Design of consensus toxins and their use for the discovery of broadly neutralizing antibodies

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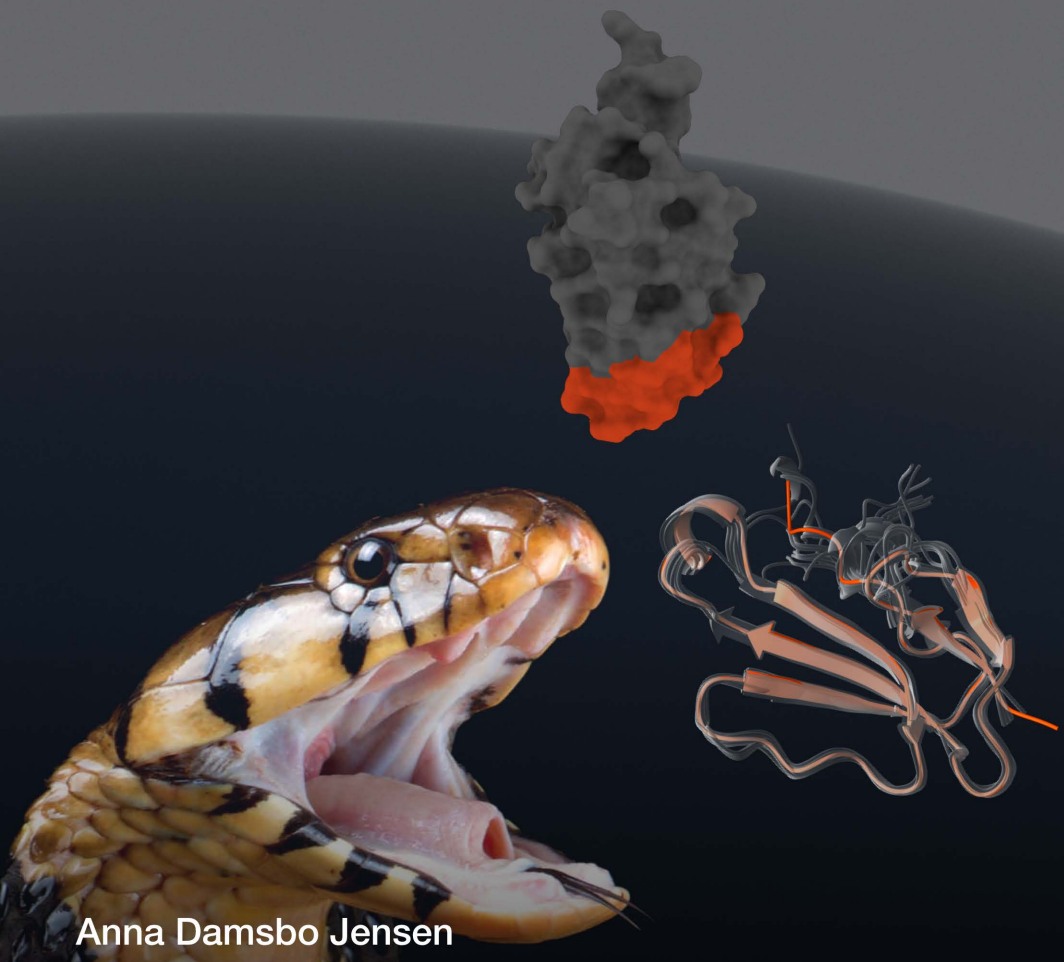

Anna Damsbo Jensen

PhD Thesis

July 2023

Department of Biotechnology and Biomedicine

Technical University of Denmark

Design of consensus toxins and their use for the discovery of broadly neutralizing antibodies

PhD thesis by

Anna Damsbo Jensen

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Technical University of Denmark

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Preface

The research presented in this PhD thesis was conducted between August 2020 and July 2023 at the Department of Biotechnology and Biomedicine at the Technical University of Denmark. The main supervisor was Prof. Andreas H. Laustsen-Kiel, and the co-supervisor was Prof. Lars Ellgaard. The Villum Foundation funded the PhD under grant number 0025302.

Electrophysiology experiments presented as a part of Article II and Manuscript II were conducted in collaboration with Sophion Bioscience in Ballerup, where Dr. Kim Boddum supervised the research.

The figures included in Chapter 1 and 6 were created using Biorender.com.

Acknowledgments

I am deeply grateful to my PhD supervisor, Prof. Andreas H. Laustsen-Kiel, for providing me with this incredible opportunity to pursue my doctoral studies. Your support, guidance, and belief in my abilities have been instrumental in shaping the course of this research. Your encouragement and challenging discussions have pushed me to strive for excellence and work harder, making me a better scientist.

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I am thankful to the institutions and foundations that have supported my research journey, including the Villum Foundation, Department of

Biotechnology and Biomedicine at the Technical University of Denmark, Department of Biology at the University of Copenhagen, Sophion Bioscience, Torben og Alice Frimodts Fond, William Demant Fonden, Knud Højgaards Fond, Otto Mønsteds Fond, Augustinus Fonden, and Danmark-Amerika Fonden.

I would like to give a special shout-out to my pub quiz team, "You're a Quizzard, Harry," for providing a fun and refreshing break each week at Kompasset.

I also want to give a special thanks to my loving boyfriend Sam for being my anchor, providing me with encouragement, and ensuring I was well-fed throughout this stressful period. Your unwavering support helped me maintain some semblance of sanity during this challenging time. Additionally, thank you for your assistance in helping me design the cover for my thesis; your contribution has made it even more special and meaningful to me.

Lastly, I express my deepest gratitude to my family for their endless support throughout my entire life. Your love, belief in me, and encouragement have been the foundation of my success.

This thesis and all its accomplishments would not have been possible without the collective efforts, encouragement, and support of all those mentioned and many others who have touched my life. I am truly humbled and grateful for the many influences that have shaped my academic and personal growth.

Abstract

Snakebite envenoming remains a significant global health challenge, impacting millions of people annually, particularly in regions with limited access to medical care. The primary treatment for snakebite envenoming is antivenom, which consists of polyclonal antibodies derived from the immunization of large animals with snake venom. However, this traditional method poses challenges, including the need to extract venom from snakes, the use of big animals in the manufacturing process, the resulting antivenoms can potentially cause adverse reactions, and these products possess limited cross-reactivity against venoms from species whose venom was not included in the immunization procedure. This thesis explores alternative strategies for developing a fundamentally new type of antivenom, with an emphasis on the utilization of recombinant toxin expression and antibody discovery techniques.

The central objective of this research is to innovate antivenom development by eliminating the need to extract venom from snakes and instead enable the heterologous expression of toxins and the discovery of antibodies *in vitro*. This work focused on α-neurotoxins from the three-finger toxin superfamily. These are crucial components of most elapid venoms and known for their potent neurotoxic effects, which are mediated by the ability of this functional group of toxins to bind and inhibit nicotinic acetylcholine receptors on the postsynaptic side of neuromuscular junctions, ultimately manifesting as paralysis in victims.

In the first part of the work behind this thesis, *Escherichia coli* and *Komagataella phaffii* (previously known as *Pichia pastoris*) were employed as hosts for the recombinant expression of α-cobratoxin, which is a well-studied α-neurotoxin. This comparison of different expression systems aimed to evaluate the efficiency and functionality of the recombinantly expressed toxins. The findings demonstrated the potential of both *E. coli* and *K. phaffii* in producing α-cobratoxin, presenting promising alternatives to traditional venom extraction methods, although the work also indicated that further optimization is warranted to obtain even higher quality toxins.

Afterward, α-cobratoxin was utilized as an antigen to discover single-chain variable fragments (scFvs) as toxin-targeting antibodies using phage display technology. Notably, our process leveraged recombinant α-cobratoxin, which was equally useful as antigen as native α-cobratoxin in the discovery process. This approach represents the first entirely *in vitro* antibody discovery strategy to discover antibodies targeting snake toxins. The study thus showcased the

viability of using recombinant snake toxins for antibody discovery, offering a potentially less hazardous discovery platform with enhanced molecular control for antivenom development, devoid of the need for snake venoms.

To broaden the neutralization capacity of toxin-neutralizing antibodies, the use of consensus α-neurotoxins was employed during a phage display-based discovery campaign involving an immune nanobody library. Here, such toxins were designed, expressed, and used as antigens to discover nanobodies with cross-neutralizing properties against snake toxins from different species of elapids from several different genera. These nanobodies demonstrated surprisingly high affinity and broad neutralizing capacities and may therefore be promising leads for the further development of future antivenom therapies.

In conclusion, the research presented in this thesis significantly propels antivenom development forward, establishing a more efficient, sustainable, and accessible approach to antivenom development, which I hope will pave the way for improved snakebite envenoming management worldwide. Moreover, the knowledge and insights generated in the work behind this thesis may offer valuable guidance to future researchers in their pursuit of broadly neutralizing antibodies and nanobodies beyond the field of snake envenoming. More specifically, it is my personal belief that the concepts and methodologies developed here hold significant promise in various scientific domains, including the development of consensus proteins and broadly neutralizing antibodies in areas, such as infectious disease research, cancer therapy, drug discovery, and diagnostics, where cross-reactivity may be a key aspect for successful clinical, industrial, and basic research applications.

Sammenfatning

Slangebid er fortsat en alvorlig global sundhedsudfordring, der rammer millioner af mennesker hvert år, især i regioner med begrænset adgang til lægehjælp. Den primære behandling af slangebid er modgift, som består af polyklonale antistoffer, der stammer fra immunisering af store dyr med slangegift. Men denne traditionelle behandlingsmetode er forbundet med udfordringer, herunder behovet for at udvinde gift fra slanger, brugen af store dyr i produktionsprocessen, at den producerede modgift potentielt kan forårsage alvorlige bivirkninger, og at disse modgifte har begrænset krydsreaktivitet over for gift fra arter, hvis gift ikke indgik i immuniseringsproceduren. Denne afhandling undersøger alternative strategier til udvikling af en ny type modgift, med vægt på brugen af rekombinant toksinekspression og teknologier til opdagelse af antistoffer.

Det overordnede mål med denne forskning er at innovere udviklingen af modgift ved at eliminere behovet for at udvinde gift fra slanger og i stedet muliggøre heterolog ekspression af toksiner og opdagelse af antistoffer *in vitro*. Denne afhandling fokuserede på α-neurotoksiner fra superfamilien af tre-finger-toksiner. Disse er afgørende komponenter i de fleste elapid-gifte og kendt for deres potente neurotoksiske virkninger, som medieres af toksiners evne til at binde og hæmme nikotiniske acetylcholinreceptorer på den postsynaptiske side af neuromuskulære forbindelser, hvilket i sidste ende manifesterer sig som lammelse hos ofrene.

I den første del af arbejdet bag denne afhandling blev *Escherichia coli* og *Komagataella phaffii* (tidligere kendt som *Pichia pastoris*) anvendt som værter for den rekombinante ekspression af α-cobratoxin, der er et velstuderet α-neurotoksin. Denne sammenligning af forskellige ekspressionssystemer havde til formål at evaluere effektiviteten og funktionaliteten af de rekombinant udtrykte toksiner. Resultaterne viste, at både *E. coli* og *K. phaffii* har potentiale til at producere α-cobratoxin, hvilket er lovende alternativer til traditionelle metoder til giftudvinding, selvom arbejdet også viste, at yderligere optimering er nødvendig for at opnå toksiner af endnu højere kvalitet.

Efterfølgende blev α-cobratoxin brugt som et antigen til at opdage enkeltkædede variable fragmenter (scFvs) som målrettede antistoffer mod toksiner ved hjælp af phage display-teknologi. Vores proces udnyttede især rekombinant α-cobratoxin, som var lige så nyttigt som antigen som naturligt α-cobratoxin i opdagelsesprocessen. Denne tilgang repræsenterer den første

komplette *in vitro*-antistofopdagelsesstrategi til at opdage antistoffer rettet mod slangetoksiner. Undersøgelsen viste således, at det er muligt at bruge rekombinante slangetoksiner til antistofopdagelse, hvilket giver en potentielt mindre farlig opdagelsesplatform med forbedret molekylær kontrol til udvikling af modgift, uden behov for slangegifte.

For at udvide neutraliseringskapaciteten af toksin-neutraliserende antistoffer blev der anvendt konsensus α-neurotoksiner under en phage display-baseret opdagelseskampagne, der involverede et immun nanobody-bibliotek. Her blev sådanne toksiner designet, udtrykt og brugt som antigener til at opdage nanobodies med krydsneutraliserende egenskaber mod slangetoksiner fra forskellige arter af elapider fra flere forskellige genera. Disse nanobodies viste overraskende høj affinitet og bred neutraliseringskapacitet og kan derfor være lovende for den videre udvikling af fremtidige antivenom-terapier.

