against other toxin classes and their preclinical validation.
- Proof of concept demonstrations around recombinant antivenom production and formulation have started to be performed with promising results.

culmination of these issues has disincentivized many large manufacturers from antivenom production, with production largely performed by public sector producers, the consequence of which has resulted in chronic shortages and absence of reliable antivenom products in many regions [8,19].

When snakebite envenoming was added to the World Health Organization's list of Neglected Tropical Diseases in 2017, a global strategy to reduce snakebite burden was developed [20] with a primary focus on the implementation of improved availability of reliable antivenoms to alleviate chronic shortages in the most critical regions, alongside improving clinical decision making and better health worker training and education. Longer term, the strategy advocates for investing in innovative research for new therapeutics which may alleviate the existing shortcomings of conventional antivenom products, further improving availability, affordability and safety [20]. Partially in response to this, there has been a sharp increase in funding for the application of cutting-edge research techniques to snakebite envenoming, especially for research into alternative therapeutics to conventional antivenom [21]. Research in this space has predominantly focused on two major areas: (i) generic inhibitors of toxin class function and (ii) recombinant, also known as synthetic, antivenoms. Generic toxin inhibitors are not the focus of this review, and we point the reader to interesting reviews on this topic [22–24]. This paper will instead focus on the recent progress made in the development of recombinant antibody-based antivenoms, which, when achieved, will likely be defined mixtures of highly characterized anti-toxin monoclonal antibodies (mAbs).

mAbs are individual immunoglobulins, produced through the cloning or synthesis of unique antibody encoding genes, and subsequently recombinantly expressed in laboratories. Due to their high target specificity, these therapeutics have become a staple pharmaceutical class for tackling cancer and immunological disorders [25]. Their recombinant nature means they can be modified to have desirable pharmacological properties, such as increased half-lives and engineering prevent off-target effects and silence of undesirable effector functions [26].

The first mAbs against snake venom toxins were isolated in the 1980s using hybridoma technology [27]. By the mid-2010s, mAb therapeutics for diseases such cancers and immune disorders had become mainstream, and animal-free and high-throughput technologies for mAb discovery increasingly accessible [28]. Alongside routine detailed characterization of snake venoms using proteomics and transcriptomics [9,29], these developments enabled the prospect of developing a fully recombinant antivenom to seem feasible and achievable. Several concept papers were published, theorizing what such an antivenom would look like, how much they could cost, and what would be required to produce such a product [30–33]. In short, it is agreed that recombinant antivenoms will consist of a cocktail (also described as an 'oligoclonal mixture') of a yet-to-be determined number of highly characterized mAbs [31,34]. The requirement for a cocktail of mAbs is due to snake venoms consisting of multiple different and distinct toxin families that need to be neutralized during envenoming. To reduce the number of mAbs required in a recombinant antivenom, and to increase its geographic utility, each constituent mAb would have to possess potent neutralizing ability and broad target reactivity within its target toxin class. It is hoped that by formulating recombinant antivenoms with the most potent and broadly neutralizing anti-toxin mAbs, engineered to possess desirable characteristics (such as humanization to reduce adverse reactions and modifications for superior pharmacokinetics), many of the serious shortcomings of conventional antivenoms, such as poor dose efficacy, high adverse reaction rates and constrained species and geographic utility, would be overcome (Figure 1).

In the last decade, laboratories from more than 30 public and private institutions (based on the manuscripts covered in this review), often working in impressive international collaborative teams, have turned their attention to the discovery of mAbs for envenoming with a view to developing a recombinant antivenom in earnest. This manuscript will attempt to summarize the collective practical progress achieved in development of recombinant antivenoms to date, from discovery and characterization of broadly neutralizing anti-toxin antibodies through to the more recent proof of concepts of antivenom mAb cocktails and oligoclonal recombinant antivenom production. The basis of this review was literature obtained from searching Google Scholar and PubMed between September and November 2024 with the search terms 'recombinant antivenom,' 'monoclonal antivenom' and 'monoclonal antibody' and 'snakebite envenoming.'

2. Methods for mAb discovery

The first technique for mAb discovery was developed in 1975 with the invention of hybridoma technology [35] which subsequently led to the discovery of the first clinically used therapeutic mAb [36]. In the last 50 years an ever-growing toolkit of methods for mAb discovery has been developed [37], progressing from *in vivo* (first generation) methods that rely on animal immunizations through to *in vitro* (second generation) techniques using antibody libraries, and we are at the beginning of the third-generation methods using *in silico* approaches for computational *de novo* antibody design and engineering [38,39]. In the context of recombinant antivenoms, the majority of research historically used hybridoma technology to discover novel mAbs against a range of different toxin families, while in the last decade yeast- and phage- display

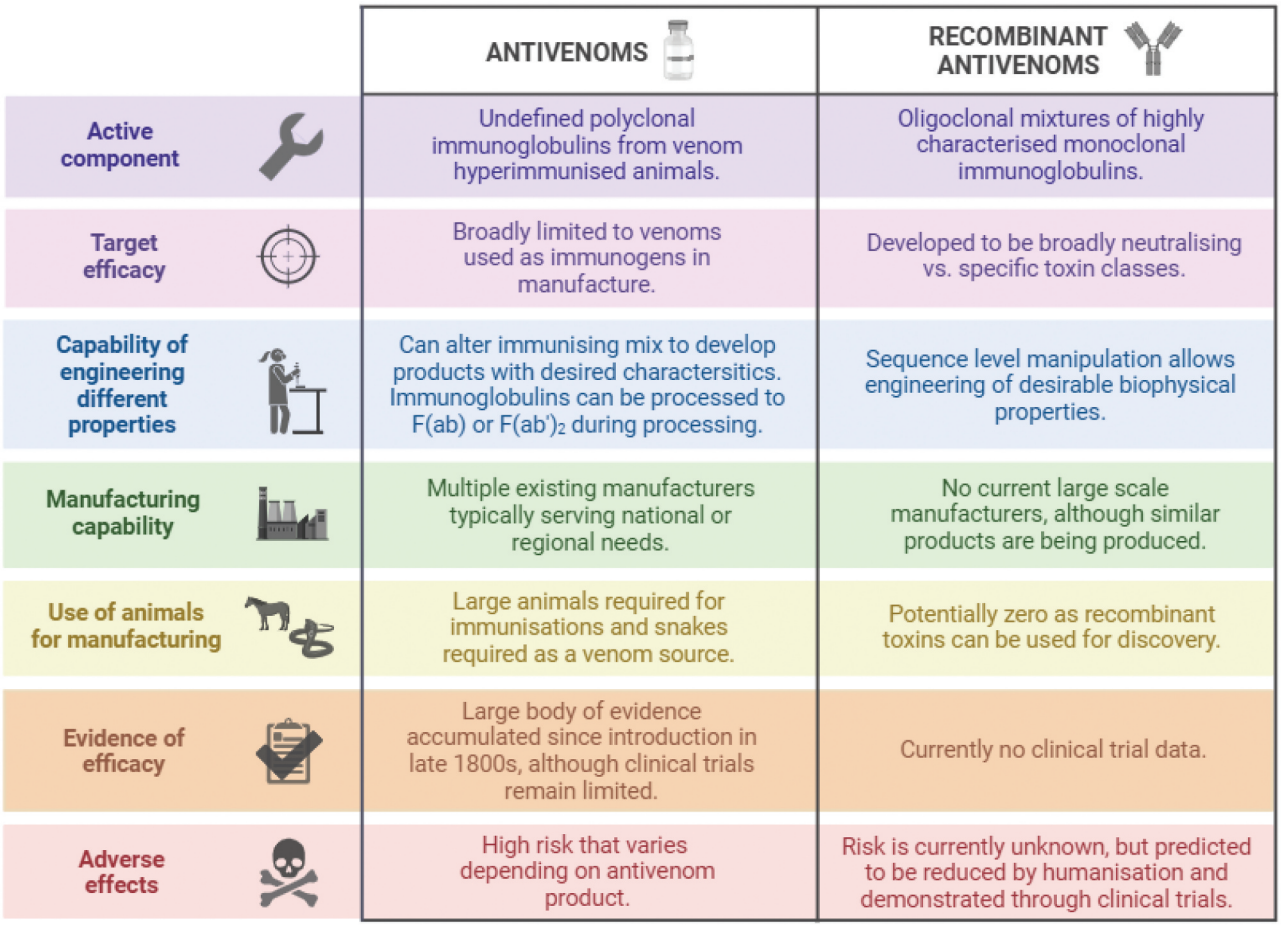


Figure 1. Comparison of key characteristics of existing conventional antivenoms and recombinant antivenoms. Created in BioRender. Casewell, N. (2025) https://BioRender.com/h82z648.

have become the dominant methods used to discover novel mAbs against snake venom toxins (Table 1). Beyond these, other techniques that may hold potential for developing recombinant antibody-based antivenoms include ribosome [73], bacterial [74] and mammalian cell [75] display of antibodies/antibody fragments, as well as B cell screening technologies from immunized sources (for review, see Pedrioli et al, [76]). However, to the best of our knowledge, these approaches have not yet been used to discover novel anti-snake venom mAbs.

2.1. In vitro *antibody display methods*

In the context of describing antibody display methods, we will simply refer to the displayed immune proteins as antibodies; however, in principle, this may refer to whole antibodies or antibody fragments (i.e. single-chain variable fragments [scFv], single-domain antibodies or other formats of immunoglobulins). *In vitro* antibody display methods employ combinatorial antibody libraries as the basis for the antibodies that are displayed on the cell/phage surface or ribosome. The genetic material for antibody libraries can be cloned into suitable expression plasmids either by amplifying the B cells from immunized or nonimmune 'naïve' donors (to produce immune or nonimmune libraries, respectively), or can be synthetically designed and produced *in vitro*. In the context of cell or phage-display, genes encoding antibody sequences are genetically fused to a gene encoding a cell surface protein within a plasmid [78–80].

Antibody display methods in general share similar methodological approaches to isolate antibodies against the protein of interest. The combinatorial antibody library is transfected into recipient cells and expression of antibodies is induced. The antibody-displaying cells/phages may first be exposed to an off-target antigen to remove nonspecific binders, and unbound cells/phages are collected and subsequently incubated with the target protein, which may be immobilized or in-solution. Typically, the target protein will be modified to include an affinity tag such as biotin to enable immobilization of the target protein or to isolate the in-solution target protein using magnetic biotin-binding beads. The bound cells/phages are then released from the target protein and can be grown for subsequent rounds, often with lower concentrations of target protein to enrich for high affinity binders, and finally the plasmids from the cells/phages are isolated to determine the gene encoding the antibody. In this way, phenotype is linked to genotype.

Antibody display libraries offer fully *in vitro* platforms from which to rapidly isolate novel mAbs within just a few weeks, and unlike immunization-based approaches can be used to isolate

Table 1. Characteristics of anti-toxin mAbs.