Afslutningsvis vil jeg sige, at den forskning, der præsenteres i denne afhandling, i høj grad driver udviklingen af modgift fremad og etablerer en mere effektiv, bæredygtig og tilgængelig tilgang til udvikling af modgift, som jeg håber vil bane vejen for en forbedret håndtering af slangebid over hele verden. Desuden kan den viden og indsigt, der er genereret i arbejdet bag denne afhandling, give værdifuld vejledning til fremtidige forskere i deres søgen efter bredt neutraliserende antistoffer og nanobodies uden for området for slangegift. Mere specifikt er det min personlige overbevisning, at de koncepter og metoder, der er udviklet her, er meget lovende inden for forskellige videnskabelige domæner, herunder udvikling af konsensusproteiner og bredt neutraliserende antistoffer inden for områder som forskning i infektionssygdomme, kræftbehandling, lægemiddelopdagelse og diagnostik, hvor krydsreaktivitet kan være et nøgleaspekt for vellykkede kliniske, industrielle og grundlæggende forskningsapplikationer.

Publications

Article I – Strategies for heterologous expression, synthesis, and purification of animal venom toxins. Esperanza Rivera-de-Torre, Charlotte Rimbault, Timothy P. Jenkins, Christoffer V. Sørensen, Anna Damsbo, Natalie J. Saez, Yoan Duhoo, Celeste Menuet Hackney, Lars Ellgaard and Andreas H. Laustsen. Frontiers in Bioengineering and Biotechnology, 9, (2022).

Manuscript I – A comparative study of the performance of *E. coli* and *K. phaffii* for expressing α-cobratoxin. Anna Damsbo, Charlotte Rimbault, Anneline Vlamynck, Ida Bisbo, Esperanza Rivera-de-Torre, Andreas H. Laustsen.

Article II – A single-chain variable fragment selected against a conformational epitope of a recombinantly produced snake toxin using phage display. Charlotte Rimbault, Pelle D. Knudsen, Anna Damsbo, Kim Boddum, Hanif Ali, Celeste M. Hackney, Lars Ellgaard, Markus-Frederik Bohn, Andreas H. Laustsen. New Biotechnology, 76, (2023)

Manuscript II - Discovery of broadly neutralizing nanobodies using designed consensus antigens. Anna Damsbo, Esperanza Rivera-de-Torre, Anneline Vlamynck, Ida Bisbo, Tulika Tulika, Kim Boddum Andreas H. Laustsen

Project aim

The aim of this project was twofold. First, to explore and optimize the expression of α-neurotoxins through heterologous systems, reducing the dependency on venomous sources for toxin purification. Second, the project aimed to design and utilize consensus toxins as antigens to discover cross-reactive antibodies with broad neutralization capabilities against snake toxins from various species. By focusing on recombinant techniques and consensus toxin design, the objective was to advance the development of innovative antivenom therapies with the potential to offer more effective and accessible treatments for venomous snakebites.

Abbreviations

3FTX	Three-finger toxins
BLI	Biolayer interferometry
CDR	Complementarity-determining region
DEFLIA	Dissociation-enhanced lanthanide fluorescence immunoassay
Fab	Antigen-binding fragment
IgG	Immunoglobulin G
LC	Long-chain
nAChR	Nicotinic acetylcholine receptor
NC	Non-conventional
PDI	Protein disulfide isomerase
PLA_2	Phospholipases
SC	Short-chain
scFv	Single-chain variable fragment
SVMP	Snake venom metalloproteases
SVSP	Snake venom serine proteases
V_HH	Variable heavy-chain antibodies
WHO	World Health Organization

Chapter 1 – Introduction

1. Snakebite envenoming

1.1 A neglected disease

Despite not being considered in our daily lives in most of Europe, snakebite envenoming represents a significant public health issue worldwide. In 2017, the World Health Organization (WHO) reinstated snakebite envenoming as a neglected tropical disease.[1] This category encompasses a group of infections or conditions that primarily affect the world's poorest populations and have received little attention in terms of research and development. This list includes viral and parasitic diseases such as dengue fever, rabies, and leishmaniasis.[2,3] The magnitude of the issue becomes apparent when considering that the monthly death toll from venomous snakebites exceeds the total number of fatalities recorded during the West African Ebola crisis from 2014 to 2016, which was the largest Ebola outbreak in history that most remember.[4,5] This highlights the alarming impact of snakebite envenoming on public health and emphasizes the urgent need for improved strategies in antivenom development and snakebite management.[4]

Snakebite envenoming imposes a considerable burden of morbidity and mortality, with conservative estimates indicating tens of thousands of deaths annually. In addition to the lives lost, survivors often face permanent disabilities and socioeconomic hardships, leaving approximately 400,000 victims with permanent sequelae such as amputation or disfigurements.[6] The accurate scale of snakebite envenoming is likely even higher, with estimates suggesting that between 1.2 and 5.5 million people suffer from snakebites each year.[7] However, these numbers are likely underestimated due to the lack of comprehensive reporting systems that record such data. Available data is often limited to victims who manage to access healthcare facilities, leaving a significant proportion unaccounted for.[8]

Beyond its immediate health consequences, snakebite envenoming exerts a far-reaching influence on communities and societies, especially in rural and developing regions where snakebites are more prevalent and access to timely medical care is challenging.[9,10] The aftermath of a snakebite can plunge a family into financial instability, perpetuating cycles of poverty and amplifying existing socioeconomic disparities.[11] Moreover, snakebites disproportionately affect vulnerable and disadvantaged populations, exacerbating the challenges faced by marginalized communities.[12] Limited healthcare access, lack of awareness,

and inadequate antivenom availability compound the burden on these populations, intensifying the socioeconomic impact of snakebite envenoming.[13]

Snakebite envenoming represents a significant global health problem in terms of mortality and the long-term socioeconomic impact on affected individuals and communities. Understanding the complex nature of snakebite envenoming requires a comprehensive exploration of the venom composition and the toxins responsible for the deleterious effects.

1.2 Snake venoms and their toxins

Snake venoms are a mixture of salts, small organic molecules, peptides, and proteins commonly known as toxins.[14] These toxins constitute a diverse and complex arsenal that varies significantly among the approximately 700 species of venomous snakes, which are categorized into four main families: Viperidae, Elapidae, Atractaspididae, and Colubridae.[15] The Viperidae (vipers, adders, and pit vipers) and Elapidae (cobras, mambas, kraits, Australasian venomous snakes, and sea snakes) are the most medically relevant families, responsible for the majority of snakebite envenomings worldwide.[15]

The clinical manifestations of snakebite envenoming depend highly on the specific snake species involved, which is directly related to the venom composition.[16] Venom can induce various toxic effects, including neurotoxicity, hemotoxicity, cytotoxicity, and myotoxicity.[6] The composition of venom exhibits considerable variation not only between different snake species but also within the same species, influenced by factors such as age, sex, diet, and geographical location (Figure 1).[17–19]

Snake venom encompasses a wide array of toxins, constituting a complex amalgamation that exhibits remarkable diversity, although the exact number may vary.[20,21] These toxins exhibit diverse characteristics in terms of their structure, toxicity, and sometimes enzymatic activity. Achieving a consensus among researchers in this field can be challenging due to the complexity and variability of snake venoms. Nonetheless, a widely accepted classification includes four major categories: Phospholipases A_2 (PLA_2), snake venom metalloproteases (SVMPs), snake venom serine proteases (SVSPs), and three-finger toxins (3FTxs).[6] This classification system serves as a valuable framework for organizing and understanding the different types of toxins present in snake venoms, based on their structural features and biological effects.[22]

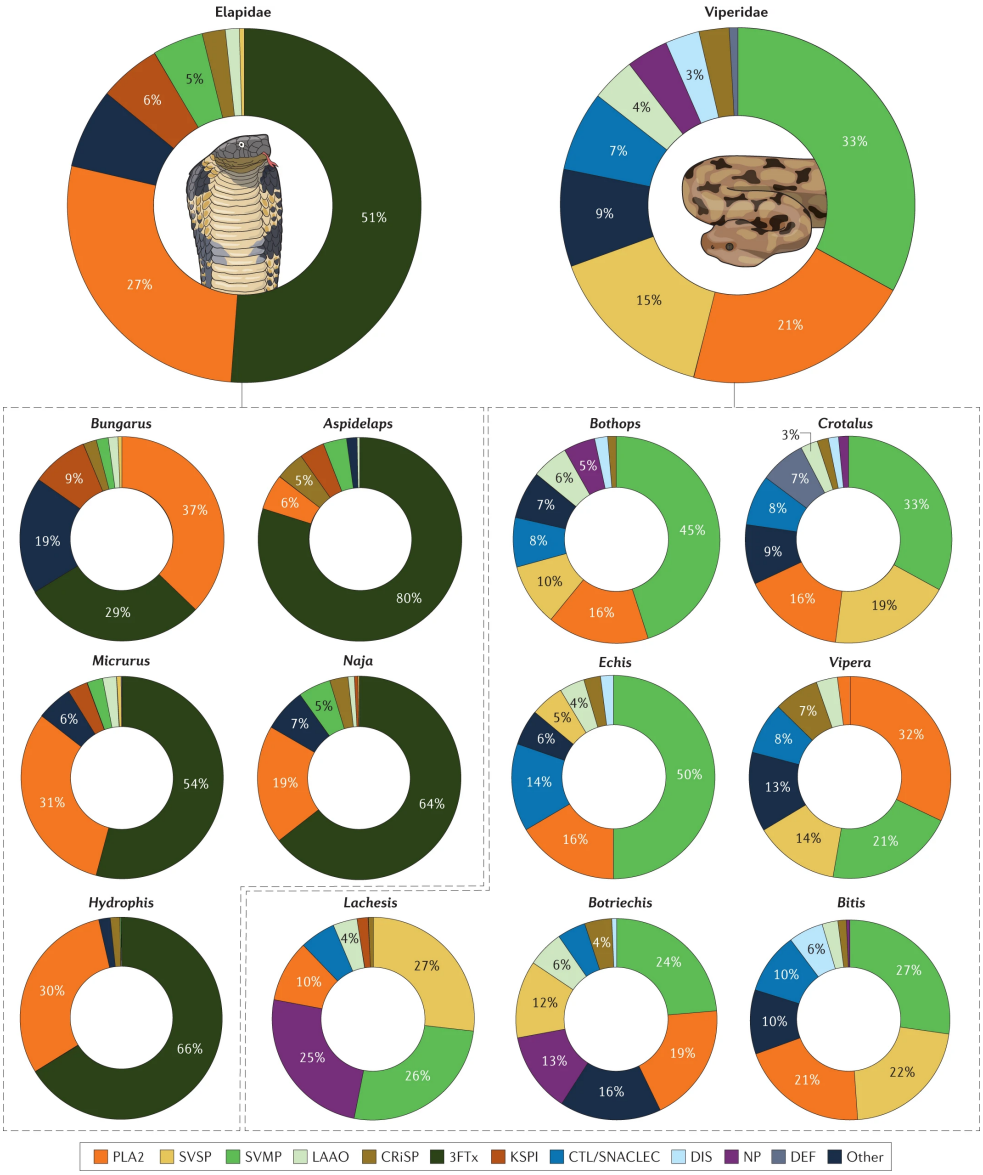

Figure 1. The composition of venom from snake species belonging to the Elapidae or Viperidae family. *The charts show the composition of snake venom from two families: Elapidae (elapids) and Viperidae (viperids). Each entry in the charts represents a group of proteins, and only those with an average abundance of more than 1% are shown. The data comes from proteomic studies conducted over the past 15 years. The smaller charts break down the venom composition at the genus level, focusing on well-studied genera. Figure is from Oliveira, A. L. et al (2022) and reproduced with permission from Springer Nature.*[17]

This thesis focuses on a subgroup family of 3FTxs known as α-neurotoxins.[23] These toxins are of paramount importance due to their ability to induce paralysis and respiratory failure, leading to asphyxiation through the paralysis of the respiratory muscles.[24]

Although 3FTxs can be found in the venoms of all snake families, they are particularly abundant in Elapid venoms. Remarkably, they constitute a significant proportion, ranging from 64% to 95%, of all toxins present in the venoms of renowned snakes such as shield-nosed cobras (*Aspidelaps* spp.), green mambas (*Dendroaspis angusticeps*), the king cobra (*Ophiophagus hannah*), and the entire genus of true cobras (*Naja* spp.).[17,25–27]

2. Three-finger toxins and α-neurotoxins

2.1 The family of 3FTxs

Three-finger toxins (3FTxs) are a medically relevant group of non-enzymatic proteins found in snake venoms, known for their diverse range of biological activities and unique structural characteristics.[28] These toxins derive their name from their distinctive three-finger fold structure, which features a protein scaffold consisting of three β-stranded loops that resemble fingers, converging at a hydrophobic core held together by four conserved disulfide bridges (Figure

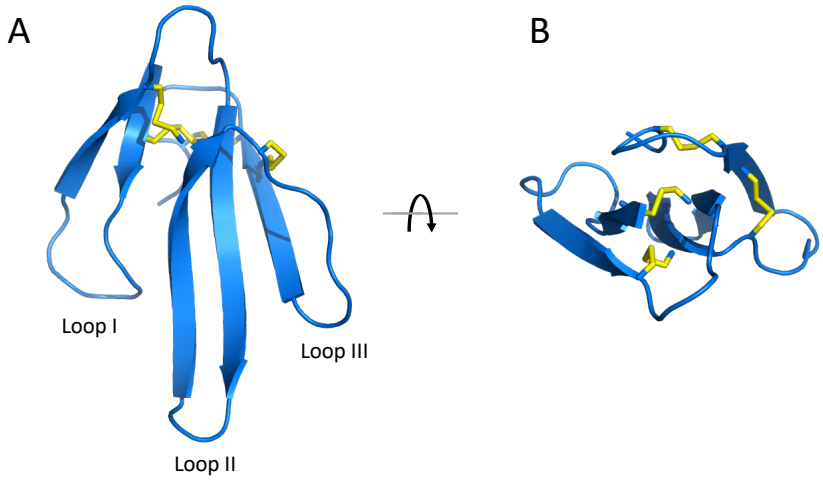

Figure 2. The structure of a 3FTX (three-finger toxin). *Panel A provides a frontal view, highlighting the presence of three loops resembling fingers. Panel B presents a top view, showcasing the four disulfide bonds that connect the various loops, contributing to the structural stability of the protein. (PDB ID: 5EBX)*

2). This arrangement results in a flat, leaf-like shape with a slight concavity.[29,30]

The broad spectrum of biological activities exhibited by 3FTxs highlights their functional versatility. These toxins exert various pharmacological actions, including cytotoxicity, coagulotoxicity, cardiotoxicity, neurotoxicity, and acetylcholinesterase inhibition.[29,31–36] The diverse toxic effects exerted by 3FTxs arise from differences in their primary sequences, unique pharmacophores, and variations in the length, loops, and flexibility.[37] Additional disulfide bridges and extensions of the N- and C-termini further contribute to the distinct functions observed among different 3FTxs.[38–44]

2.2 α-neurotoxins and their role in snakebite envenoming

Among the diverse range of 3FTxs, a subgroup of particular interest is the α-neurotoxins. These toxins have a crucial function in snakebite envenoming, especially when bitten by an elapid, by exerting their action postsynaptically at neuromuscular junctions.[45] They achieve this by binding with high affinity to the cholinergic receptor at the motor end plate in muscle fibers, effectively hindering the binding of acetylcholine.[45] Consequently, this provokes flaccid paralysis, which can lead to respiratory failure and asphyxiation due to the immobilization of the respiratory muscles.[23] α-neurotoxins can be further categorized based on their structural characteristics.[46]

One structural classification widely accepted by the toxinology scientific community divides α-neurotoxins into short-chain α-neurotoxins (SC-α-neurotoxins), long-chain α-neurotoxins (LC-α-neurotoxins), non-conventional α-neurotoxins (NC-α-neurotoxins), and atypical α-neurotoxins, each with variances in primary and tertiary structure.[46] SC-α-neurotoxins typically consist of approximately 60-62 residues and contain four conserved disulfide bridges. In comparison, LC-α-neurotoxins are larger, ranging from 66 to 75 residues, and possess an additional disulfide bond at the tip of the second loop. They also display a shorter first loop and a longer C-terminal tail. NC-α-neurotoxins are approximately 62-68 residues long and have a fifth disulfide bridge in the first loop. In comparison, atypical α-neurotoxins exhibit considerable variation in length, ranging from 57 to 87 residues, and may contain an additional disulfide bond in either the first or second loop (Figure 3).[37,47]

It is worth noting that some α-neurotoxins can exist in a dimeric form.[41,42,48] These dimeric α-neurotoxins can either be covalently-bound dimers with one or more disulfide bridges or non-covalently bound dimers held together by hydrophobic interactions or hydrogen bonds.[49,50] The dimerization of α-neurotoxins leads to the diversification of target selectivity by enabling novel

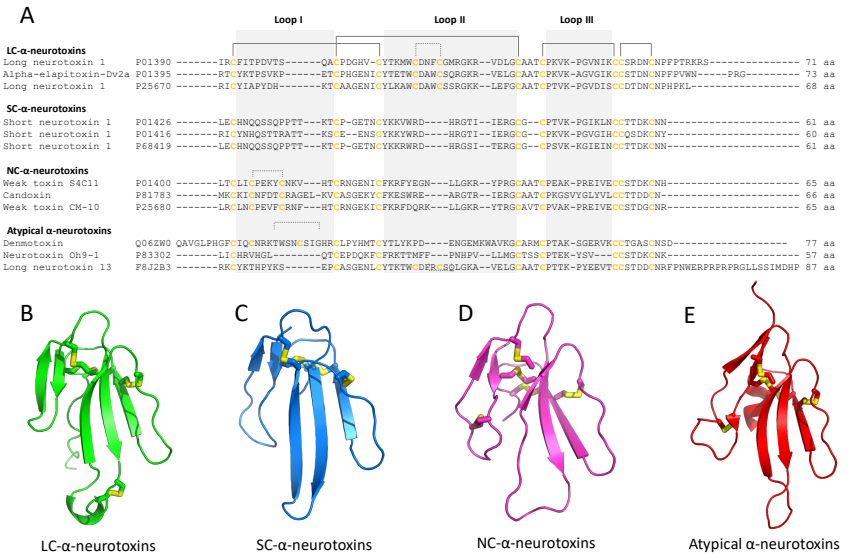

Figure 3. Variations of α-neurotoxins*. A) The figure displays a sequence alignment of the four distinct groups of α-neurotoxins. Cysteine residues, denoted by a yellow color, are highlighted along with the corresponding disulfide bond pattern. The three loops of the α-neurotoxins are indicated by a light grey shading. The displayed toxins are Long neurotoxin 1 (Naja Nivea), Alpha-elapitoxin-Dv2a (Dendroaspis viridis), Long neurotoxin 1 (Aspidelaps scutatus), Short neurotoxin 1 (N. pallida), Short neurotoxin 1 (D. polylepis), Short neurotoxin 1 (N. nivea), Weak toxin S4C11 (N. melanoleuca), Candoxin (Bungarus candidus), Weak toxin CM-10 (N. Nivea), denmotoxin (Boiga androphili), Neurotoxin Oh9-1 (Ophiophagus Hannah), Long neurotoxin 13 (Drysdalia coronoides). B) Structure of Long neurotoxin 1 (P01390, predicted by Alphafold). C) Structure of Short neurotoxin 1 (P01426, PDB ID: 1IQ9). D) Structure of the Weak toxin S4C11 (P01400, predicted by Alphafold). E) Structure of Denmotoxin (Q06ZW0, PDB ID: 2H5F)*

structural conformations that allow interactions with new receptor subtypes not targeted by their monomeric constituents.[31,51]

The primary target of all α-neurotoxins is the nicotinic acetylcholine receptor (nAChR).[37] This receptor plays a critical role in transmitting signals between nerve cells and muscle cells, specifically at the neuromuscular junction. By binding to the nAChR, α-neurotoxins disrupt the normal function of this receptor, leading to the inhibition of muscle contraction.[52]

2.3 Primary target of α-neurotoxins – The nicotinic acetylcholine receptor

The nAChR is a crucial component of the nervous system, responsible for transmitting signals from motor nerves to muscles and facilitating neurotransmission within the sympathetic and parasympathetic nervous systems.[53] It is composed of five subunits that can assemble in both homo- and heteropentameric configurations, forming a pseudo-symmetrical arrangement

surrounding a central transmembrane ion channel (Figure 4).[54] Each subunit consists of a large extracellular domain, contributing to the formation of ligand-binding sites at the subunit interfaces between a primary α-subunit and a complimentary α-subunit or a non-α subunit.[55] The transmembrane domain of each subunit consists of four α-helices that line the ion channel pore permeable to sodium (Na^+) and potassium (K^+) while the intracellular domain exhibits variable structures.[53]

The assembly of different subunits generates diverse nAChR subtypes with distinct pharmacological, physiological, and clinical significance. For instance, the postsynaptic skeletal muscle nAChR primarily consists of the combination $(\alpha1)_2\beta1\varepsilon\delta$ ($(\alpha1)_2\beta1\gamma\delta$ in fetal muscle nAChR).[55] Neuronal nAChRs, on the other

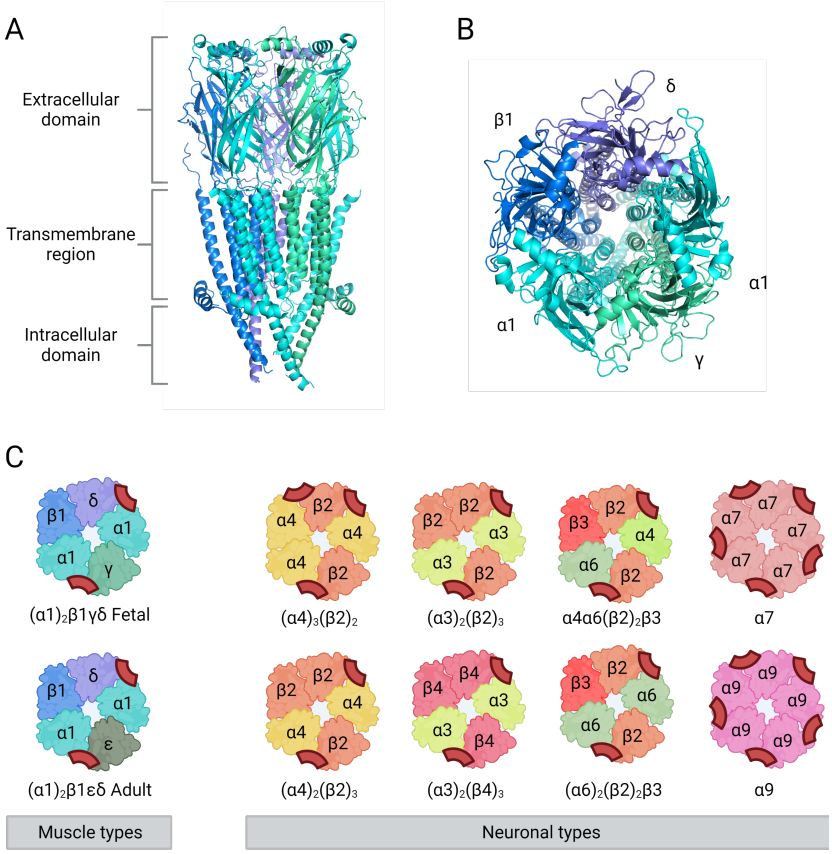

Figure 4. Structure of nicotinic acetylcholine receptor (nAChR). *A) Cryo electron microscopy structure of the (α1)2β1εδ) nAChR, depicted in three dimensions (PDB ID: 6UWZ). B) Top view of the receptor showing the subunit arrangement. C) Illustration of the various subunit combinations responsible for muscle or neuronal types of nAChR. The ligand binding sites are indicated with red shapes. Figure is adapted from Nirthanan (2020).*[37]

hand, encompass various pentameric combinations of α2 to α10 and β2 to β4 subunits and exist both as homo- and heteropentamers (Figure 4C).[54,56] These neuronal nAChR subtypes play significant roles in the central nervous system, modulating processes such as cognition, memory, pain perception, and addiction, and are also found in extra-neuronal locations, where they contribute to the modulation of key cellular signaling pathways.[57–61]

Under normal physiological conditions, the transmission of signals through the nAChR follows a well-defined process. When a nerve impulse reaches the pre-synaptic terminal, it triggers the release of the neurotransmitter acetylcholine (ACh) into the synaptic cleft.[62] ACh then diffuses across the synaptic cleft and binds to the ligand-binding sites on the extracellular domain of the nAChR. This binding event induces a conformational change in the receptor, leading to the opening of the ion channel pore within the transmembrane domain (Figure 5).[63]

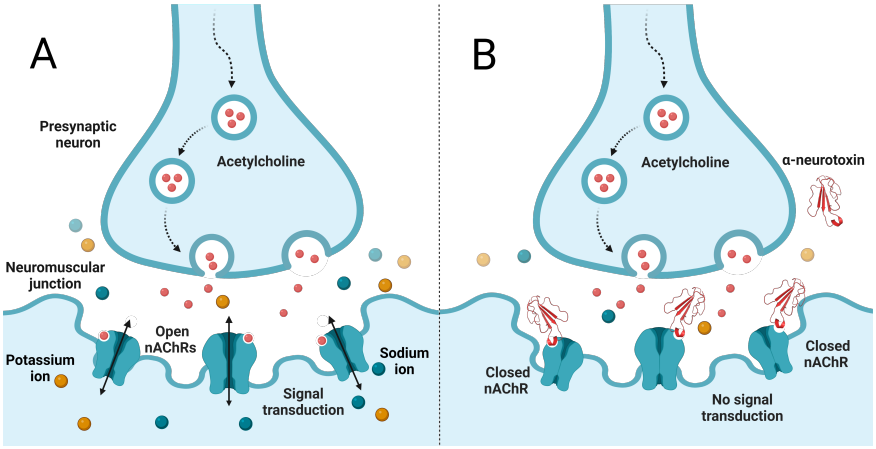

Figure 5. α-neurotoxins prevent ACh to bind to nAChR. *A) At the neuromuscular junction, the release of ACh from the presynaptic terminal triggers its binding to the nAChR. This binding event leads to the opening of the receptor, enabling the influx of ions. B) However, the presence of α-neurotoxins disrupts this process by binding to the nAChR. Consequently, the binding of ACh and the subsequent transmission of nerve signals are blocked.*