							In vivo[e]					
							Local		Lethal			
mAb(s)	Discovery method	mAb format[a]	Toxin Target[b]	Species tested against	WHO Region[c]	*in vitro*[d]	p.i.	Res.	p.i.	Res.	Notes	Ref.
95mat5	Synthetic-HYDL	IgG	Lc-α-NTx	*D. polylepis, N. kaouthia, O. hannah*	AFR, SEAR, WPR	Y	n/a	n/a	Y*	Y +	*neutralized lethality vs. *D. polylepis* in preincubation and rescue models. Partial neutralization of *O. hannah* induced lethality in preincubation.	[40]
Centi-3FTX-D09	Immunized-HPDL	Fab	Lc-α-NTx	*B. caeruleus, D. polylepis, D. viridis, L. colubrina, N. haje, N. kaouthia, N. naja, N. nivea, N. scutatus, N. sputatrix, O. hannah, O. microlepidotus, O. scutellatus, P. textilis*	AFR, SEAR, WPR	Y	n/a	n/a	Y*	n/a	*neutralized lethality vs. *N. kaouthia, N. naja, N. haje, N. nivea, O. scutellatus, D. polylepis, O. hannah*. Achieved partial neutralization of *L. colubrina, B. caeruleus, N. scutatus, D. viridis, P. textillis, O. microlepidoutus, N. sputatrix.*	[41]
2554_01_D11	Naïve-HPDL	IgG	Lc-α-NTx	*D. polylepis, N. kaouthia, O. hannah*	AFR, SEAR, WPR	Y	n/a	n/a	Y*	Y +	*neutralized lethality vs. *N. kaouthia*, extended TTD for *D. polylepis* and *O. hannah*. + partially neutralized lethality vs. *N. kaouthia*	[42]
TPL0027_01_F7	Naïve-HPDL	scFv	CTX	*N. melanoleuca, N. mossambica, N. nigricollis,*	AFR	Y*	n/a	n/a	n/a	n/a	*neutralized toxin induced cell cytotoxicity	[43]
2551_01_A12	Naïve-HPDL	IgG	Lc-α-NTx	*D. polylepis, N. kaouthia, N. melanoleuca*	AFR, SEAR	Y*	n/a	n/a	n/a	n/a	*neutralized toxin function in patch clamp experiments	[42]
363_01_F07	Naïve-HPDL	IgG	DTX	*D. polylepis*	AFR	n/a	n/a	n/a	Y*	n/a	*neutralized lethality via intracerebroventricular route when used in cocktail with mAbs 365_01_G06, and 367_01_H01.	[44]
67_01_H01	Naïve-HPDL	IgG	DTX	*D. polylepis*	AFR	n/a	n/a	n/a	Y*	n/a	*neutralized lethality via intracerebroventricular route when used in cocktail with mAbs 363_01_F07 and 365_01_G06.	[44]
365_01_G06	Naïve-HPDL	IgG	DTX	*D. polylepis*	AFR	n/a	n/a	n/a	Y*	n/a	*neutralized lethality via intracerebroventricular route when used in cocktail with mAbs 363_01_F07 and 367_01_H01	[44]
361_01_F07	Naïve-HPDL	IgG	DTX	*D. polylepis*	AFR	n/a	n/a	n/a	Y*	n/a	*neutralized lethality via intracerebroventricular route when used in cocktail with mAbs 365_01_G06 and 367_01_H01	[44]
TPL0004_01_A11	Naïve-HPDL	IgG	PLA_2 (GI)	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*demonstrated specific binding to PLA_2	[45]
C37	Naïve-HPDL	scFv	undefined	*M. lebetina*	EMR	Y	n/a	n/a	N*	n/a	*extended TTD compared to venom alone	[46]
C69	Naïve-HPDL	scFv	undefined	*M. lebetina*	EMR	Y	n/a	n/a	N*	n/a	*extended TTD compared to venom alone	[46]
H6	Naïve-CPDL	V_HH	SVMP	*B. atrox*	AMR	n/a	Y*	n/a	N +	n/a	*neutralized local hemorrhagic activity. + cocktail of H6/H8/H9/M85 did not prevent lethality but did reduce hemorrhagic pathology	[47]

(*Continued*)

Table 1. (Continued).

mAb(s)	Discovery method	mAb format[a]	Toxin Target[b]	Species tested against	WHO Region[c]	*in vitro*[d]	*In vivo*[e] Local p.i.	Local Res.	Lethal p.i.	Lethal Res.	Notes	Ref.
H8	Naïve-CPDL	V_HH	SVMP	*B. atrox*	AMR	n/a	Y*	n/a	N*	n/a	*neutralized local hemorrhagic activity. + cocktail of H6/H8/H9/M85 did not prevent lethality but did reduce hemorrhagic pathology	[47]
H9	Naïve-CPDL	V_HH	SVMP	*B. atrox*	AMR	n/a	Y*	n/a	N*	n/a	*neutralized local hemorrhagic activity. + cocktail of H6/H8/H9/M85 did not prevent lethality but did reduce hemorrhagic pathology	[47]
M85	Naïve-CPDL	V_HH	PLA_2 (GII)	*B. atrox*	AMR	n/a	Y*	n/a	N*	n/a	*neutralized local myotoxic activity. + cocktail of H6/H8/H9/M85 did not prevent lethality but did reduce hemorrhagic pathology	[47]
anti-Ba/Bn	murine hybridoma	IgG	SVSP	*B. alternatus, B. neuwiedi*	AMR	Y*	n/a	n/a	n/a	n/a	*neutralized coagulant activity *in vitro*	[48]
TPL0629_01_D11	Naïve-CPDL	V_HH	Sc-α-NTx	*M. diastema, M. fulvius*	AMR	Y	n/a	n/a	N*	n/a	*lethality prevented when used as cocktail with TPL0637_01_A07	[49]
TPL0637_01_A07	Naïve-CPDL	V_HH	PLA_2 (GI)	*M. diastema, M. fulvius*	AMR	Y	n/a	n/a	N*	n/a	*lethality prevented when used as cocktail with TPL0629_01_D11	[49]
1.1E	Naïve-HPDL	scFv	PLA_2 (GII)	*B. alternatus, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi, L. muta*	AMR	Y*	n/a	n/a	n/a	n/a	*neutralized hemolysis *in vitro*	[50]
1.4E	Naïve-HPDL	scFv	PLA_2 (GII)	*B. alternatus, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi, L. muta*	AMR	Y*	n/a	n/a	n/a	n/a	*neutralized hemolysis *in vitro*	[50]
2.1E	Naïve-HPDL	scFv	PLA_2 (GII)	*B. alternatus, B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi, L. muta*	AMR	Y*	n/a	n/a	n/a	n/a	*partially neutralized hemolysis *in vitro*	[50]
C13, C24, C39, C43, and C45	Naïve-HPDL	scFv	undefined	*N. oxiana*	EMR	n/a	n/a	n/a	Y*	Y*	*partial protection of lethality when cocktail of mAbs used, mAbs were Nt tested individually.	[51]
HuscFv15	Naïve-HPDL	scFv	SVMP	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*neutralized fibrinogenolytic activity	[52]
HuscFv20	Naïve-HPDL	scFv	SVMP	*N. kaouthia*	SEAR	Y	n/a	n/a	n/a	n/a	*neutralized fibrinogenolytic activity	[52]
HuScFv24	Naïve-HPDL	scFv	Lc-α-NTx	*N. kaouthia*	SEAR		n/a	n/a	Y*	n/a	*partial neutralization of lethality	[53]
E10	Naïve-HPDL	scFv	PLA2	*E. carinatus*	SEAR	N	Y*	n/a	Y +	n/a	*neutralized local myotoxic activity. + extended TTD compared to venom alone	[54]
E113 [55]	Naïve-HPDL	scFv	PLA2	*E. carinatus*	SEAR	N	Y*	n/a	Y +	n/a	*neutralized local myotoxic activity. + extended TTD compared to venom alone	[54]
N194	Naïve-HPDL	scFv	PLA_2 (GI)	*N. naja*	SEAR	N	Y*	n/a	Y +	n/a	*neutralized local myotoxic activity. + extended TTD compared to venom alone	[54]

(*Continued*)

Table 1. (Continued).

mAb(s)	Discovery method	mAb format[a]	Toxin Target[b]	Species tested against	WHO Region[c]	*in vitro*[d]	*In vivo*[e] Local p.i.	Local Res.	Lethal p.i.	Lethal Res.	Notes	Ref.
N248	Naïve-HPDL	scFv	PLA_2 (GI)	*N. naja*	SEAR	N	Y*	n/a	Y +	n/a	*neutralized local myotoxic activity. + extended TTD compared to venom alone	[54]
MABaP1–3	murine hybridoma	IgG	SVMP	*B. asper*	AMR	Y	Y*	n/a	n/a	n/a	*neutralized local hemorrhage vs. purified toxin, but not whole venom	[56]
MABaP1–6	murine hybridoma	IgG	SVMP	*B. asper*	AMR	Y	Y*	n/a	n/a	n/a	*neutralized local hemorrhage vs. purified toxin, but not whole venom	[56]
MABaP1–8	murine hybridoma	IgG	SVMP	*B. asper*	AMR	Y	Y*	n/a	n/a	n/a	*neutralized local hemorrhage vs. purified toxin, but not whole venom	[56]
2552_02_B02	Naïve-HPDL with chain shuffling	IgG	Lc-α-NTx	*N. kaouthia*	SEAR	Y	n/a	n/a	Y	n/a	*neutralized toxin function in patch clamp experiments	[55]
DRFS2, DRFL6, DRFL8, DRFS1, DRFS3, DFRS6, DRFS9, DRFS13, DRFL1, DRFL3	immunised ChPDL	scFv	PLA_2 (GII)	*D. russelii formosensis*	SEAR	N	n/a	n/a	Y*	n/a	*partially neutralized lethality when tested as a cocktail of all 10 scFvs.	[57]
S1	immunized ChPDL	scFv	CTX	*N. atra*	WPR	Y*	n/a	n/a	n/a	n/a	*neutralized venom induced cell cytotoxicity	[58]
P4G6a	murine hybridoma	IgG	undefined 3FTX	*N. ashei*	AFR	Y*	n/a	n/a	n/a	n/a	*inhibition ELISA	[59]
P6D9a	murine hybridoma	IgG	undefined 3FTX	*N. ashei*	AFR	Y*	n/a	n/a	n/a	n/a	*inhibition ELISA	[59]
P6D9	murine hybridoma	IgG	undefined 3FTX	*N. ashei*	AFR	Y*	n/a	n/a	n/a	n/a	*inhibition ELISA	[59]
H11	Naïve-HYDL	IgG	SVMP	*E. pyramidum leakeyi*	AFR	Y*	n/a	n/a	n/a	n/a	*neutralized prothrombin activation	[60]
G12F3	Naïve-HPDL	scFv	PLA_2 (GI)	*N. oxiana*	EMR	Y			Y*		*provided 'appreciably greater' protection than contol in 0.5 LD_{50} challenge, although data not shown.	[77]
N24	Naïve-CPDL	V_HH	undefined	*N. oxiana*	EMR	N	n/a	n/a	Y*	n/a	*neutralized lethality	[61]
KC329718	Naïve-CPDL	V_HH	PLA_2 (GII)	*B. jararacussu*	AMR	N	n/a	Y*	n/a	n/a	*mice receiving mAb 30 minutes post venom injection displayed significant reduction in tissue damage and inflammatory cell count	[62]
B7	Naïve-HPDL	scFv	undefined	*B. jararacussu, C. d. terrificus*	AMR	Y	n/a	n/a	Y*	n/a	*extended TTD compared to venom alone	[63]
C11	Naïve-HPDL	scFv	undefined	*B. jararacussu, C. d. terrificus*	AMR	Y	n/a	n/a	Y*	n/a	*extended TTD compared to venom alone	[63]
E9	Naïve-HPDL	scFv	undefined	*B. jararacussu, C. d. terrificus*	AMR	Y	n/a	n/a	Y*	n/a	*extended TTD compared to venom alone	[63]
24a6	HCAb murine hybridoma	IgG	SVSP	*C. rhodostoma, E. ocellatus*	SEAR, AFR	n/a	n/a	n/a	n/a	n/a	developed to demonstrate high-through put mAb discovery	[64]

(*Continued*)

Table 1. (Continued).