Upon opening of the ion channel, the nAChR facilitates the movement of positively charged ions, particularly Na^+, into the cell, while K^+ ions exit.[62,64] This results in a net inward flow of positively charged ions. In the neuromuscular junction, this influx of cations generates the endplate potential, an electrical signal that propagates along the muscle fiber and initiates muscle contraction. Similarly, in the neuronal nAChR present in the central nervous system, this influx of cations induces an excitatory response in the postsynaptic neuron, facilitating the transmission of the nerve impulse. It is important to note that the nAChR acts as a non-selective cation channel, enabling the passage of

multiple types of positively charged ions. It exhibits permeability to Na^+ and K^+ ions, and certain subunit combinations also allow the passage of Ca^{2+}.[65,66]

The binding of ACh to the nAChR is transient, and once the ACh molecules are released from the binding sites, the receptor returns to its resting conformation, ready for subsequent neurotransmission events.[62]

However, in the presence of α-neurotoxins, the normal functioning of the nAChR is disrupted.[29,37,67] These toxins bind to the orthosteric ligand-binding sites, meaning the same binding site as ACh, of the nAChR, preventing ACh from binding. This prevents the opening of the ion channel, thereby inhibiting the influx of cations and disrupting the normal signal transmission process.[55,68]

The potent binding affinity and selectivity of α-neurotoxins for the nAChR allow them to exert their paralyzing effects by specifically targeting the receptors present in the neuromuscular junction. This interference with acetylcholine transmission leads to muscle paralysis, ultimately causing respiratory failure and potentially resulting in asphyxiation.[39,68] These toxins possess a high affinity and selectivity for the nAChR, allowing them to specifically target and block neuromuscular transmission. A common binding core of amino acids present in the toxin's structure, including positively charged and aromatic residues, facilitates the recognition of the nAChR by α-neurotoxins.[39]

One notable feature of α-neurotoxins is the presence of a highly conserved arginine residue located at the tip of the second loop.[69–71] This arginine residue plays a critical role in toxin binding and interaction with the nAChR. It mimics the shape and physicochemical properties of acetylcholine, the natural ligand of the receptor, by occupying the orthosteric ligand-binding site on the nAChR. By binding to this site, α-neurotoxins prevent acetylcholine from effectively binding and opening the receptor channel.[31,52]

The positively charged and aromatic residues present in the toxin's structure interact with complementary residues on the nAChR, further stabilizing the toxin-receptor complex.[39,70,71] This interaction involves electrostatic interactions and hydrophobic interactions between the toxin and specific regions of the receptor. The binding of α-neurotoxins to the nAChR induces conformational changes in the receptor, effectively blocking the channel and inhibiting the transmission of signals.[39,70,71]

2.4 α-neurotoxins beyond the neuromuscular junction

While the primary target of α-neurotoxins is the muscle nAChR, it is important to note that these toxins can also affect other subtypes of nAChRs found in the central nervous system, extending their effects beyond the neuromuscular

junction.[37] For instance, certain LC-α-neurotoxins can inhibit several different types of neuronal nAChRs, which is likely attributed to the longer second loop and C-terminal tail present in these toxins. Moreover, some LC-α-neurotoxins can also block the $GABA_A$ receptor and muscarinic AChR *in vitro*, while select NC-α-neurotoxins can inhibit muscarinic AChR *in vitro*.[43,72–77] This broad range of receptor targets underscores the complexity and versatility of α-neurotoxins. However, SC-α-neurotoxins display remarkable specificity and exclusively bind to the muscle nAChR.[39] These toxins demonstrate a high degree of selectivity for the muscle nAChR and do not interact with other subtypes of nAChRs or additional receptor systems.

The ability of α-neurotoxins to interact with various receptor targets highlights their multifaceted nature and potential implications in different physiological processes, making the preferential targets for antivenom development due to their medical relevance.[17] The selective inhibition of neuromuscular transmission by α-neurotoxins leads to muscle paralysis and respiratory failure.[28] Additionally, the interaction of these toxins with various receptor types, such as neuronal nAChRs, GABAA receptors, and muscarinic AChR, implies potential effects on a wider range of central nervous system functions. These may include cognition, memory, pain perception, and modulation of cellular signaling pathways, thereby making them compelling candidates for drug development.[35,37,78–80]

Given the significance of α-neurotoxins in snakebite envenoming and their potential impact on human health, they represent an essential target for the development of antivenom therapies.[81] Understanding the structural characteristics, receptor interactions, and biological activities of α-neurotoxins is crucial for the design and production of effective antivenom formulations that can neutralize the toxic effects of these toxins and prevent severe envenomation.[82]

3. Traditional antivenom

Traditional antivenom has its origin more than 125 years ago due to the pioneering work of scientists such as Albert Calmette, Camille Phisalix, and Vital-Justin-Bertrand.[83–85] This breakthrough therapy has played a vital role in mitigating the effects of snakebite envenoming. The production of traditional antivenom involve the immunization of large animals and the subsequent purification of antibodies from their plasma (Figure 6).[86]

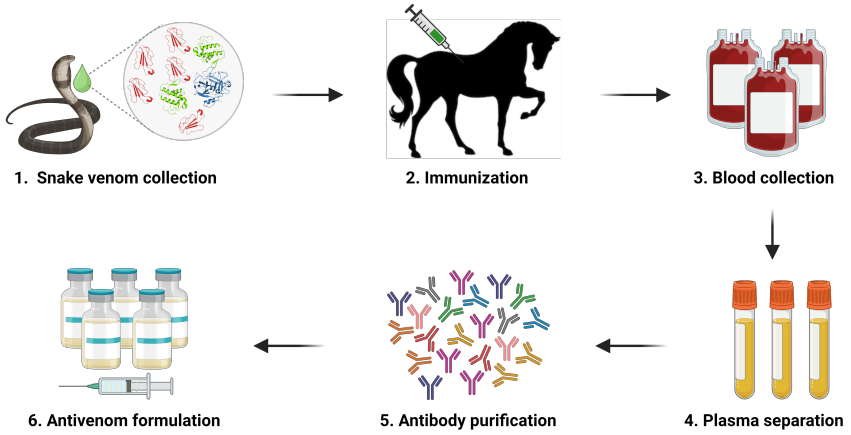

Figure 6. Production of traditional antivenom involves several steps. *First, snake venom is collected from the desired species. Next, a large mammal, usually a horse, is immunized by repeated injections of the venom. During a specific timeframe, the animal's immune system generates antibodies targeting the venom. Blood is subsequently collected from the immunized animal, followed by a separation of the plasma and purification of the antibodies. These purified antibodies constitute the final antivenom product.*

While traditional antivenom has played a crucial role in saving the lives of people that has suffered from snakebite envenoming, it is not without limitations.[86,87] One of the major challenges lies in generating antibodies that efficiently neutralize all medically relevant snake venom toxins.[88–90] The efficacy of the antivenoms heavily relies on the immunogenicity and the abundance of the toxin in the venoms used for immunizations. Certain toxins, such as the α-neurotoxins and other 3FTxs, are not very immunogenic, or they are not present in a sufficient concentration in the venom to effectively trigger an immune response in the immunized animal.[91] Consequently, traditional antivenoms often contain a limited proportion of antibodies capable of neutralizing venom effects. This is particularly problematic since toxins with low immunogenicity can be medically relevant, as they can exert toxic effects at low doses.[92–94]

Traditional antivenoms, which are polyclonal mixtures, consist of a vast but unknown number of unique antibodies.[95] While they primarily target toxins they were raised against, they also recognize numerous other proteins from foreign pathogens encountered by the antivenom production animal throughout its life. Among these antibodies, only approximately 10 to 40% specifically target the toxins in the venom, leaving the majority to focus on non-snake toxin molecules or proteins.[96] Consequently, higher dosages of antivenom are often necessary to achieve venom neutralization. Moreover, the specific antibody clones present in

a given antivenom product can vary between batches, as different antivenom batches are typically sourced from distinct production animals.[97] This inherent variability adds complexity and challenges to antivenom efficacy and consistency.

Traditional antivenoms are typically not administered without a diagnosis due to the risk of severe adverse reactions.[98,99] The immunogenicity of antivenoms can lead to serum sickness, an immune complex-mediated hypersensitivity reaction.[100] These adverse reactions can range from mild symptoms such as fever and rash to severe manifestations including anaphylaxis, renal impairment, and cardiovascular complications.[6,99] Moreover, the limited percentage of antibodies in the antivenom that specifically target snake toxins necessitates higher dosages for effective venom neutralization, which, unfortunately, also increases the potential risks of side effects.[96]

Species-specificity is yet another limitation of traditional antivenoms which arises from the fact that they are typically developed using venom from a specific snake species. While these antivenoms are effective against the toxins produced by that particular snake species, they may not be effective against the toxins from other snake species.[88–90] This poses substantial challenges in regions with a high diversity of venomous snakes, where snakebite cases can involve various species. In such regions, it becomes necessary to have multiple antivenom formulations available to cover a wide range of snakebite cases. An accurate diagnosis to identify the snake species responsible for the bite is of high importance, as administering the wrong antivenom can lead to ineffective treatment and potentially worsen the condition of the snakebite victim.

In addition to the challenges associated with generating effective antivenoms, there are other drawbacks of traditional antivenom production. One significant concern is the cost associated with manufacturing and distributing antivenom on a large scale. It is a complex and labor-intensive process to produce antivenom with various stages such as venom collection, animal immunization, antibody purification, and quality control. These processes require specialized facilities, equipment, and skilled personnel, all of which contribute to the overall cost of antivenom production. The high cost of manufacturing antivenom can have profound implications for regions with limited healthcare resources, particularly in developing countries where snakebite envenomation is a significant health problem. The expense associated with antivenom production can make it unaffordable or economically unsustainable for healthcare systems and individuals in these regions. [11,101] Consequently, access to lifesaving antivenom treatments becomes a challenge, leading to inadequate or delayed treatment for snakebite victims.[102,103] Moreover, the reliance on milking

venomous snakes to obtain the venom needed for antivenom production raises ethical considerations and logistical challenges.[101,104,105] Milking snakes for venom extraction requires expertise, specialized handling techniques, and appropriate facilities to ensure the safety of both the snakes and the personnel involved. [106] Additionally, the process can be time-consuming and may yield limited quantities of venom, further complicating large-scale production.

Furthermore, the geographical distribution and availability of traditional antivenom in regions affected by snakebite envenomation present significant challenges.[107,108] Remote areas with a high incidence of snakebites often face difficulties in accessing timely and sufficient antivenom supplies. Factors such as limited healthcare infrastructure, inadequate transportation, and storage requirements (e.g., need for cold chain) can impede the effective distribution of antivenom to those in need.[109–111]

These limitations underscore the need for continuous research and innovation in the field of antivenom development. Efforts are underway to address these challenges and improve the efficacy, accessibility, and affordability of antivenom.[112] Advancements aim to enhance the cross-reactivity and polyvalency of antivenoms, allowing for broader neutralization of venom toxins. Additionally, developments in biotechnology, such as the use of recombinant antibodies, hold promise for the future of antivenom therapy.[85,113,114]

4. Recombinant antivenom

4.1 Antibodies and antibody fragments

Antivenom therapy revolves around the use of antibodies, which are large proteins employed by the immune system to recognize and neutralize specific molecules or proteins known as antigens. Among the various antibody isotypes, the immunoglobulin G (IgG) is the most prevalent, accounting for approximately 75% of the antibodies found in serum.[115,116] An IgG consists of two heavy and two light chains, each comprising constant and variable regions. The variable regions of both the heavy and the light chains are together responsible for antigen binding (Figure 7).