							In vivo[e]					
							Local		Lethal			
mAb(s)	Discovery method	mAb format[a]	Toxin Target[b]	Species tested against	WHO Region[c]	*in vitro*[d]	p.i.	Res.	p.i.	Res.	Notes	Ref.
49d8	HCAb murine hybridoma	IgG	CTL	*C. rhodostoma, E. ocellatus*	SEAR, AFR	n/a	n/a	n/a	n/a	n/a	developed to demonstrate high-through put mAb discovery	[64]
50b8	HCAb murine hybridoma	IgG	CTL	*C. rhodostoma, E. ocellatus*	SEAR, AFR	n/a	n/a	n/a	n/a	n/a	developed to demonstrate high-through put mAb discovery	[64]
57h6	HCAb murine hybridoma	IgG	PLA_2 (GII)	*C. rhodostoma, E. ocellatus*	SEAR, AFR	n/a	n/a	n/a	n/a	n/a	developed to demonstrate high-through put mAb discovery	[64]
62e9	HCAb murine hybridoma	IgG	SVSP	*C. rhodostoma, E. ocellatus*	SEAR, AFR	n/a	n/a	n/a	n/a	n/a	developed to demonstrate high-through put mAb discovery	[64]
62f10	HCAb murine hybridoma	IgG	PLA_2 (GII)	*C. rhodostoma, E. ocellatus*	SEAR, AFR	n/a	n/a	n/a	n/a	n/a	developed to demonstrate high-through put mAb discovery	[64]
71e2	HCAb murine hybridoma	IgG	PLA_2 (GII)	*C. rhodostoma, E. ocellatus*	SEAR, AFR	n/a	n/a	n/a	n/a	n/a	developed to demonstrate high-through put mAb discovery	[64]
126_01_B08	Naïve-HPDL	scFv	PLA_2 (GII)	*B. asper, D. russelii, E. c. sochureki, E. leucogaster, E. ocellatus*	AMR, SEAR, EMR, AFR	Y*	n/a	n/a	n/a	n/a	* partial neutralization of the cytotoxic effects of venom PLA_2 on cells	[65]
127_02_H06	Naïve-HPDL	scFv	PLA_2 (GII)	*B. asper, D. russelii, E. c. sochureki, E. leucogaster, E. ocellatus*	AMR, SEAR, EMR, AFR	Y*	n/a	n/a	n/a	n/a	* partial neutralization of the cytotoxic effects of venom PLA_2 on cells	[65]
125_01_D09	Naïve-HPDL	scFv	PLA_2 (GII)	*E. c. sochureki, E. leucogaster, E. ocellatus*	EMR, AFR	Y*	n/a	n/a	n/a	n/a	* partial neutralization of the cytotoxic effects of venom PLA_2 on cells	[65]
127_02_A02	Naïve-HPDL	scFv	PLA_2 (GII)	*B. asper, E. leucogaster, E. ocellatus*	AMR, AFR	Y*	n/a	n/a	n/a	n/a	* partial neutralization of the cytotoxic effects of venom PLA_2 on cells	[65]
TPL0039_05_E02	Naïve-HPDL	IgG	PLA_2 (GII)	*B. asper*	AMR	Y	Y	Y*	n/a	n/a	*inhibited creatine kinase increase when injected i.m. alongside toxin	[66]
TPL0039_05_B12	Naïve-HPDL	IgG	PLA_2 (GII)	*B. asper*	AMR	Y	Y*	N*	n/a	n/a	*IgG (with LALA + YTE Fc mutation) is protective when adminstered intramuscularly alongside toxin or venom, but enhances toxin activity in rescue models.	[66]
TPL0039_05_F04	Naïve-HPDL	IgG	PLA_2 (GII)	*B. asper*	AMR	Y*	n/a	n/a	n/a	n/a	*neutralizes myotoxin II induced cell cytotoxicity	[66]
TPL0039_05_G08	Naïve-HPDL	IgG	PLA_2 (GII)	*B. asper*	AMR	Y	N*	n/a	n/a	n/a	*failed to inhibit creatine kinase increase when injected i.m. alongside toxin	[66]
TPL0039_05_B04	Naïve-HPDL	IgG	PLA_2 (GII)	*B. asper*	AMR	Y*	n/a	n/a	n/a	n/a	*neutralized myotoxin II induced cell cytotoxicity	[66]
TPL0039_05_A03	Naïve-HPDL	IgG	PLA_2 (GII)	*B. asper*	AMR	Y*	n/a	n/a	n/a	n/a	*neutralized myotoxin II induced cell cytotoxicity	[66]
5	Naïve-CPDL	V_HH	Lc-α-NTx	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*demonstrated specificity for α-cobratoxin	[67]
10	Naïve-CPDL	V_HH	Lc-α-NTx	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*demonstrated specificity for α-cobratoxin	[67]
12	Naïve-CPDL	V_HH	Lc-α-NTx	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*demonstrated specificity for α-cobratoxin	[67]

(*Continued*)

Table 1. (Continued).

mAb(s)	Discovery method	mAb format[a]	Toxin Target[b]	Species tested against	WHO Region[c]	*in vitro*[d]	*In vivo*[e] Local p.i.	Local Res.	Lethal p.i.	Lethal Res.	Notes	Ref.
MAJar1	murine hybridoma	IgG	SVMP	*B. jararaca*	AMR	Y	Y*	n/a	n/a	n/a	*partial inhibition of local hemorrhagic activity	[68]
MAJar2	murine hybridoma	IgG	SVMP	*B. jararaca*	AMR	Y	N*	n/a	n/a	n/a	*No inhibition of local hemorrhagic activity	[68]
MAJar3	murine hybridoma	IgG	SVMP	*B. jararaca*	AMR	Y	Y*	n/a	n/a	n/a	*neutralized local hemorrhagic activity	[68]
MAJar4	murine hybridoma	IgG	SVMP	*B. jararaca*	AMR	Y	Y*	n/a	n/a	n/a	*partial inhibition of local hemorrhagic activity	[68]
MAJar5	murine hybridoma	IgG	SVMP	*B. jararaca*	AMR	Y	N*	n/a	n/a	n/a	*No inhibition of local hemorrhagic activity	[68]
MAJar6	murine hybridoma	IgG	SVMP	*B. jararaca*	AMR	Y	Y*	n/a	n/a	n/a	*partial inhibition of local hemorrhagic activity	[68]
MAJar7	murine hybridoma	IgG	SVMP	*B. jararaca*	AMR	Y	N*	n/a	n/a	n/a	*No inhibition of local hemorrhagic activity	[68]
TPL0197_01_C08	Naïve-HPDL	IgG	Lc-α-NTx	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*demonstration of pH dependent binding of antigens for improved cellular antibody recycling	[69]
C2	Naïve-CPDL	V_HH	Lc-α-NTx	*N. kaouthia*	SEAR	Y	n/a	n/a	Y	Y*	*neutralized lethality in rescue model of envenoming	[70]
C43	Naïve-CPDL	V_HH	Lc-α-NTx	*N. kaouthia*	SEAR	Y	n/a	n/a	Y*	n/a	*partial neutralization of lethality	[70]
C19	Naïve-CPDL	V_HH	Lc-α-NTx	*N. kaouthia*	SEAR	Y	n/a	n/a	Y*	n/a	*partial neutralization of lethality	[70]
C20	Naïve-CPDL	V_HH	Lc-α-NTx	*N. kaouthia*	SEAR	Y	n/a	n/a	Y*	n/a	*neutralized lethality	[70]
VHH-P3-1	Naïve-H_CPDL	V_HH	PLA_2 (GI)	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*partial neutralization of enzymatic activity	[71]
VHH-P3-3	Naïve-H_CPDL	V_HH	PLA_2 (GI)	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*partial neutralization of enzymatic activity	[71]
VH-P3-7	Naïve-H_CPDL	V_HH	PLA_2 (GI)	*N. kaouthia*	SEAR	Y*	n/a	n/a	n/a	n/a	*partial neutralization of enzymatic activity	[71]
aNAH1	Naïve-CPDL	V_HH	undefined	*N. atra*	WPR	Y*	n/a	n/a	Y +	n/a	*competitive binding ELISA vs. whole venom. +partial neutralization of lethality	[72]
aNAH6	Naïve-CPDL	V_HH	undefined	*N. atra*	WPR	Y*	n/a	n/a	Y +	n/a	*competitive binding ELISA vs. whole venom. +partial neutralization of lethality	[72]
aNAH7	Naïve-CPDL	V_HH	undefined	*N. atra*	WPR	Y*	n/a	n/a	Y +	n/a	*competitive binding ELISA vs. whole venom. +partial neutralization of lethality	[72]
aNAH9	Naïve-CPDL	V_HH	undefined	*N. atra*	WPR	Y*	n/a	n/a	Y +	n/a	*competitive binding ELISA vs. whole venom. +partial neutralization of lethality	[72]

[a]Only mAbs which were the primary focus of each paper are listed. Individual papers may have described many more mAbs, for example as initial results from discovery screens, which were not further characterized or deselected. [b]HYDL = human yeast display library. HPDL = human phage display library. CPDL = camelid phage display library. H_CPDL = humanised camelid phage display library. ChPDL = Chicken phage display library. HCAb = human heavy chain only antibody. [c]mAb format: the most characterized mAb format used in each publication is listed. Within relevant publications other formats of each mAb may have been investigated. [d]Toxin target abbreviations: Lc-α-NTx = long chain alpha neurotoxin, Sc-α-NTx = short chain alpha neurotoxin, CTX = cytotoxin, 3FTX = three finger toxin, CTL = c-type lectin, SVSP = snake venom serine protease, SVMP = snake venom metalloproteinase, PLA_2 (GI) = Group I phospholipase A_2, PLA_2 (GII) = Group II phospholipase A_2, undefined = the toxin target was not specified in the paper. [e]WHO geographical regions in which the snakes whose venoms were tested are naturally found. AMR = Americas, AFR = Africa, SEAR = Southeast Asia, EMR = Eastern Mediterranean, WPR = Western Pacific Region. [f]Denotes if any *in vitro* venom or toxin specific experiment was performed using the described mAbs. [g]Local *in vivo* is defined as any experiment where the venom challenge is to cause local effects and not lead to lethality. [h]Lethal *in vivo* is defined as any experiment where the venom challenge is intended to cause death. P.I. = preincubation of mAb and venom. Res. = mAb is administered after venom administration. Y = yes, *N*= no, n/a = experiment not performed. ^this table is not intended to be exhaustive, and we apologize to colleagues in advance in the instance we may have missed contributions.

antibodies against poorly immunogenic antigens. Although *in vitro* antibody display methods often result in antibodies with lower affinity than immunization approaches, due to the lack of affinity maturation during the initial discovery phase, this can be overcome by *in vitro* affinity maturation techniques, as reviewed in Li et al [81].

2.2. Phage display

The first antibody display library using bacteriophages ('phages') was described by McCafferty et al in 1990 [78], where the authors fused scFvs to the M13 phage pIII coat protein 'G3P' and isolated scFv binders to the antigen of interest. Antibody phage display typically uses the M13 bacteriophage, a non-lytic filamentous phage that specifically infects *Escherichia coli* with F-pili [82]. Gene sequences encoding antibody fragments or single-domain antibodies (larger immunoglobulins are more challenging due to limitations of prokaryotic protein expression systems [83]) are fused to the G3P phage surface coat protein, which recognizes the bacterial F pilus to facilitate infection of bacteria [84]. For monovalent display, the antibody sequences and G3P protein are contained on a minimal phagemid which only contains the genetic information to produce antibody-G3P proteins. For replication an M13 helper phage is co-infected into bacteria alongside the minimal phagemid to provide the essential genes for phage assembly (Figure 2, left panel [85]). The antigen, immobilized or in solution, is incubated with the phage library, washed to remove nonspecific binders, and finally bound phages are eluted (Figure 2, left panel [85]). The eluted phages are then infected into *E. coli* along with helper phage for propagation of the antigen-recognizing phages, and subsequent rounds of biopanning are performed until the library is sufficiently enriched for high affinity antibodies [85].

Advantages of phage display libraries are that phage libraries can contain up to 1×10^{11} unique antibodies, which is 100-fold greater than most yeast display libraries [80], biopanning is typically performed using standard lab equipment, and the method is relatively simple and low-cost [85]. Additionally, the use of an amber codon between the antibody fragment and G3P in the minimal phagemid enables facile progression from screening phages to evaluating soluble antibody fragments [86]. When phages are expressed in amber suppressor strains of *E. coli* the amber codon is translated into a glutamine, enabling production of the G3P-antibody fusion, however when the same sequence is expressed in non-amber suppressor strains the amber codon is read as a stop signal and produces soluble antibody fragment without G3P fusion [87]. This can greatly speed up the isolation of antibody fragments for further evaluation, compared to cloning the fragments into other expression plasmids. Potential disadvantages of phage display predominantly stem from the use of a prokaryotic system to express immunoglobulins – e.g. potential selection bias of antibodies due to difficulty of bacteria

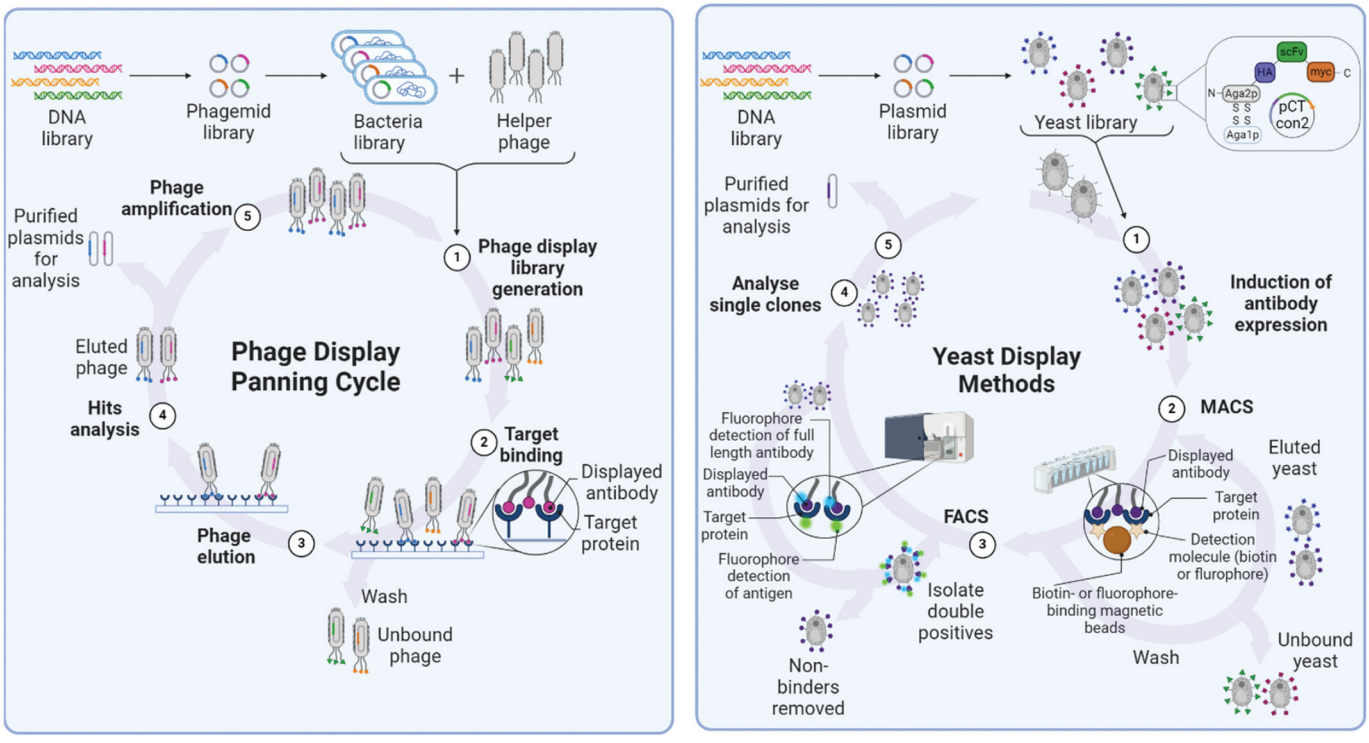


Figure 2. An overview of phage and yeast display methods. (left) Phage display biopanning cycle. A minimal phagemid library containing antibody-encoding sequences is transformed into suitable bacteria along with helper phage. Phages are assembled in the bacteria and isolated for subsequent biopanning. Isolated phages are incubated with the target antigen, either in solution or immobilized, washed to remove unbound phage, and bound phages are then collected by elution away from the target antigen. The eluted phages can then be re-infected into bacteria for another panning cycle, or the phagemids may be purified for sequence analysis and downstream cloning and protein expression. (right) Yeast display screening strategy. A plasmid library containing antibody-encoding sequences genetically fused to a yeast surface protein is transformed into suitable yeast. Expression of the antibodies on the cell surface is induced and yeast may then be incubated with off-target antigens to remove non-specific binders. Remaining yeast are then incubated with the target antigen bound to magnetic beads, and bound yeast are then collected by magnetic separation (MACS). The collected yeast can then be re-grown for another selection cycle, or proceed to FACS to isolate high-affinity binders. After sufficient enrichment the antibody-encoding plasmids are isolated for downstream cloning and protein expression. Created in BioRender. Casewell, N. (2025) https://BioRender.com/c29z341.

in folding eukaryotic antibody fragments [79] and production of misfolded proteins [88].

2.3. Yeast surface display

Yeast surface display (YSD) of antibodies was first described by Boder & Wittrup in 1997, in which the authors displayed a single scFv tethered to an agglutinin subunit (Aga2p) on the cell wall of *Saccharomyces cerevisiae* [79]. The authors then performed affinity maturation of the scFv using a high-mutant strain of yeast that randomly mutated the scFv, followed by kinetic selections using fluorescence-activated cell sorting (FACS) to identify scFv with higher affinity toward the initial target [79]. Subsequently, a wide range of antibody formats ranging in size from single-domain antibodies to full IgG have been utilized in YSD, and methods utilizing other anchoring proteins and species of yeast have been developed [89]. We will briefly describe the methodology associated with YSD as described by Kang, Lax and Wittrup using galactose-inducible display libraries [89].

Yeast harboring a combinatorial DNA library (Figure 2, right panel) are first grown in glucose media for propagation and the surface expression of antibodies is induced by changing the media to a galactose-rich media (Figure 2, right panel). Magnetic activated cell sorting (MACS) may first be used to deplete the library to a suitable size (less than 10^7) for FACS. Induced, antibody-expressing yeast are first incubated with magnetic beads (biotin-binder beads or fluorophore-binding beads, as appropriate for the labeling of the target protein) and at this stage can concurrently be incubated with off-target antigens. After incubation, the beads are placed on a magnet to isolate off-target binders or those that bind nonspecifically to beads, while the unbound fraction containing negatively selected yeast is collected and incubated with the magnetic beads that have been coated with protein of interest (labeled with biotin or fluorophore). After incubation, magnetic separation is used to separate the beads with bound yeast, while unbound yeast are washed away. The bound yeast are then cultured as previously, and subsequent rounds of MACS may be performed until the library size is depleted sufficiently for FACS. Multiple rounds of FACS are performed with decreasing amounts of the target protein (fluorescently labeled), alongside fluorescent labeling of an epitope tag on the displayed antibody as a measure of antibody expression. After the final rounds of FACS plasmid DNA is isolated from the yeast cells and sequenced to identify unique clones for downstream evaluation.

An advantage of using YSD for mAb discovery, as opposed to phage and bacteria, is that protein expression in yeast uses eukaryotic molecular machinery that is more similar to mammalian cells in terms of post-translational modifications, producing proteins that are glycosylated [88] and correctly folded [90]. The incorporation of FACS into the YSD workflow allows for quantitative screening to isolate high-affinity and stable binders, by concurrently measuring both target protein binding and antibody expression levels [91], as opposed to the non-discriminate antibody enrichment methods employed in phage display [80].