Apart from full-length antibodies, smaller antibody formats are available, including for example the antigen-binding fragment (Fab) and the single-chain variable fragment (scFv).[117] Fab fragments contain the whole light chains and the variable region and the first constant domain of the heavy chain. ScFv is engineered as single-chain molecules, where an artificial linker is used to connect the variable domain of the heavy and light chain, thus retaining their antigen-binding capabilities. Both Fabs and scFvs can be produced through

recombinant methods. Fabs, being a derivative of IgGs, can also be generated by the cleavage of native antibodies using an enzyme called papain, thus maintaining the natural pairing of their variable regions. In contrast, scFvs are artificially engineered by linking the variable regions of the heavy and light chains, a configuration that doesn't occur naturally.[118] The single-chain format of scFv allows for more flexibility in engineering and easier production, while the Fab fragment offers better stability and binding affinity due to the presence of both variable domains.[119] In certain species like camels, llamas, and sharks, a unique class of antibodies composed solely of heavy chains can be found. By isolating the variable regions of these heavy-chain antibodies, variable heavy-chain antibodies (V_HH) or nanobodies can be obtained (Figure 7).[120]

Each antibody format presents its own unique advantages and challenges for therapeutic applications. IgGs have been widely used due to their well-established efficacy and ability to neutralize snake venom toxins

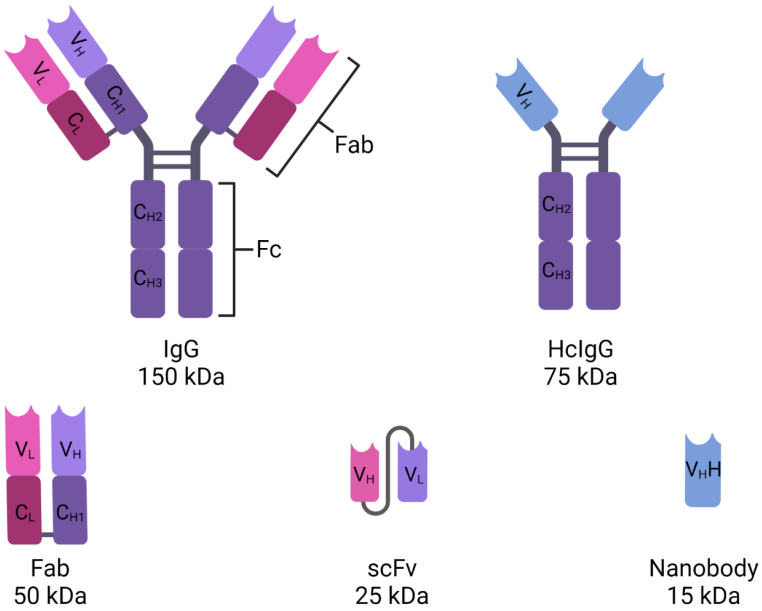

Figure 7. Different antibody formats. *Immunoglobulin G (IgG) consists of two heavy and two light chains with constant (C) or variable (V) regions, making it bivalent with two antigen-binding sites. The IgG can be divided into two regions: the fragment crystallizable (Fc) and the fragment antigen-binding (Fab). Fab fragments, while monovalent, retain the antigen-binding determinants of IgGs. A heavy chain-only IgG (HcIgG) from camelid species only consists of two heavy chains containing C and V regions. The IgG can be reformatted into different structures that maintain the ability to recognize antigens, such as the Fab fragment or the even smaller single-chain variable fragments (scFv) that only consist of the two variable regions linked by an artificial linker. The variable region from HcIgG can be isolated as Variable Heavy domain of heavy chain (V_HH) also known as nanobody.*

effectively.[116,121,122] Their bivalent nature allows for increased binding avidity, ensuring robust toxin neutralization. Furthermore, IgGs exhibit long circulating half-lives, thanks to their ability to recycle through the neonatal Fc receptor (FcRn), enabling them to provide prolonged protection against venom toxins after administration.[123] However, the use of IgGs in antivenom formulations also presents pharmacokinetic and pharmacodynamic challenges. Their large size may limit tissue penetration, potentially affecting their ability to access toxins that permeate fast within tissues or reach specific target organs affected by venom components.[95,113,124,125] Regarding the manufacturing perspective, IgGs are typically expressed in mammalian cell lines, which can be more challenging and expensive for large-scale production.[126,127]

Fab fragments, with their smaller size, possess the ability to penetrate tissue more effectively than IgGs, allowing them to access toxins that may be inaccessible to IgGs due to limited tissue penetration.[124] However, their monovalent nature may reduce neutralization potency and their shorter half-life may require more frequent dosing.[128] Nevertheless, the price associated with frequent dosing is buffered by the reduction in the manufacture cost in comparison with IgGs, as Fabs can be produced recombinantly in *E.* coli, although they are more commonly expressed in mammalian cells to ensure proper chain pairing.[129–131] They can also be generated directly from IgGs enzyme digest.[118,132]

The smallest format capable of retaining the binding properties of an human IgG is the scFv. scFvs, are a monovalent format that offer advantages in terms of ease of production and manipulation.[119] They are a versatile tool for investigating toxin binding *in vitro* and *ex vivo*, but they are typically not used as therapeutics due to their lower stability and susceptibility to aggregation, which could affect their efficacy.[133] ScFvs can be used as in discovery campaigns and afterward, the variable regions responsible for antigen binding can be grafted into Fabs or IgGs to improve neutralization potency.[119] ScFvs can be produced recombinantly in various expression systems, including bacteria and yeast.[134–136 133]

Nanobodies have shown promise in various therapeutic applications due to their small size and unique stability.[120,137] Their compact structure allows for superior tissue penetration, which could be advantageous in targeting venom toxins that act in specific anatomical compartments.[138,139] However, nanobodies' monovalent nature may lead to reduced binding affinity compared to IgGs, potentially impacting their neutralization potency. Additionally, their smaller size may result in faster renal clearance, necessitating more frequent administrations to maintain therapeutic levels.[140] Nevertheless, the easy

expression with high yields in bacteria or yeast combined with their stability makes them an attractive antibody format.[141,142]

4.2 Next-generation antivenom

Recombinant antivenom, an alternative to traditional animal plasma-derived antivenom, holds significant promise to create a more efficient and potentially cost-effective antivenom with fewer side effects.[104,143] There are two opposed approaches to discover antibodies: top-down and bottom- up.[95] The difference between top-down and bottom-up approaches in antibody discovery lies in the composition and control over the generated antivenom products. The top-down approach involves expressing a polyclonal pool of antibodies, resulting in an antivenom product with limited characterization, while the bottom-up approach focuses on identifying and expressing a defined panel of monoclonal antibodies, allowing for a carefully designed composition and minimum batch-to-batch variation. Oligoclonal recombinant antivenoms generated through the bottom-up approach offer the potential for a higher proportion of therapeutically active antibodies, potentially enabling lower dosing and manufacturing costs in the longer run.[113,144,145]

This thesis focuses on a "bottom-up" discovery process, where antibodies specifically targeting a chosen toxin are identified, providing complete control

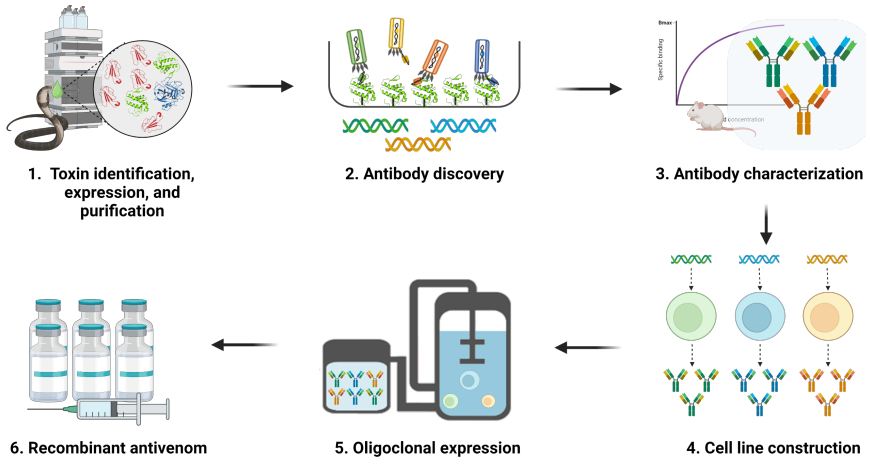

Figure 8. The "bottom-up" approach for the development of recombinant antivenom involves a series of steps. *First, medically significant toxins present in the venom are identified and purified. These purified toxins serve as the targets for antibody discovery. Once the antibodies are identified, they are further characterized for their effectiveness in neutralizing the toxins. To facilitate large-scale production, cell lines capable of expressing these antibodies are created. The expression of antibodies can be achieved using bioreactors or other suitable systems. The resulting product is a defined oligoclonal antibody mixture, which forms the basis of the new type of antivenom.*

over the components of the antivenom (Figure 8). To discover such antibodies, several techniques are available, including hybridoma technology, and display technologies, such as yeast display and phage display. In this project, the phage display method was utilized.[146–150]

Antibody phage display technology involves linking phenotype (binding capability represented by the displayed antibody) and genotype (DNA sequence encoding the antibody).[106–108] Large libraries can be constructed by isolating RNA or DNA encoding the antibody variable regions from B cells derived from immunized animals or human donors. The variable regions are then cloned into phage display vectors that encode a fusion protein of the antibody fragment and a phage coat protein.[151] The phage display campaign involves several rounds of selections: 1) Introducing the phage library to an antigen, 2) binding of phages displaying antigen-binding antibody fragments, 3) washing to remove unbound phages, 4) elution of specifically bound phages, and 5) amplification of the selected phages. Repeating these rounds 2-3 times enriches a pool of phages capable of binding the target antigen (Figure 9).[152,153]

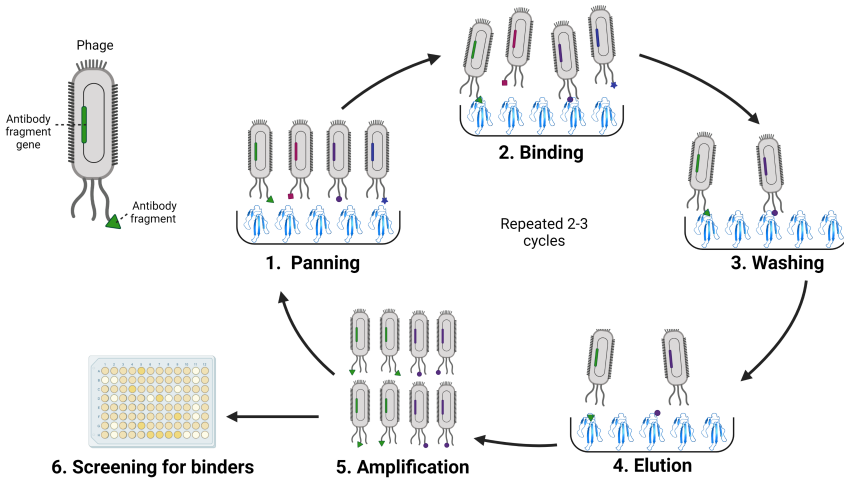

Figure 9. Phage-display technology. *1) A library of phages is subjected to panning against an antigen, allowing for 2) binding of phages displaying antibody fragments that can specifically bind to the antigen. 3) Unbound phages are subsequently washed away, followed by 4) elution of the specifically bound phages. 5) The eluted phages are then amplified by infecting E. coli. This cycle is iterated 2 to 3 times. After phage amplification, the phages are screened to identify potential binders.*

Antibody phage display libraries can originate from various sources. In a naïve library, the antibodies are derived from B cells that have not undergone natural affinity maturation. As a result, the library encompasses a broader range of antibodies with diverse specificities. In contrast, an immunized library is

generated from B cells obtained from donors who have been immunized with the target antigen. This approach enables the selection of antibodies that have been specifically elicited in response to the antigen of interest.[151,153] A naïve library usually contains antibodies with a broader diversity, while an immunized library is already optimized for certain antigens.[153–155] In this project, a naïve scFv library (Article II) and an immunized llama nanobody library (Manuscript II) were employed to discover antibodies with specificity for the target toxins.

The understanding of various antibody formats, such as IgG, Fab fragments, scFv, and nanobodies, plays a crucial role in designing targeted therapies. Recombinant antivenom, an alternative to traditional animal plasma-derived antivenom, has shown promise in this regard.[101,105,114] By leveraging techniques like phage display, antibodies can be identified with high specificity and affinity for particular toxins through iterative selection rounds based on different criteria. These criteria may include high affinity, cross-reactivity, or pH-sensitive antigen binding properties.[153] Utilizing both immunized and naïve libraries, researchers can further enhance the development of effective recombinant antivenom therapies with improved toxin specificity and therapeutic potential.[121,122,140]

4.3 Selecting the antibody format with the most relevant therapeutic properties

In recombinant antivenom development, antibody optimization plays a crucial role in addressing key challenges, such as improving pharmacokinetics, increasing stability, improving affinity, reducing immunogenicity, and increasing cross-reactivity.[156] Pharmacokinetics refers to how antibodies are absorbed, distributed, metabolized, and excreted in the body.[157] In addition to antibody optimization, the choice of antibody format, whether it be full-length IgG or nanobody, can significantly impact the therapeutic efficacy and potential applications of recombinant antivenom development, making it an important consideration in the overall design process.[133]

Balancing specificity and affinity is a crucial aspect of antibody optimization.[158] The antibody affinity represents the strength of the binding interaction between the antibody and its target, while the specificity refers to the ability of antibodies to selectively recognize and bind to their target.[159] There is indeed a tight relationship between both characteristics, and a compromise may sometimes be necessary to develop broadly neutralizing antibodies. However, the ultimate goal is to produce broadly neutralizing antibodies with high affinity to enhance the antivenom's neutralization potency.[121]

Addressing the challenge of immunogenicity is also essential in the process of antibody optimization for therapeutic applications. The use of antibodies as therapeutics can trigger immune responses in patients, compromising treatment efficacy and potentially leading to adverse effects.[160] To mitigate immunogenicity, various strategies are employed, such as humanization or fully human antibody design, to minimize immune reactions. Such evaluations are crucial to ensure the safety and efficacy of antibody-based therapies and minimize the risk of unwanted immune responses.[161]

In the pursuit of optimizing therapeutic antibody pharmacokinetics, it is crucial to select antibodies with pH-dependent antigen-binding characteristics. These antibodies exhibit lower affinity under low pH conditions, such as the acidic environment in endosomes, allowing for efficient recycling within the cellular system. [153] By releasing their cargo (toxin) for degradation and subsequently being recycled for further rounds of toxin neutralization, these antibodies reduce the overall dose needed and extend the duration of antibody action.[101,162] This strategy may improve in development of effective and long-lasting antibody-based therapies for neutralizing snake venoms.