YSD can be used to display a wide range of antibody formats from small single-domain antibodies to whole IgG [88]. Similar to phage display, amber suppression can be used in YSD to switch between antibody display on the cell wall and secretion of soluble antibodies, which removes the requirement for subcloning for downstream antibody analysis [92]. Finally, YSD is a highly effective method for affinity maturation to improve the binding properties of the antibodies, and using homologous recombination, mutant libraries can be efficiently created [93]. Antibody gene sequences can be mutated using error-prone PCR [93], DNA shuffling [94] or error-prone DNA replication in yeast [95].

Although the display of multiple copies of antibody on the cell wall is advantageous to normalize antigen binding, this can also present a disadvantage, particularly when screening for target proteins that are oligomers, as multivalent high-avidity but low affinity binders could be selected [88]. As discussed previously, the diversity of yeast-display libraries are often orders of magnitude lower than that of phage libraries [88]; however, the functional expression of these libraries at the protein level may be greater in yeast [89].

2.4. Considerations in the use of in vitro antibody-display libraries for development of recombinant snake antivenoms

In recent years, several groups have reported the use of phage- and yeast-display to discover novel mAbs against snake venom toxins [40,42,44,60,65,70,96,97], using both immune and nonimmune libraries and different antibody formats (Table 1). Two different approaches for the discovery of broadly neutralizing antivenom mAbs have been successfully employed; use of consensus toxins [40,98] and use of cross-panning (alternating toxins between rounds of phage display biopanning [42,43,65]). Beyond venom variation, other important considerations for applying these techniques include the degree of labeling, as some venom toxins are relatively small molecular weight proteins (as described in [99]) and the potential for some toxins to have cytotoxic activity toward the cells displaying antibodies.

3. Highly characterized anti-long-chain α-neurotoxin mAbs

A substantial portion of recent recombinant antivenom research has focused on venom neurotoxins, and particularly the neutralization of a single sub-class of neurotoxin from the three-finger toxin (3FTx) family, namely, the long-chain α-neurotoxins (Lc-α-NTxs) [40–42,61,100,101]. Reasons for the focus on Lc-α-NTxs mAbs in recent years are primarily due to their amenability to purifcation and production, as well as their associated clinical importance in many elapid venoms [9]. In particular, Lc-α-NTxs have proven to be relatively straightforward to express recombinantly [40], enabling sufficient quantities of toxins with appropriate affinity tags necessary for use in the antibody library screening to be obtained easily. This, coupled with their simple mechanism of action (detailed below) enabling the development of several *in vitro* functional assays, has

allowed for straightforward screening and demonstration of preclinical activity of anti-Lc-α-NTxs mAbs *in vitro* and *in vivo*.

Lc-α-NTxs belong to a class of 3FTxs which are grouped together due to their shared target of the muscle-type nicotinic acetylcholine receptor (nAChR) located on the post-synaptic membrane of the neuromuscular junction, named the snake venom α-neurotoxins (α-NTxs) [102]. nAChRs are pentameric receptors consisting of five α-subunits (homomeric) or a mixture of α and non-α subunits (heteromeric) activated by the binding of the neurotransmitter acetylcholine (ACh) which is released from the pre-synaptic motor neuron terminal [103]. Muscle-type nAChRs are heteromeric, consist of a specific set of subunits in a specific arrangement and are located exclusively on the post-synaptic membrane of the neuromuscular junction [104]. Lc-α-NTxs bind to the ACh-binding site (orthosteric site) located on the extracellular N-terminal region of the muscle-type nAChR and prevent the binding of ACh, therefore preventing activation of the nAChR [105]. The orthosteric site consists of six loops with A, B, and C contributed from a principal subunit (α subunit) and D, E, and F from a complementary subunit (non-α subunit or α in homomeric receptors) [106,107]. Inhibition of the muscle-type nAChR commonly results in weakness of the facial muscles but in more severe cases paralysis of the skeletal and respiratory muscles resulting in a serious medical emergency [108]. Lc-α-NTxs differ from other classes of α-NTxs in their structure, number of disulfide bonds and amino acids [102]. These differences translate into different nAChR binding properties compared to other α-NTxs as they generally have a high affinity and longer dissociation times [109,110].

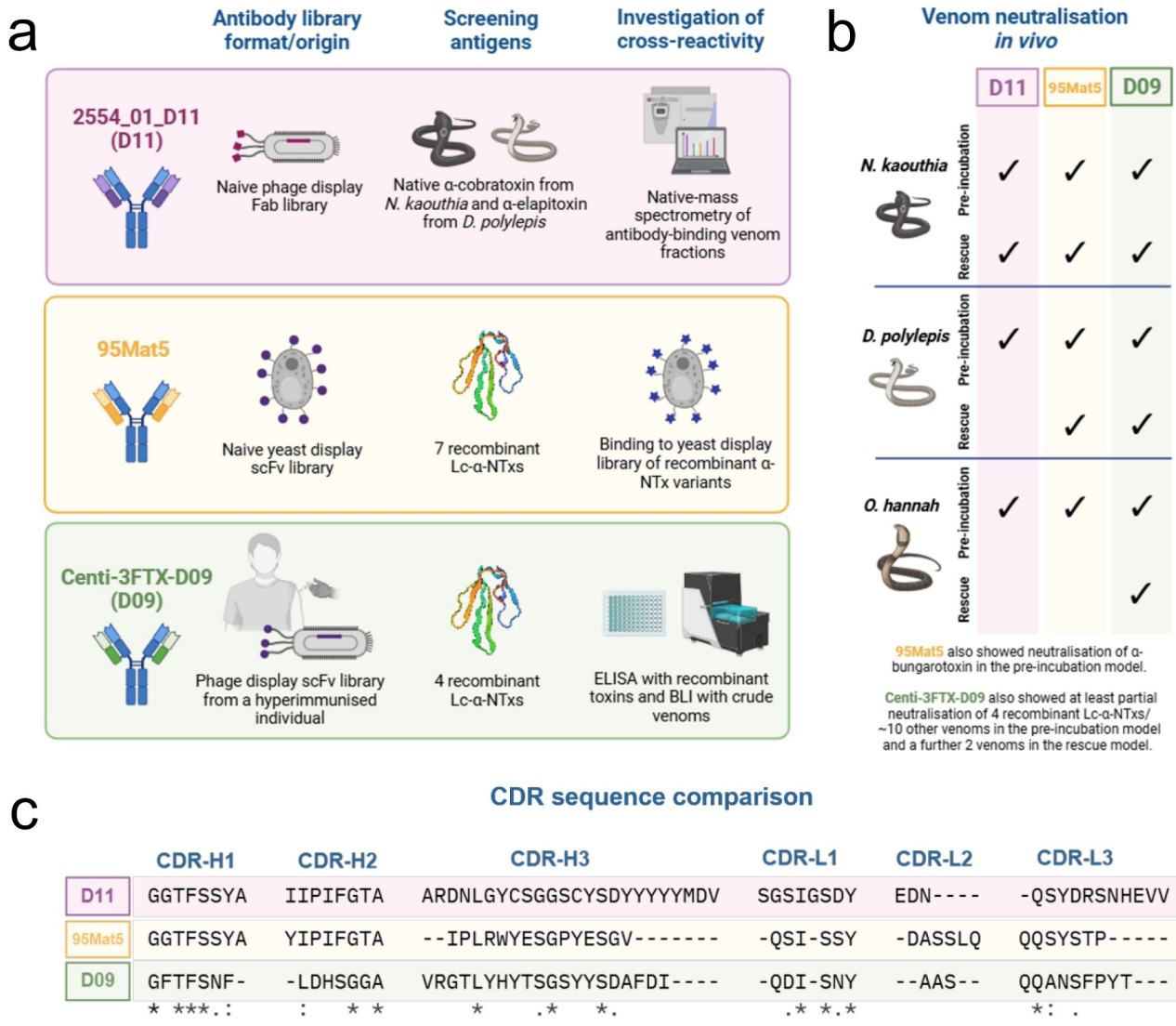
Many medically important species of elapid snakes across different continents produce venom which contain Lc-α-NTxs. Namely members of the cobra (*Naja* spp.), king cobra (*Ophiophagus* spp.), mamba (*Dendroaspis* spp.), krait (*Bungarus* spp.), and coral snake (*Micrurus* spp.) genera in addition to Australian elapids such as the Taipan (*Oxyuranus* spp.) [9,111]. Each species produces a specific isoform of Lc-α-NTx and their sequence and structure are well-conserved across species [112]. Neurological effects consistent with the inhibition of muscle-type nAChRs are common in clinical reports of envenomation by many of these species [108] and whole venom has been demonstrated to inhibit nAChRs in *in vitro* [113] and *ex vivo* studies [114], while Lc-α-NTxs isolated from these venoms or expressed recombinantly have also shown inhibition of nAChR activity [40,42,113,115]. Lc-α-NTxs have also been shown to be important drivers of neurotoxic effects *in vivo* in the venoms of elapids from different continents such as *Dendroaspis polylepis* [116] and *Naja melanoleuca* from sub-Saharan Africa [117], and *Naja kaouthia* [118] and *Naja naja* from Asia [119].

The presence of Lc-α-NTxs in the venom of many medically important elapid species and their importance in the development of neurotoxicity post envenomation, as well as the long-recognized low potency of many antivenoms against neurotoxins [120], establishes them as a key target for both existing antivenoms and novel therapeutics. There have been several approaches to produce neutralizing synthetic antibodies that show effective neutralization of Lc-α-NTx-containing venoms, one such is the discovery of single-domain antibodies produced via antibody library screening against toxins from the venom of a single species [61,100,101]. Candidates that have progressed the furthest by demonstration of neutralization of multiple venoms *in vivo* have been created in the full IgG format, each using different sources of antibody libraries, methods of screening, and evaluation techniques (Figure 3(a)) [40–42].

The first broadly neutralizing anti-Lc-α-NTx mAb was developed through a sequential panning approach. Initial screening of a naïve phage display library of scFvs produced a mAb, 368_01_C05, capable of prolonging the survival of mice dosed with α-cobratoxin isolated from the venom of *N. kaouthia* [55]. This mAb served as a scaffold to engineer mAbs with enhanced Lc-α-NTx affinity and broader species recognition, a process known as 'affinity maturation.' To achieve this, phage-display libraries were created where the heavy chain of the mAb was fixed, and the light chain was mutated or 'shuffled.' This approach led to the development of the mAb '2554_01_D11' [42]. In addition to α-cobratoxin, α-elapitoxin isolated from the venom of *D. polylepis* was also used in different rounds of the antibody screening process and whole venoms (*D. polylepis*, *N. kaouthia*, *N. melanoleuca*, *N. naja,* and *Ophiophagus hannah*) were used to examine cross-reactivity of 2554_01_D11 by examining the binding to elapid venom fractions followed by mass spectrometry analysis of antibody-bound fractions. *In vitro* neutralization of either isolated Lc-α-NTxs or a venom fraction containing Lc-α-NTxs was confirmed before demonstration of prolonged survival in a murine preclinical model where 2× LD_{50} of either *N. kaouthia*, *O. hannah,* or *D. polylepis* venom or 2× LD_{50} of α-cobratoxin was preincubated with mAb for 30 minutes at 37°C ('preincubation model' as outlined by the WHO guidelines for preclinical assessment of antivenom efficacy [121]). Delayed administration of mAb ('rescue model' [122]) ten minutes post administration of 2× LD_{50} *N. kaouthia* venom also resulted in prolonged survival [42]. It should be noted that mAb doses were formulated as molar ratios based on the calculated α-NTx content of each venom rather than mass ratios to whole venom.

A similar approach involving the screening of a synthetic yeast display library of Fabs resulted in the production of mAb '95Mat5' [40]. Recombinant Lc-α-NTxs were created, as opposed to screening against isolated native toxins as used by Ledsgaard et al [42], and seven recombinant toxins were used in the library screening process. A candidate antibody was then affinity matured using a mutagenesis approach [123] as opposed to chain shuffling used by Ledsgaard et al. [42], resulting in 95Mat5. A comprehensive investigation of 95Mat5 cross-reactivity was carried out by creation and screening of a yeast display library of 828 3FTx variants followed by demonstration of neutralization *in vitro* against recombinant Lc-α-NTxs as well as α-bungarotoxin isolated from the venom of *Bungarus multicinctus*. Prolonged survival was demonstrated *in vivo* in the murine preincubation model against 2× LD_{50} α-bungarotoxin, *N. kaouthia* venom, *D. polylepis* venom and *O. hannah* venom. The rescue model showed full protection for 24 hours against 2× LD_{50} *N. kaouthia* and *D. polylepis* venoms with a 20 minute time delay [40].

Finally, an approach involving the generation of antibody libraries from the immune cells of an individual human subject



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CDR sequence comparison

	CDR-H1	CDR-H2	CDR-H3	CDR-L1	CDR-L2	CDR-L3
D11	GGTFSSYA	IIPIFGTA	ARDNLGYCSGGSCYSDYYYYYMDV	SGSIGSDY	EDN----	-QSYDRSNHEVV
95Mat5	GGTFSSYA	YIPIFGTA	--IPLRWYESGPYESGV-------	-QSI-SSY	-DASSLQ	QQSYSTP-----
D09	GFTFSNF-	-LDHSGGA	VRGTLYHYTSGSYYSDAFDI----	-QDI-SNY	--AAS--	QQANSFPYT---
	* ***.:	: * *	* .* *.	.* *.*		*: .

Figure 3. Comparative summaries of anti-Lc-α-NTx mAbs: 2554_01_D11 (D11) [42], 95Mat5 [40] and Centi-3FTX-D09 (D09) [41]. (a) Overview of the antibody library screening process, source of antigens used to screen libraries and method of screening for mAb cross-reactivity. (b) Breadth of *in vivo* neutralization of anti-lc-α-NTx mAbs. Ticks represent whether neutralization was observed *in vivo* by observation of prolonged survival only and do not represent the degree of neutralization or survival. Subcutaneous administration of venom for D11 and 95Mat5, intraperitoneal for D09. (c) Clustal Omega [49] amino acid alignment of CDR of D11, 95mat5 and D09. "*" indicates positions with fully conserved residues, ":" indicates conservation between residues with similar properties."." indicates conservation between residues with weakly similar properties. D11 CDR H1/H2/H3 sequences obtained from [55]. Created in BioRender. Casewell, N. (2025) https://BioRender.com/m62g435.

hyperimmunized against multiple species of venomous snakes, including species from the elapid family, resulted in the production of the mAb 'Centi-3FTX-D09' [41]. Libraries were screened using four recombinant Lc-α-NTxs identified in elapid species from different genera (*Naja nivea, Bungarus caeruleus*, *Oxyuranus scutellatus* and *D. polylepis*) and screened for cross-reactivity using ELISA with the four recombinant toxins and biolayer interferometry with 20 elapid venoms. Following this, Centi-3FTX-D09 was extensively assessed *in vivo* with varying degrees of protection observed against approximately 19 elapid venoms at a 1× LD_{90} dose in the murine preincubation model and full protection against five elapid venoms (*D. polylepis*, *O. hannah*, *N. kaouthia*, *N. nivea,* and *Naja haje*) in a rescue model with a ten minute time delay (all venoms administered via intraperitoneal route, Centi-3FTX-D09 via intravenous route) [41].

Comparison of the amino acid sequences of complementarity-determining regions (CDRs) of 2554_01_D11, 95mat5 and Centi-3FTX-D09 reveals striking similarity and apparent convergence of several residues between the three independently discovered antibodies (Figure 3). In particular, 50% of the residues in CDR H1 region of all three mAbs are identical, while 38% of the residues of CD1 L1 are conserved. When comparing 95Mat5 to Centi-3FTX-D09 alone, conservation of CDR L1 between the two mAbs increases to 67%. Indeed, 95Mat5 and Centi-3FTX-D09 share broad conservation across all CDRs, with 23/56 matching amino acid residues. Of particular note, all three antibodies possess long CDR H3 regions (15 to 24 amino acids). Furthermore, all the other cross-reactive mAbs detected during the discovery process of 95Mat5 similarly had long CDR H3 regions, all of which contained a [W/Y]YxxGxY motif [40], which is similarly present in

the CDR H3 of Centi-3FTX-D09, although not 2554_01_D11. Khalek et al. speculate that the overall similarities demonstrated between mAbs generated through independent and diverse methods are suggestive of a limited number of approaches available to obtain broad recognition of Lc-α-NTxs [40].

The convergence of CDR sequences is also reflected in the crystal structures of 95Mat5 and Centi-3FTX-D09 with Lc-α-NTxs, which revealed similarities between the antibody-toxin and nAChR-toxin interactions [40,41]. Examination of the Fab of 95Mat5 in complex with a recombinant Lc-α-NTx from *O. hannah* and comparison with the structure of α-bungarotoxin-nAChR complex [105] revealed key Tyr residues on the heavy and light chains of 95Mat5 that interact with Arg and Phe residues on the toxin that mimic the interaction with analogous nAChR Tyr residues and α-bungarotoxin Asp/Phe residues. These observations, along with the observation that CDR H3 of 95Mat5 approximates loop C of the nAChR orthosteric binding site, provides a rationale for the broad cross-reactivity of the mAb. Crystal structures of the Centi-3FTX-D09 Fab with the four recombinant Lc-α-NTxs used for library screening revealed a similar explanation for its cross reactivity where Tyr and Asp residues from the heavy chain in similar positions to the ones identified in 95Mat5 interact with similar Arg and Phe residues on the toxin [41] (Figure 3).

4. Recombinant mAbs for other snake venom toxin families

While the most thoroughly described mAbs have been focused against Lc-α-NTxs, it remains that snake venoms are composed of multiple different proteinaceous toxin families with distinct modes of action and widely varying pharmacological effects [2,9]. In this section, we summarize and discuss the substantial progress on antibody discovery against various other snake venom toxin families (Table 1).

4.1. Short-chain three finger toxins (Sc-α-NTxs)

Sc-α-NTxs are a vital neurotoxic component of many elapid venoms and therefore mAbs capable of neutralizing their pathology will be a crucial component of any recombinant antivenom. Despite this, Sc-α-NTxs have been largely neglected in antibody discovery campaigns so far. At the time of writing, there is just a single characterized mAb (TPL0629_01_D11), which has been demonstrated to neutralize Sc-α-NTx lethality *in vivo* in preincubation and rescue experiments [49]. Notably, the shared structural and sequence similarities between Sc-α-NTxs and Lc-α-NTxs [102] as well as their shared target, which characterized anti-Lc-α-NTxs mAbs appear to mimic [40,41], suggests that mAbs capable of cross-neutralizing both Sc-α-NTxs and Lc-α-NTxs may be achievable, although no such antibodies have been discovered to date.

4.2. Cytotoxic three finger toxins (CTX)

CTX are another key component of many African elapid venoms [124], responsible for extensive dermonecrosis [2]. Recombinant mAbs toward these toxins have been less investigated than their Lc-α-NTxs counterparts, restricted to a handful of described recombinant mAbs which were examined *in vitro* [43,58,59]. The most notable study was that of Ahmadi et al., who described scFvs which could neutralize CTX from three species of African cobra *in vitro* [43]. The focus of *in vitro* investigation in these studies is primarily due to CTX not being responsible for lethal envenoming pathology in humans. However, *in vivo* investigation of neutralizing local envenoming can be attempted through local envenoming assays, such as minimum necrotic dose assays [121], and will ultimately be required for the translation of these mAbs and their inclusion in recombinant antivenoms.

4.3. Phospholipase A_2 (PLA_2)

Snake venom PLA_2s have extremely broad pharmacological functions, with notable structural differences between group I (elapid) PLA_2 and group II (viperid) PLA_2 [125]. Recombinant mAb discovery for group II PLA_2s has been well established, particularly for central and south American pit vipers, with 19 mAbs described of varying potency and cross-species neutralizing efficacy discovered for both viperid enzymatic and non-enzymatic PLA_2s [48,50,62,63,65,66,126,127] (Table 1). In the Americas, group II PLA_2 are largely responsible for extensive myonecrosis and tissue destruction, a condition for which conventional antivenoms shows limited capability of halting progression [16]. Recently, Prado et al [62] described a V_HH mAb, KC329718, which showed promising potential in neutralizing or inhibiting a range of local envenoming effects, including myotoxicity, cell damage, and significant reductions in inflammatory cell counts, when administered 30 minutes post-intramuscular injection of *Bothrops jararacussu* venom.

While discovery of mAbs against group II PLA_2 has been well established, discovery of antibodies against group I PLA_2s, which include the highly life-threatening pre-synaptic PLA_2 neurotoxins, has been notably less advanced [49,54]. Four mAbs have been discovered for *N. kaouthia* PLA_2s; however, none have been tested *in vivo* [45,71], while mAbs discovered against other *Naja* species PLA_2s have demonstrated disappointing activity *in vivo* [54,77] (Table 1). At the time of writing, only a single anti-group I PLA_2 mAb which can convincingly neutralize venom-induced lethality *in vivo* has been described. mAb TPL0637_01_A07, discovered by Benard-Valle and colleagues, was capable of preventing lethality in a rescue model of mice challenged with a lethal dose of PLA_2 purified from the venom of *Micrurus fulvius*, the eastern coral snake [49]. While a promising start, similarly to Sc-α-NTxs, the medical importance of Group I PLA_2s means that discovery of mAbs against these classes will have to be substantially expanded in the near future.