In the context of neutralizing snake venoms across different species, the development of cross-binding antibodies capable of targeting multiple similar antigens becomes essential.[163,164] Rather than focusing on individual toxins, some researchers aim to identify antibodies that can target whole families of toxins, such as 3FTxs, PLA_2s, SVMPs, and SVSPs. This rational approach ensures a more comprehensive coverage against the diverse array of toxins found in snake venoms, enabling the creation of more effective and versatile antivenom therapies.[164,165]

If one considers the scenario in sub-Saharan Africa, where 18 medically relevant elapid snakes listed as category 1 by WHO exist.[81] Assuming each snake produces 5-10 distinct toxins that require neutralization, an antivenom targeting all snakes in the region would need to neutralize a staggering range of 90-180 toxins. To overcome this challenge, the use of broadly neutralizing antibodies is vital.

Broadly neutralizing antibodies offer the potential to reduce the number of antibodies required in the antivenom formulation. Instead of relying on a large number of antibodies (e.g., 90-180), researchers aim to develop antivenoms that can effectively neutralize venom from several different snakes using only 5-25 antibodies.[82,87,121,165] Such a reduction in the number of antibodies to produce not only streamlines the manufacturing process, reducing time and complexity,

but also enhances the economic viability of antivenom production by lowering associated costs.[101]

Broadly neutralizing antibodies can be obtained through various approaches. One strategy to enhance the diversity and cross-reactivity of antibodies in phage display selection is through a technique called cross-panning.[153] Cross-panning involves in successive rounds of selection. This exposure increases the probability of identifying antibodies that can, for example, recognize multiple antigens within the same toxin family. .[87,121,153] Additionally, designed toxins that resemble the average characteristics of a toxin family (consensus toxins) can be used as antigens to enable the discovery of antibodies that recognize common epitopes shared among different toxins.[153] The latter strategy was utilized in Manuscript II to discover broadly neutralizing antibodies.

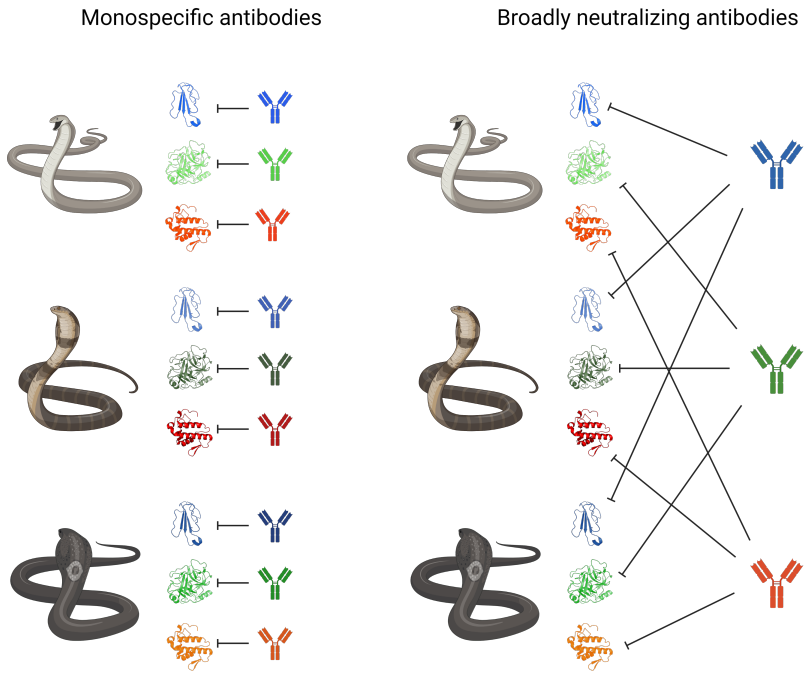

Figure 10. Broadly neutralizing antibodies can target similar toxins across snake species. *Instead of targeting each individual toxin produced by a snake, a more efficient approach involves the use of broadly neutralizing antibodies. These antibodies have the capability to neutralize multiple similar toxins found in various snake species. By employing such antibodies, a broader spectrum of venom components can be effectively neutralized.*

5. Consensus toxins

Consensus toxins are designed from conserved and homologous native sequences, representing a synthetic approach to capture the essential features and functional properties of a specific class of toxins.[166] Rather than creating chimeric proteins, which combine different regions or domains from unrelated toxins, consensus toxins are designed solely from the naturally occurring similarities and variations within a particular group of toxins.[167]

To obtain a consensus toxin, multiple sequence alignment is performed to identify conserved regions shared among similar proteins.[166,167] These conserved regions serve as the basis for designing a synthetic construct that preserves the common elements found in the native toxins.[168]

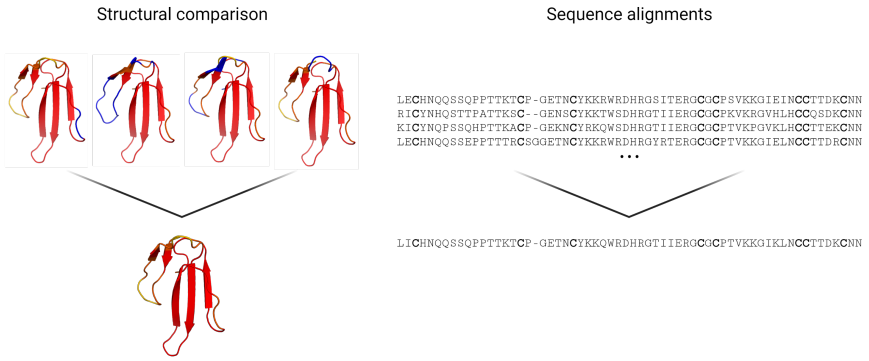

Figure 11. Designing consensus toxins. *The figure illustrates the concept of consensus toxins, which are synthetic proteins created by combining multiple natural toxins. The blue color in the structural comparison indicates regions with higher variability, while the red indicates more conserved regions across the structures. An example of a sequence alignment of SC-α-neurotoxins is shown, comparing the individual toxin sequences to the consensus sequence.*

In the context of this project, two consensus toxins were designed for α-neurotoxins found in African elapids. Specifically, an SC-α-neurotoxin and an LC-α-neurotoxin consensus toxins were designed, recombinantly expressed, and purified for their use in phage display-based antibody discovery campaigns. Previous studies have successfully employed consensus SC-α-neurotoxins to immunize horses, as de La Rosa et al. (2019) demonstrated.[169] The immunization efforts in these studies resulted in the generation of polyclonal antibodies capable of neutralizing venom from different elapid species. This finding highlights the potential of consensus toxins to elicit a broad immune response, leading to the production of antibodies with cross-reactivity against various venom components.[169]

Regarding the nature of broadly neutralizing antibodies, it is relevant to consider whether their effectiveness stems from polyclonality, involving the contribution of multiple specific monoclonal antibodies, or from the inherent broadly neutralizing properties of a few dominant monoclonal antibodies.[95] While the previous studies employing consensus toxins in horses have shown successful venom neutralization, it is yet to be determined whether the observed broadly neutralizing effect is primarily attributed to a few potent monoclonal antibodies or the collective action of a diverse set of monoclonal antibodies targeting different epitopes.[95,169]

It is worth noting that snake consensus toxins have not been previously utilized in phage display techniques. However, in other contexts, consensus toxins derived from spider toxins have been employed in phage display libraries.[170] This approach allows the identification of antibodies with the capacity to neutralize both spider and scorpion toxins.

Consensus toxins may revolutionize antivenom development by enabling the discovery of antibodies with a broader spectrum of activity. By focusing on conserved regions, this approach expands the possibilities for generating antibodies that can recognize and neutralize multiple toxins. This technical strategy has great potential for enhancing the effectiveness and versatility of antivenom, providing a more comprehensive solution for treating snakebite envenomation by enabling the production of antibodies with broader specificity. By generating antibodies that can neutralize venom from multiple snake species, a single antivenom product could cover a wider range of snakebites, reducing the need for multiple treatments specific to different snake species. This not only simplifies the decision-making process for healthcare professionals but also has the added benefit of potentially reducing the occurrence of side effects associated with administering multiple antivenom treatments. With a more targeted and comprehensive approach, this advanced antivenom holds promise for improving patient outcomes while minimizing the risks of adverse reactions, making it a significant advancement in snakebite treatment.

6. Recombinant protein expression

6.1 Different strategies to express proteins

The conventional approach used to investigate venoms and toxins relies on natural sources, such as animals that produce these compounds.[6,171,172] However, this approach poses numerous limitations, including difficulties in purifying specific toxins from the venom, low abundance of certain toxins, and challenges associated with obtaining the venom itself.[80,173–175] Furthermore,

exploring dormant genes within the natural sources is not feasible with the traditional approach.[176,177]

An alternative strategy is the recombinant production of toxins, which offers several advantages.[178] Firstly, it allows for the generation of highly pure toxin samples, eliminating impurities that may be present in natural sources. Additionally, recombinant expression often yields higher quantities of toxins compared to purification from natural sources. Furthermore, recombinant production provides the opportunity to design and engineer unnatural or artificial toxins with specific modifications enabling the exploration of toxin variations and mutants.[168,178]

In this project, the emphasis is placed on consensus toxins, which are synthetic proteins. Consequently, the production of these consensus toxins necessitates their expression through recombinant methods.

Previous studies have attempted to express α-neurotoxins using various expression systems, including different *Escherichia coli* expression systems and human embryonic kidney (HEK) cells.[179–182] However, the expression of α-neurotoxins remains a challenge, and few studies have thoroughly investigated whether the expressed toxins maintain structural integrity and functional properties comparable to their native counterparts. One of the primary

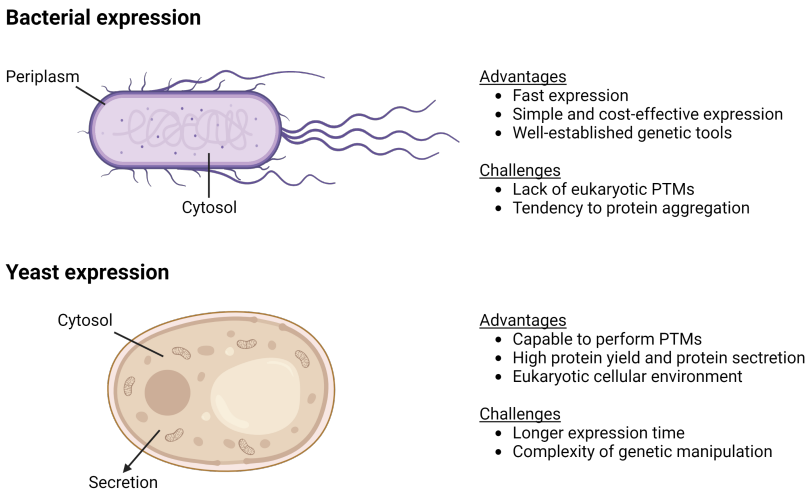

Figure 12. Advantages and challenges when using bacterial or yeast expression systems. *Bacterial expression commonly occurs in either the cytosol or periplasm, offering a fast and established system. However, bacteria may lack the necessary machinery for post-translational modifications (PTMs). In contrast, yeast expression allows proteins to be expressed in the cytosol or secreted into the media, benefiting from the yeast's greater capacity for PTMs and enabling the expression of more complex proteins. Nevertheless, yeast systems require longer expression times and genetic manipulation is more challenging compared to bacteria.*

challenges in expressing α-neurotoxins is their complex disulfide bond formation.[37,178,183] Disulfide bonds play a critical role in maintaining both the structural integrity and functional properties of these toxins. Therefore, the search for an optimal expression system for α-neurotoxins continues to be a key focus in this field.

In order to overcome these challenges, ongoing efforts are being made to identify the most suitable expression system for α-neurotoxins. Within the scope of this project, two distinct expression systems were employed: *E. coli* and *Komagataella phaffii* (formerly known as *Pichia pastoris*). For a comprehensive understanding of the advantages and limitations associated with these expression systems, a detailed discussion can be found in Article I, which extensively explores the intricacies of *E. coli* and *K. phaffii*, as well as other expression platforms (Figure 12).

6.2 Expression systems employed in this project

The *E. coli* expression system offers several advantages, including high protein yield, ease of genetic manipulation, and cost-effectiveness.[178,184] To optimize the expression of α-neurotoxins in *E. coli*, two different approaches were employed.

The first approach involved the use of the SHuffle strain, which is designed to enhance disulfide bond formation (Figure 13A).[185] This strain provides a favorable intracellular environment for the expression of disulfide-rich proteins. It expresses disulfide bond isomerases (DsbC) that facilitate proper protein folding by reshuffling disulfide bonds, increasing the likelihood of correct folding and functionality of the expressed α-neurotoxins.[186]

The second approach in the *E. coli* expression system utilized a modified CyDisCo system (Figure 13B). This system involves the co-expression of sulfhydryl oxidase (Erv1p) and two protein disulfide isomerases (PDIs), one

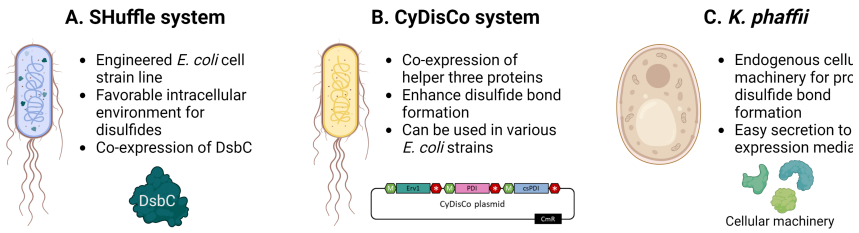

Figure 13. The three expression system utilized during this project. *A) The SHuffle is an engineered cell strain that creates a more favorable intracellular environment for the formation of disulfides. B) The CyDisCo system employs a helper plasmid, which express three proteins that enhance the disulfide bond formation. C) K phaffii is a eukaryotic cell that contains endogenous machinery that can help with the proper folding of disulfides.*

from humans and one from cone snails.[187,188] The modified CyDisCo system aims to enhance correct disulfide bond formation in *E. coli*.[189] It is notable that this system does not require a specific *E. coli* strain and can be used with any strain carrying the helper plasmid, providing flexibility in the choice of *E. coli* strains for expression.[190]

In addition to *E. coli*, the *K. phaffii* expression system was also employed (Figure 13C). *K. phaffii* is a yeast expression system that possesses cellular machinery capable of efficient disulfide bond formation.[191] This system offers advantages in terms of post-translational modifications and proper protein folding, making it a suitable choice for expressing complex disulfide-rich toxins, including the α-neurotoxins and the consensus toxins for these.[192,193]

The production of structurally correct and pure toxins is of utmost importance in the context of recombinant antivenom development, as phage display relies solely on molecular recognition without the aid of the immune system. Achieving this requires the use of expression systems and optimization strategies that facilitate the generation of recombinant toxins closely resembling their native counterparts in both structure and function, while ensuring high levels of purity.

By obtaining recombinant toxins that closely mimic their native counterparts, there is a significant increase in the likelihood of the discovered antibodies effectively recognizing and neutralizing the native toxins. This approach enhances the efficacy of subsequent immunization processes, as the antibodies generated have a higher probability of targeting the relevant toxin epitopes. The result is the development of antivenoms with improved therapeutic potential and a greater capacity to counteract the venom's harmful effects.

7. An innovative approach to snakebite antivenom development

Snakebite envenoming poses a significant health risk in regions with venomous snakes. Conventional antivenom has limitations in terms of efficacy, specificity, and availability. To overcome these challenges, researchers are exploring innovative approaches for antivenom development. This includes optimizing antibodies to enhance their potency and broaden their specificity, enabling them to neutralize a wide range of snake toxins. Additionally, the use of consensus toxins, synthetic molecules designed from conserved sequences, aids in the discovery of broadly neutralizing monoclonal antibodies that can target multiple toxins across different snake species. Recombinant expression systems, such as *E. coli* and *K. phaffii*, are utilized to produce structurally intact and functional α-neurotoxins and consensus toxins. These techniques offer advantages such as higher purity, increased yield, and the ability to modify and

design toxins for research purposes. The combined efforts of antibody optimization and recombinant protein expression contribute to the development of more effective antivenoms, improving our understanding of snake venom composition and providing innovative solutions for snakebite envenoming.

Chapter 2 – Article I

Strategies for heterologous expression, synthesis, and purification of animal venom toxins, *Frontiers in Bioengineering and Biotechnology*, 2022, Volume 9

This scientific review provides a comprehensive overview of the advantages, drawbacks, and challenges of animal toxin expression using different heterologous expression systems. It discusses various strategies for expressing toxins in different types of cells, including bacteria, yeast, insect, and mammalian cells. Additionally, it explores alternative approaches such as cell-free biosynthesis and peptide synthesis that do not involve cellular expression. The review also covers the use of tags and fusion proteins to facilitate expression, enhance solubility, and standardize purification. This article highlights the advantages and disadvantages of these systems in toxin production and discusses the potential applications of native as well as designed recombinant toxins, such as their usefulness in the discovery of broadly neutralizing antibodies and their potential use as toxin-derived drugs. Finally, it addresses the importance of biosafety considerations when working with highly bioactive proteins.

In relation to the research performed behind this thesis, the review provides a comprehensive analysis of the benefits of using bacterial and yeast systems for heterologous expression of toxins. It also discusses the advantages of recombinant toxin production over purification from natural sources.

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REVIEW
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Strategies for Heterologous Expression, Synthesis, and Purification of Animal Venom Toxins

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Animal venoms are complex mixtures containing peptides and proteins known as toxins, which are responsible for the deleterious effect of envenomations. Across the animal Kingdom, toxin diversity is enormous, and the ability to understand the biochemical mechanisms governing toxicity is not only relevant for the development of better envenomation therapies, but also for exploiting toxin bioactivities for therapeutic or biotechnological purposes. Most of toxinology research has relied on obtaining the toxins from crude venoms; however, some toxins are difficult to obtain because the venomous animal is endangered, does not thrive in captivity, produces only a small amount of venom, is difficult to milk, or only produces low amounts of the toxin of interest. Heterologous expression of toxins enables the production of sufficient amounts to unlock the biotechnological potential of these bioactive proteins. Moreover, heterologous expression ensures homogeneity, avoids cross-contamination with other venom components, and circumvents the use of crude venom. Heterologous expression is also not only restricted to natural toxins, but allows for the design of toxins with special properties or can take advantage of the increasing amount of transcriptomics and genomics data, enabling the expression of dormant toxin genes. The main challenge when producing toxins is obtaining properly folded proteins with a correct disulfide pattern that ensures the activity of the toxin of interest. This review presents the strategies that can be used to express toxins in bacteria, yeast, insect cells, or mammalian cells, as well as synthetic approaches that do not involve cells, such as cell-free biosynthesis and peptide synthesis. This is accompanied by an overview of the main advantages and drawbacks of these different systems for producing toxins, as well as a discussion of the biosafety considerations that need to be made when working with highly bioactive proteins.

Keywords: animal toxins, venom, neurotoxin, heterologous expression, recombinant toxins, recombinant protein expression, bioinsecticide, toxin-inspired drug

1 INTRODUCTION

Animal venoms present a treasure trove of biologically active compounds that have evolved to perform highly specialized biochemical tasks, particularly in the contexts of defense against predators and prey capture (Arbuckle et al., 2017; Rivera-de-Torre et al., 2020). These venoms are complex mixtures of peptides and proteins displaying toxic activity, commonly known as toxins, salts, and small metabolites, such as neurotransmitters and nucleosides. To deliver their toxins, animals have evolved different types of piercing structures, such as fangs in snakes, stingers in scorpions, or chelicerae in spiders; by causing physical damage to the skin of prey and perceived predators. Venomous animals deliver their toxins inside the body of their victims, thereby surpassing physical barriers that would normally protect against foreign substances. Moreover, many venoms contain proteolytic enzymes such as metalloproteases, hyaluronidases, and disintegrins that may digest extracellular matrix proteins, causing necrosis to the victim and easing the access of other toxins to their final targets. Other venom components can then compromise cell viability by damaging the cell membrane (e.g., phospholipases A_2 and pore-forming proteins), affect cell signaling pathways by blocking or activating ion channels (i.e., neurotoxins) (Calvete, 2017; Rivera-de-Torre et al., 2019), or interfere with the blood homeostasis either *via* procoagulant activities (Isbister, 2009) or vasodilatation (Kakumanu et al., 2019). Toxin diversity is thus enormous across the animal kingdom, and it is important to understand the underlying mode of action of medically relevant toxins on their targets in order to devise and evaluate novel therapeutic interventions that serve to neutralize their effects (Salvador et al., 2017; Chinnasamy et al., 2020) or to exploit their bioactivities for therapeutic or biotechnological purposes (Brown and Alewood, 2001; Holford et al., 2018).

To date, most toxin research has relied on sourcing toxins directly from animal venoms (Ahmadi et al., 2020). However, a given toxin represents only a small percentage of the whole venom, which means a low purification yield *via* classic processes such as fractionation. Moreover, the purity of the target toxin is often suboptimal when isolated from whole venoms, complicating subsequent research (Rohou et al., 2007). Toxins are not only scarce and difficult to obtain from the natural source; some toxins are not even present in the venom as they are encoded by dormant genes. Fortunately, the ever-increasing availability of genomic, transcriptomic, and proteomic data from venomous animals has allowed the discovery of dormant or low-expression genes (Palagi et al., 2013; Rivera-de-Torre et al., 2018; Herzig et al., 2020; Walker et al., 2020).

Given the challenges of obtaining rare and low-abundance toxins, other approaches must be taken for procuring animal toxins to fully exploit the potential that lies within their diversity. In this relation, heterologous expression of toxin genes in a laboratory setting presents an exciting and promising alternative to extracting animal toxins from their natural source. This process involves the expression of genes or part of them in a host organism that does not express such genes intrinsically and comes with many advantages. For instance, heterologous expression allows for high yield toxin production while ensuring homogeneity and avoiding cross-contamination with other venom components. Ensuring purity is especially important because toxins are usually part of multigene families, which is why the separation of isoforms by classic chromatographic fractionation might not yield sufficiently pure toxins for particular experiments. Also, heterologous expression strategies minimize the need for animal use in venom research, thereby reducing the risks of accidental envenomations and the stress of animal handling. Thus, heterologous expression also supports the 3Rs in animal research: replacement, reduction, and refinement (Hallen et al., 2007; Valle et al., 2015; Calvete, 2017).

Additionally, the heterologous production of recombinant toxins is not restricted to natural versions of the toxins. The process of expressing toxins heterologously can take advantage of the plethora of molecular biology tools available to design and produce new toxins with unique and desirable properties, which are not present in nature. For example, consensus toxins are artificially designed toxins resembling an average sequence of a collection of natural toxins that might possibly be useful as antigens to obtain broadly neutralizing antibodies that can cross-neutralize multiple native toxin isoforms (de la Rosa et al., 2018, 2019). Moreover, toxins can be modified to modulate their target selectively to induce a therapeutic rather than a harmful toxic effect (Liu et al., 2016; Almeida et al., 2017, 2019).

In this review, we present the possibilities offered by the principal heterologous expression systems (bacteria, yeast, insect cells, and mammalian cells) for the heterologous expression of toxins as well as strategies for producing toxins without cells, such as cell-free biosynthesis or chemical synthesis of peptides. We also discuss the most useful molecular biology features that should be considered to enhance purification and exploit downstream applications. Finally, we highlight some of the most promising research efforts involving toxin expression, e.g., antivenom research, development of bioinsecticides, toxin-derived drug development, and the bioethical considerations surrounding such research activities.

2 CLASSIFICATION OF TOXINS

Designing a successful toxin expression strategy starts with the analysis of the target toxin characteristics. The biochemical and biophysical features of the target toxin may limit the selection of the most appropriate expression host system. Therefore, accurate classification of toxins is key to predict toxin characteristics, as many homologous toxins possess similar biophysical properties.

Due to the breadth and long history of toxinology, toxin classification has become complex since the most classical categories based on toxic activity coexist with the latest classifications based on protein structure. One of the most basic toxin classifications relies on their ecological role, since toxins serve a distinct purpose and primarily help fulfill three functions: 1) prey capture, 2) defense against predators, and 3) intraspecific competition, for each of which a given toxin has

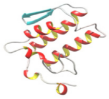
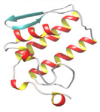
Name	Myotoxin II	β-bungarotoxin	Phospholipase A_2
Species	*Bothrops asper*	*Bungarus multicinctus*	*Naja nigricollis*
UniprotKB	P24605	P00617	P00605
3D structure			
Toxic activity	Myotoxic	Anticoagulatory	Neurotoxic

FIGURE 1 | Comparison of Myotoxin II from *Bothrops asper*, β-bungarotoxin from *Bungarus multicinctus* and a phospholipase A_2 (PLA_2) from *Naja nigricollis*. They are homologous proteins that cluster together due to sequence similarity and share an archetypical PLA_2 fold. Nevertheless, they differ in their toxic activity.

evolved to perform a highly specialized task (Casewell et al., 2013). This abundance of biochemical opportunities has resulted in the enormous diversity of weaponized proteins and peptides that now exist in nature (Casewell et al., 2013). Scientists have categorized toxins based on different variables such as structure similarity and domain homology (Tasoulis and Isbister, 2017). For instance, considering their structural homology, toxins can be grouped in families, including three-finger toxins, cysteine-rich secretory proteins, disintegrins, L-amino acid oxidases, hyaluronidases, metalloproteases, natriuretic peptides, phospholipase A_2s, C-type lectins, and venom Kunitz-type toxins, to name some. However, one can also group toxins based on their toxic activity, i.e., which physiological system they target (e.g., the cardiovascular, nervous, or immune system), what the specific protein activity is (e.g., myotoxic, neurotoxic, or cardiotoxic), or which pharmacological target they have (e.g., the nicotinic acetylcholine receptor, or voltage-gated sodium/potassium channels) (Fry et al., 2009, 2012; Zhang, 2015). Naturally, structural and functional classifications are interrelated, and some specific folds are directly related to certain toxic activities, e.g., Kunitz-type toxins are usually neurotoxins. However, toxins that cluster together based on structural homology do not necessarily cluster based on function. For instance, while myotoxin II from *Bothrops asper* (P24605), beta-bungarotoxin from *Bungarus multicinctus* (P00617), and PLA_2 from *Naja nigricollis* (P00605) are all PLA_2s, they differ widely in their activity. Indeed, P24605 shows myotoxic, P00617 anticoagulatory, and P00605 neurotoxic activity (**Figure 1**).