4.4. Snake Venom Metalloproteinases (SVMPs)

SVMPs are the primary toxin of many viperid venoms [9,128], responsible for coagulopathy, hemorrhage and tissue damage, and are therefore an essential target for neutralization of any recombinant antivenom. Despite this, discovery of synthetic mAbs against SVMPs has generally lagged behind that of smaller toxins. Just a handful of studies have examined anti-

SVMP mAbs (Table 1). V_HH mAbs discovered against hemorrhagic P-III SVMPs from *Bothrops atrox* were able to effectively neutralize local hemorrhagic pathology in mice, but unable to neutralize the lethal effects of whole venom, likely caused by non-SVMP toxin families present in this venom [47]. Similarly, scFvs capable of neutralizing *in vitro* SVMP activity of *B. jararacussu* and *Crotalus durissus terrificus* were unable to prevent lethality in mice, although they prolonged survival time against lethal challenge with each venom [63].

P-III SVMPs in particular are large multi-domain proteins [128], and thus understanding neutralizing-epitope regions of these toxins is particularly important and can help understand structure-function relationships. A recent notable study demonstrated that of 72 mAbs isolated which bound to recombinant P-IIIa SVMP Ecarin, only three were capable of complete neutralization of prothrombin activation [60]. Examination of the three mAbs revealed largely shared sequences suggesting they bound to a common epitope, revealed by cryo-EM to be in the non-catalytic cysteine-rich region. Similarly, hybridoma-generated mAbs against P-IIIb SVMP Jararhagin, from *B. jararaca*, were only capable of functional neutralization if they bound in the C-terminal portion of the non-catalytic disintegrin domain [68]. The results of both these studies highlight the relevance of non-catalytic domains as potential targets for discovery of neutralizing mAbs. In contrast, scFvs capable of neutralizing the fibrinogenolytic activity of elapid *N. kaouthia* P-IIIc SVMP, Kaouthiagin, were demonstrated to bind on or near the catalytic site of the Met domain [52], while a hybridoma-generated mAb capable of neutralizing functional activity of the P-I SVMP BaPI, which consists of solely a catalytic metalloproteinase domain, was previously described [129].

5. Bottlenecks and translational development of recombinant antivenoms

Several strategies have been specifically developed and validated with small molecular weight toxins, such as Lc-α-NTxs, to rapidly and reliably discover anti-venom mAbs with broad utility and high potency, and are now routinely being performed against various toxin classes in laboratories globally [40,42,47,60,101]. However, despite the success with discovery of mAbs against elapid post-synaptic Lc-α-NTxs [40–42] and viperid II PLA_2s [47,65,66,127], specific challenges and bottlenecks persist for the discovery of broadly neutralizing mAbs for multiple snake venom toxin classes. As previously mentioned, there is currently a significant lack of mAbs targeting pre-synaptic PLA_2s which play a major role in a large proportion of snakebite deaths worldwide [108], for which only a single promising mAb has been described [49]. Notably, discovery of broadly neutralizing mAbs for many viperid toxin classes lags substantially behind that of elapid toxin classes (Table 1). For example, mAbs against snake venom serine proteases, a major class of hemotoxic viper venom toxin, are underrepresented [48,64], while another major viperid toxin class, the C-type lectins and C-type lectin-like proteins have just two mAbs described which have not yet been demonstrated to have any functional potential beyond recognition [64]. Reasons for the slower progress in discovery for other toxin classes are diverse. For some toxin classes, medium or high throughput *in vitro* assays for screening antibody functionality remain lacking [130]. For other toxins, such as CTXs and SVMPs, recombinant expression, which is useful to obtain adequate quantities of pure toxins with a range of tags for discovery campaigns, remains challenging, either due to toxic effects exerted on expression hosts or extensive disulfide bonding and poor yields [131]. Furthermore, it is not yet clear if mAbs for common yet minor venom components such as hyaluronidases, L-amino acid oxidases and CRISPs [9], which are *assumed* to have minor or accessory roles in envenoming pathophysiology, yet are regularly neutralized in function by antivenoms, will be required for complete neutralization of envenoming pathology in humans, thus de-prioritizing discovery of mAbs for these classes. It may be that transition from a 'shotgun' undefined conventional antivenom approach toward a highly targeted recombinant antivenom approach may unmask unknown pathologies caused by undefined venom components previously neutralized by conventional antivenoms [132]. The recent development of a target product profile for development and potential formulation of a pan-African recombinant antivenoms [133] will hopefully aid research efforts on filling outstanding gaps in mAb discovery and what specific milestones remain to be accomplished in order to further progress recombinant antivenoms toward their potential initial clinical use.

In vivo models of envenoming remain problematic in terms of aiding antibody discovery and translational progression [66,134]. The standard preincubation model of envenoming lethality continues to be widely used in describing the majority of anti-toxin mAbs to date (Table 1), despite its well-known issues with regard to not being reflective of clinical snakebite scenarios, which may impact on the translational development of antivenom mAbs [66,122,134]. Use of 'real-world scenario' rescue models of envenoming, which can more accurately reflect envenoming, better considering the pharmacokinetics of venom, and the pharmacodynamics of venom and mAbs, are much more preferable for mAb discovery and *in vivo* validation [122] although they remain in the minority when it comes to examining antivenom mAb potential (Table 1). While lethality may be the worst-case scenario of envenoming, it must be remembered that different toxin classes and sub-classes exert different or multiple pathophysiological effects, some of which remain poorly understood. Some toxins may or may not contribute to lethality, or may even exert substantial species-specific (i.e. mouse vs. human) potencies or actions, or may be currently impossible to reflect in murine or other models, but are clinically relevant nonetheless [132,135]. In this context, the evaluation of mAbs ability to neutralize specific pathophysiological traits of envenoming, such as coagulopathy, dermonecrosis, or acute kidney injury, to name but a few, will require the use of existing, or development of, appropriate tailored assays and associated improvements in pathophysiological understanding, to assess mAb functionality [132,134,136].

Arguably the next phase of recombinant antivenom development is the accelerated investigation into oligoclonal mixtures of mAbs capable of broadly neutralizing the various clinically relevant toxin families present within a single snake venom [9]. The majority of studies of recombinant anti-toxin antibodies to date largely focused on neutralization of a single

toxin family. While evident that in specific circumstances a single mAb may be sufficient to neutralize the lethal effects of a specific venom [40–42], it is clear that due to the existence of multiple medically important toxins in the majority of venoms, a single mAb will not be sufficient to neutralize lethality in *in vivo* models, as evidenced in several studies [47,49]. Thus, if recombinant antivenoms are to one day become the mainstay of envenoming therapy, they will need to match or exceed the current expectations of existing polyvalent antivenoms, that is; capable of neutralizing envenoming from multiple different genera, imparting multiple pathologies via distinct toxin families. To achieve this, recombinant antivenoms will have to consist of defined, so-called oligoclonal, mixtures of distinct mAbs [30,31].

The most thorough demonstration specifically aimed at demonstrating oligoclonal cocktails was recently published by Benard-Valle et al. [49]. Here, the authors generated a mixture of two V_HH mAbs, anti-Sc-α-NTx mAb TPL0629_01_D11 and anti PLA_2 mAb TPL0637_01_A07, which had individually been demonstrated to have broad *in vitro* neutralizing potential against their targeted toxin family, and were capable of potently neutralizing the toxins they were originally biopanned against in both preincubation and rescue *in vivo* lethal challenge assays. Despite this, when injected individually in a murine preincubation *in vivo* model, TPL0629_01_D11 and TPL0637_01_A07 were not capable of neutralizing lethality of either *M. fulvius* and *Micrurus diastema* venoms. However, when injected as a cocktail, the mixture was capable of neutralizing lethality in preincubation challenge [49]. This study is the first specific proof-of-principle demonstration of the oligoclonal antibody concept, where actual recombinant antivenoms, sold in regions consisting of diverse medically important snake fauna, would have to be capable of neutralizing pathology from multiple different snake genera and multiple venom toxin families [49]. However, while demonstrating proof of principle that cocktails of mAbs will almost certainly be required for recombinant antivenoms [49], more research is required until confident estimations on how many individual mAbs may ultimately be required for a recombinant antivenom to mimic the neutralizing scope of existing conventional antivenoms.

The concept of needing cocktails of mAbs is not confined to treating snakebite envenoming, with various licensed therapeutics available which consist of two or three individual mAbs. Multiple therapeutics developed for treating SARS-CoV -2 infection at the height of the COVID-19 pandemic consisted of two individual mAbs targeting independent epitopes of the spike protein [137]. Notably, Inmazeb, an antibody cocktail used to treat Ebola, is a trivalent cocktail of the mAbs atoltivimab, maftivimab, and odesivimab, each targeting independent regions of the Ebola virus [138], with each mAb independently manufactured prior to blending into a final product. Encouragingly, the deployment of Imazeb has several features which would be similar in the deployment of a recombinant antivenom (a single dosage application, deployed intravenously in remote settings [139]) and could serve as a sensible foundation for the initial deployment of recombinant antivenoms in similar settings.

Unsurprisingly, manufacturing of multiple mAbs for a single product can substantially increase the costs associated with production [30]. Currently, for cocktail mAb therapeutics, each mAb needs individual preclinical and clinical validation, in addition to blended validation. Thus, a major translational bottleneck is the need to produce cocktails of mAbs antibodies for a recombinant antivenom to have true geographical and species utility, while simultaneously reducing the ultimate cost to end users. Recently, proof-of-concept for the strategy of mixing multiple cell lines in a single batch to manufacture tailored recombinant antivenoms was achieved [140]. Although an initial demonstration, this study provides specific evidence for a strategy which could be utilized to ultimately reduce the manufacturing costs of recombinant antivenoms and other potential therapeutics where oligoclonal antibody mixtures could find utility [140]. While oligoclonal production via traditional mammalian cell culture means is generally the accepted way forward for recombinant antivenom production, alternative methods for manufacturing recombinant antivenoms are actively being considered, noticeably in plants [45,141,142].

6. Barriers and other areas for development

While overcoming some of the technical shortcomings of conventional antivenoms, recombinant antivenoms will similarly be vulnerable to issues of accessibility widely encountered by conventional antivenoms [8]. As highlighted by the global SARS-CoV-2 pandemic and recent Ebola outbreaks, challenges relating to end user cost and accessibility of mAbs persist, with accessibility to such therapies overwhelmingly biased toward North America and Europe [143]. During the SARS-CoV-2 pandemic, REGEN-COV (casirivimab and imdevimab) was priced at $2,100 per dose [144], and while the cost per dose of Inmadev is not publicly available, Regeneron receive $67 million per year for supply of Inmadev through the US Government Biomedical Advanced Research and Development Authority (BARDA), which supplies it to Ebola patients in the Democratic Republic of Congo free of charge [145]. A 2020 manuscript predicted the cost of oligoclonal antivenom manufacturing to be in the region on USD $48–-1354 per treatment [30], with the upper estimate not too dissimilar to the cost of REGEN-COV. Although still unaffordable for the vast majority of snakebite victims, costs could be offset through proposed antivenom stockpiling and supply schemes [8], and ultimately may be more cost-effective when considering predicted improvements in dose and safety.

It is relatively easy to engineer the scFv and V_HH recombinant antibody formats used for display and discovery of broadly neutralizing anti-venom antibodies into other antibody formats or to modify desirable functions aside from neutralization [49,66]. This flexibility may allow individual mAbs within recombinant antivenoms to be engineered in an antibody format which is best-suited to neutralize their target toxin pharmacology. For instance, recombinant antivenoms could consist of a mixture of antibody formats, with smaller formats with superior tissue penetrative characteristics, such as V_HH, targeting cytotoxic-specific toxin components such as CTX within tissues, while mAbs

targeting circulating coagulopathic SVMPs may be formatted into larger formats, such IgG or V_HH-Fc [70] to take advantage of their longer serum half-lives and recycling capabilities.

Effector function is an area which is highly investigated in antibody fields, with mutations substantially affecting pharmacokinetics and retention, thus possibly aiding in the translational development of recombinant antivenoms [70,146,147]. The focus in the recombinant antivenom field the last 10 years or so has been on discovery, but a handful of reports discussing desirable characteristics other than neutralization have recently begun to emerge [66,69,70]. For example, anti-toxin recombinant mAbs with greater ability to be recycled back into the circulation when endocytosed, therefore extending half-life and improving therapeutic effect at lower doses, have been specifically sought through innovative panning strategies [69]. Existing known Fc mutations which can substantially increase IgG serum half-lives of mAbs through reduced binding to Fc receptors, such as LALA [148] and YTE [149] have also been examined in antivenom monoclonals, albeit with surprising results. Using phage display, Sørensen et al [66] discovered a scFv, named B12, which strongly bound to myotoxin II from *Bothrops asper*, which was subsequently reformatted into a human IgG1 format containing both LALA and YTE mutations, named 'B12(LALA + YTE).' During initial examination, B12(LALA + YTE) demonstrated impressive *in vitro* neutralization of myotoxin II activity and was able to completely neutralize myotoxin II toxicity in a preincubation *in vivo* challenge model. Surprisingly, when B12(LALA + YTE) was used in a rescue *in vivo* challenge model, where B12(LALA + YTE) was administered intravenously three minutes after intramuscular myotoxin II challenge, a marked increase in myotoxin II toxicity was observed, measured by a significant increase in plasma creatine kinase levels and evident kidney damage [66]. Notably, administration of B12(LALA + YTE) alone did not cause pathology, and was only observed in the presence of myotoxin II. Rescue models using B12 with only the LALA mutation, or reformatted as a Fab fragment, also did not result in increases in pathology. Thus B12(LALA + YTE) appears to result in an antibody-dependent enhancement of toxicity (ADET) of the PLA_2 myotoxin II from *B. asper*, increasing myotoxcity [66]. Sørensen et al [66] speculated these results indicated that the observed ADET may be related to an increased half-life of the antibody-toxin complex or to possible increased (due to the addition of the YTE mutation) neonatal Fc receptor (FcRn)-mediated uptake of the antibody-toxin complex. As the majority of all the antivenom mAbs discovered to date and tested *in vivo* have only been examined *in vivo* using preincubation assays rather than rescue assays, it is unknown how frequently ADET may occur, and highlights the importance of preclinical rescue experiments in the development of antivenom mAbs [122,134].

While approaches to develop mAbs with the most desirable characteristics will prove to be essential in aiding ultimate translation of a recombinant antivenom to therapeutic use, a major barrier is the continued comprehensive lack of understanding of venom pharmacokinetics and venom and antivenom pharmacodynamics in both mice and humans [66,134,150]. Ultimately, the failure rate of pharmaceuticals, including biologicals, in reaching the clinic remains high [151]. With snakebite envenoming remaining critically underfunded, thorough understanding of pharmacokinetics and pharmacodynamics of envenoming will be beneficial, if not absolutely essential, to enable proficient translation of recombinant antivenom candidates to emerge from preclinical and clinical development and to the patient bedside. Recombinant antivenoms will likely retain some of the clinical issues faced by conventional antivenoms, including being poorly efficacious in treating some of the 'treatment resistant' envenoming syndromes, such as pre-synaptic neurotoxicity and local tissue damage [16,108,152]. It is well recognized that administration of antivenom as quickly as possible following an envenoming substantially increases the chances of better patient outcomes, reducing the extent of venom induced pathophysiology and increasing the rate of patient recovery [135,152]. Despite this, even if snakebite victims do attend hospital promptly, conventional antivenom delivery is often delayed until the onset of overt symptoms due to limited supplies [8,153] and credible concerns of antivenom efficacy, safety and quality [8,13,154]. It is hoped that the assumed improved safety profiles of recombinant antivenoms, for example their humanization [31,44], will enable their use in clinic before the onset of overt symptoms and limitation of irreversible damage, thus also improving patient outcomes through treatment at the earliest possibility [135,152]. However, while many in the envenoming field are optimistic of the potential capability of recombinant antivenoms to substantially improve patient outcomes, ultimately, we will not be able to determine if recombinant antivenoms will overcome the treatment and safety limitations of existing conventional antivenoms until they are tested in clinical trials.

The issue of clinical trials for envenoming therapies, both existing and proposed, remains problematic. Investment outside of academia into demonstrating the clinical efficacy of envenoming therapies, both existing and new, is still extremely limited in comparison to other diseases [7,20,132,134]. It remains that for conventional antivenom products, the onus on demonstration of clinical efficacy is left to academics utilizing public funding to cover the cost, rather than the manufacturers themselves. As recombinant antivenoms will rightly be required to undergo clinical trials, this itself may prove to be a substantial barrier to the development of recombinant antivenom therapies if large pharmaceutical companies cannot be persuaded to invest in recombinant antivenoms. Pre-empting this issue, potentially more affordable and more rapid alternatives to traditional clinical development has been suggested, and in some cases already trialed for conventional antivenoms [5], such as small Phase 2 clinical dose-finding and safety trials or schemes to allow emergency use of unproven clinical interventions outside clinical trials, which may enable the rapid production of reliable clinical data on intervention efficacy and safety in lieu of more traditional clinical trials [132], which have been used for new mAb therapies in infectious disease settings [155].

Other initiatives to improve the attractiveness of recombinant antivenoms to pharmaceutical companies and encourage investment could be considered. For instance, unlike conventional antivenoms, recombinant antivenoms could be tailored to target broader markets, including regions with varying income levels, such as the Americas. This could encourage pharmaceutical companies to invest, as it helps spread financial risk by enabling equitable cost recovery through higher-income markets, crucially making it financially viable to supply to underserved areas. The

veterinary pharmaceutical market may also be a way to accelerate recombinant antivenom development, with frequent and suspected substantial companion and domestic animal snakebite envenoming burdens suspected [156,157], while veterinary mAb therapies continue to dramatically increase in sales [158].

Finally, an area of great excitement in the wider field of biologics is the *in silico* design of de novo protein inhibitors, which have the potential to revolutionize snakebite therapies. Vázquez-Torres et al [159] demonstrated the use of RFdiffusion to design novel protein inhibitors of 3FTxs, which showed impressive neutralization of neurotoxicity in preclinical models. To inhibit α-neurotoxins, proteins were designed that bind the toxin edge β-strands in a mechanism different to mAbs discussed in section 3, above, using a consensus Sc-α-NTx and α-cobratoxin from *N. kaouthia* as templates, and for cytotoxic 3FTxs the protein binders were designed to interact with the three-finger loops of a consensus cytotoxin. The designed proteins, due to their small size (~ 100 amino acids), have better tissue penetration than whole IgG, are thermostable, and are easily produced in bacteria at low-cost. Additionally, manuscripts reporting the *de novo* design of immunoglobulin-like domains [133] and single-domain [25] antibodies may provide alternative approaches for the discovery of anti-venom mAbs and is likely to expand substantially in the coming years in application within envenoming research.

7. Expert opinion

The progress of recombinant antivenoms being predominantly a concept less than a decade ago [31] to now, where demonstration of an experimental recombinant antivenom capable of matching the utility and scope of existing conventional antivenom is expected within a few years, has been remarkable. In particular, the development of broadly neutralizing mAbs against Lc-α-NTxs with impressive preclinical efficacy provides optimism that lessons learnt for this toxin class can be readily applied to the discovery of broadly neutralizing mAbs against the other key pathogenic toxins. Of the anti-Lc-α-NTxs mAbs detailed in Section 3 [40–42] each mAb showed promising neutralizing effects against whole venoms *in vivo* in the gold standard preincubation murine model of envenoming and in the more challenging rescue models, with *in vitro* investigations of cross-reactivity indicating the potential of neutralization of an expanded range of venoms. Furthermore, a mechanism of mAb cross-reactivity has been established that, when expanded upon, could be a pathway to the generation of a mAb that broadly neutralizes all Lc-α-NTxs.

In contrast to the progress in development of recombinant mAbs against Lc-α-NTxs and Group II PLA_2, antibody discovery campaigns have for the most part neglected Sc-α-NTxs, cytotoxic 3FTx, group I PLA_2 and SVMP toxins at present, and this is the logical and critical next-step in order to development recombinant antivenoms capable of neutralizing the breadth of toxins within a venom.

With practical stages of mAb discovery for recombinant antivenoms now much better understood and being more widely applied, it is now the time for more thought and research on how these potential new therapeutics may be manufactured and deployed. A currently unanswered question is how eventual anti-venom mAbs will be formulated for use. For example, will they be formulated on the basis of syndromic polyvalent recombinant antivenoms (i.e. neurotoxic and non-neurotoxic) or based on more familiar geographically based broad-spectrum polyvalent antivenoms? Alternatively, anti-venom mAbs could be explored for use as adjuncts to existing antivenoms, possibly boosting their potency, or alongside small molecule generic inhibitors currently under investigation. Consideration of this point has begun with the recent publication of a specific target product profile for development and potential formulation of pan-African recombinant antivenoms [133], but thought into other practicalities need to be also considered, such as identifying and engaging with potential manufacturers capable of producing recombinant antivenoms at scale and how to enable adequate supply to endemic areas. Another area we have not touched upon in this article is the immense potential of anti-venom mAbs for the development of rapid diagnostics for snakebite envenoming [160], which has the potential to revolutionize snakebite envenoming treatment through early identification of envenoming before onset of overt pathology. Regardless of the eventual application of recombinant antivenom mAbs, we anticipate that at least two or three candidate mAbs will be sufficiently developed to commence initial human safety trials within the next decade, enabling a much clearer picture of how recombinant anti-venom mAbs will be implemented to lessen the burden of snakebite envenoming.

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