Finally and especially relevant for heterologous expression, toxins can be classified based on which post-translational modifications (PTMs) they undergo, such as N-glycosylation, O-glycosylation, disulfide-bond formation, methylation, C-terminal amidation, epimerization, bromination, and hydroxylation of proline, amongst others (Walsh et al., 2005; Wood et al., 2009; Degueldre et al., 2017) (**Figure 2**). Toxins are

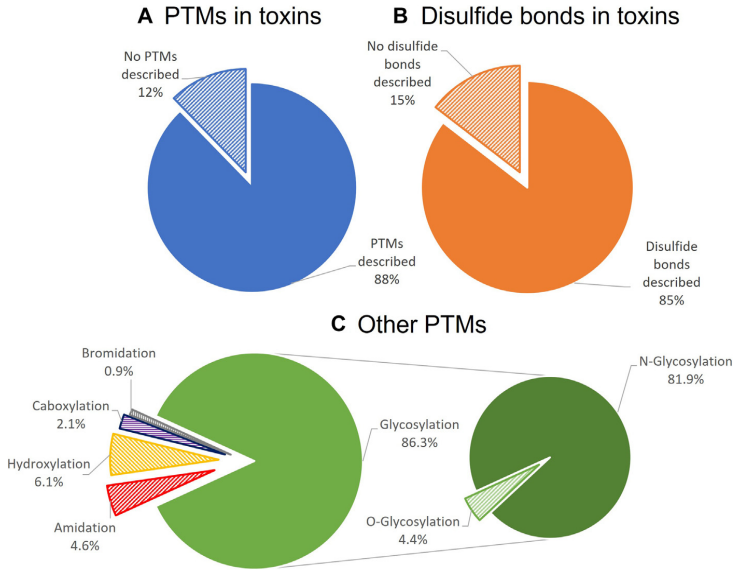


FIGURE 2 | Distribution of PTMs among toxins listed in Uniprot-Toxprot, the Animal Toxin Annotation Project. Only 12% of the listed animal toxins do not have any described PTMs **(A)**. From the toxins with PTMs, 85% had disulfide bonds **(B)**. After disulfide bonds, glycosylation, and especially N-glycosylation, is the most common PTM described for toxins **(C)**.

secreted proteins translated as preproproteins and processed in the endoplasmic reticulum (ER), where a wide variety of PTMs occur as soon as the nascent peptide is exposed to the modifying enzymes. PTMs enable great biochemical diversity of bioactive peptides and often play an essential role in activity, chemical properties, and structural stability. N-glycosylation is one of the most prevalent PTMs in toxins, and the carbohydrate moiety usually has a critical role in toxin stability and solubility. The carbohydrate moiety can also modulate protein functionality and affect enzymatic activity or target recognition (Sandro and Leandro, 2009), as has been described for metalloproteases, three-finger toxins, and serine proteases (Osipov et al., 2004; Silva-Junior et al., 2007; Sandro and Leandro, 2009). For example, the hemorrhagic rhodostoxin from the Malayan pit viper, *Calloselasma rhodostoma,* changes its substrate specificity upon deglycosylation (Tan et al., 1997). Some PTMs have even been shown to occur spontaneously, as was the case for the recombinantly expressed scorpion toxins MeKT11-1 and MeKT11-3, which in aqueous solution underwent cyclization of the N-terminal glutamine, forming pyroglutamate (Kuzmenkov et al., 2018). Even though disulfide bond formation and glycosylation can be achieved in microbial eukaryotic systems, such as yeast, most PTMs need specific enzymatic routes that not all heterologous systems can provide.

An assortment of expression host options is available, ranging from simple bacteria to the most advanced mammalian cell cultures, and choosing the most appropriate expression host for a given toxin can be informed by prior knowledge about the protein. For instance, the structural classification and the possible PTMs provide critical physicochemical information about the target toxin solubility, functionality, stability, and expected yield, narrowing the heterologous expression host options.

3 SYSTEMS FOR THE HETEROLOGOUS EXPRESSION OF TOXINS

3.1 Bacteria: *Escherichia coli*

Since the first functional recombinant protein was expressed in 1977 (Itakura et al., 1977), bacteria have been the most widely used system for heterologous expression of proteins. Consequently, over time, a whole plethora of tools have been developed to improve bacteria for protein expression in both small and large scales.

The domain Bacteria comprises a vast number of physiologically and metabolically well-characterized organisms. Fundamental research on bacterial physiology has provisioned a knowledge-based framework to rationally design processes in a sophisticated manner (Carr and Church, 2009; Choe et al., 2016). The acquired knowledge has led to a collection of genetically engineered bacterial chassis for heterologous toxin expression. Among all bacterial hosts used for recombinant protein production, *Escherichia coli* is the most widely utilized. *E. coli* adapts to a large range of physical and chemical culture conditions while accumulating recombinant proteins up to 80% of its dry weight. Using *E. coli*, it is possible to express proteins that are safe to administer as biotherapeutics, with efficient methods to remove endotoxins in place (Mamat et al., 2015; Schneier et al., 2020). Even though bacterial systems possess considerable advantages, the main challenge in producing toxins from eukaryotic organisms in bacteria is the correct formation of disulfide bonds and the incorporation of PTMs that prokaryotic systems cannot introduce. Nevertheless, many genetic tools and techniques exist for expressing recombinant proteins, such as optimized bacterial strains, co-expression with chaperones or foldases, and the use of various promoters for tightly regulated expression (**Figure 3**).

Animal toxins have been recombinantly expressed in microbial systems since the early 1990s (Boyot et al., 1990; Fiordalisi et al., 1991; Park et al., 1991; Dudler et al., 1992; Kelley et al., 1992). To date, bacterial expression still remains the preferred system for the heterologous expression of toxins, especially for small and cysteine-less toxins like actinoporins from sea anemones (Alegre-Cebollada et al., 2007). Bacterial expression has been successfully used to produce the majority of scorpion toxins produced so far (Amorim et al., 2018), and it has been widely used to express snake toxins (Clement et al., 2016; Shulepko et al., 2017; David et al., 2018; Guerrero-Garzón et al., 2018; Russo et al., 2019), conotoxins from cone snails (Yu et al., 2018; Nielsen et al., 2019; Liu et al., 2020), and spider toxins (Meng et al., 2011; Souza et al., 2012; Chassagnon et al., 2017; Wu et al., 2017). Nevertheless, as mentioned before, PTMs and notably, complex disulfide-bonding patterns pose a considerable challenge for the general use of bacterial expression systems.

Animal venoms are an extremely diverse source of cysteine-rich proteins and peptide-based toxins. Cysteines are usually involved in intramolecular disulfide bridges (Pennington et al., 1999), critical for the structural integrity of the toxins in the extracellular environment, although covalent oligomerization also occurs (Osipov et al., 2004, 2008). Finally, cysteines can also participate in toxin activity *via* disulfide tethering with their target (Gajewiak et al., 2014), further illustrating the critical importance of disulfide bonds in toxins.

Even though a few PTMs can occur spontaneously, by far, most PTMs, including disulfide bonds, require enzymatic catalysis. For example, C-terminal amidation can be critical for toxin function and folding (Benkhadir et al., 2004; Kang et al., 2011), and while prokaryotes do not possess the enzymes for the amidation pathway, this PTM has been successfully introduced in a subsequent biochemical step (Ray et al., 1993). Nevertheless, synthetic production (discussed in **Section 5**) is usually preferred for production of amidated toxins. Achieving this and other modifications *in vivo* requires co-expression of the responsible enzymes (Du et al., 2019). To further compound this problem, in many cases, the responsible enzymes remain unknown.

Considering the addressed drawbacks of bacterial expression, producing toxins in bacteria might seem suboptimal. However, the simplicity of bacterial expression systems in combination with strategies specially engineered for the production of disulfide-rich proteins can circumvent many of the inherent drawbacks. Additionally, misfolding of target proteins can lead to the formation of inclusion bodies, which might be a beneficial starting point for protein purification.

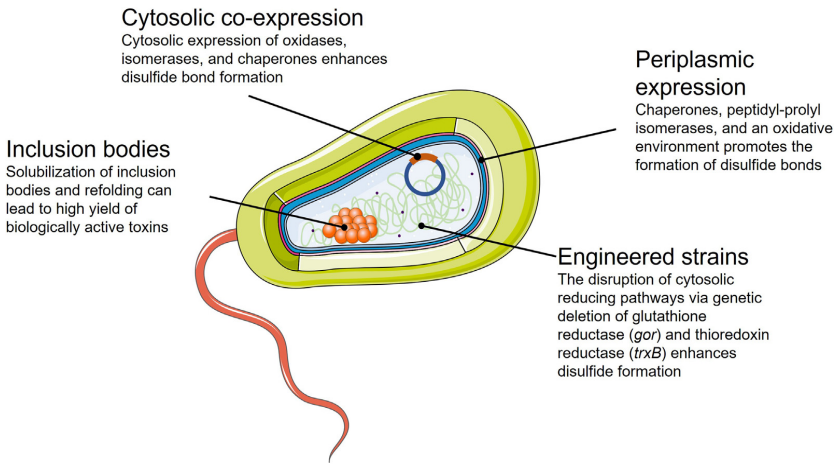


FIGURE 3 | Summary of the strategies available for the successful expression of toxins in an *E. coli* bacterial expression system.

3.1.1 Strategies for the Expression of Disulfide-Rich Toxins in *E. coli*

3.1.1.1 Periplasmic Expression

A major challenge with intracellular expression of disulfide-rich peptides in *E. coli* is the low yield of correctly folded protein due to the reducing environment of the bacterial cytoplasm. One of the commonly used solutions is to bypass the cytoplasm and have the nascent protein secreted into the periplasm of the bacterium.

In the periplasmic space, correct protein folding is promoted by the presence of chaperones, catalysts of disulfide bond formation, and peptidyl-prolyl isomerases (Goemans et al., 2014). Product secretion to the periplasm occurs when the gene of interest includes a short signal sequence at the N-terminus. The signal sequence directs the precursor proteins to the protein export systems in the cytoplasmic membrane and allows the protein to be translocated across to the periplasmic space. During translocation, the signal sequence is proteolytically removed by signal peptidases, ensuring the N-terminal authenticity of the expressed mature protein (Paetzel et al., 2002).

Periplasmic expression in combination with fusion proteins (see **Section 4** below) has been extensively used for the expression of toxins. Sequira et al. described a robust toxin expression method in which thousands of toxins were expressed in the periplasmic space fused to the protein disulfide isomerase DsbC (Sequeira et al., 2017; Turchetto et al., 2017). This strategy has been successfully applied for the expression of myotoxins (Giuliani et al., 2001), Kunitz-type toxins (He et al., 2008), hereunder dendrotoxins from snakes (Smith et al., 1997), conotoxins from cone snails (El Hamdaoui et al., 2019), neurotoxins from spiders (Chow et al., 2020), and beta-defensins from sea anemones (Anangi et al., 2012) to name some.

Unfortunately, signal sequences have unpredictable effects on the production yields of recombinant proteins, and it is not possible to predict how a given signal peptide will perform in combination with a recombinant toxin. Therefore, it is recommended to initially screen signal sequence libraries and check the secretory performance for production of the toxin of interest (Freudl, 2018).

Another related drawback of periplasmic expression is the limited yield of the expressed toxin due to the low throughput capacity of inner membrane transport and the volumetric capacity of the periplasmic compartment. However, it has recently been demonstrated that the harmonization between the target gene expression intensity and the translocon capacity is of importance in the improvement of the production yields for periplasmic expression (Schlegel et al., 2013; Baumgarten et al., 2018). Precise control of the expression intensity of the gene encoding the target protein permits the translocation machinery not to be saturated, and the protein production in the periplasm to be optimized.

3.1.1.2 Engineered Bacterial Strains and Co-Chaperone Expression

Whilst translocation of larger proteins into the periplasm can be inefficient and thus reduce yields, bacterial systems have been developed to allow high yield expression of disulfide-rich proteins within the cytoplasm, such as unique *E. coli* strains. Two genetically engineered *E. coli* strains commercially available are SHuffle® (New England Biolabs) and Origami™ (Novagen). These *E. coli* strains promote disulfide formation by disrupting the cytosolic reducing pathways *via* genetic deletion of glutathione reductase (*gor*) and thioredoxin reductase (*trxB*) (Stewart et al., 1998; Bessette et al., 1999) to create a more oxidizing environment. In addition, the SHuffle® strain expresses a periplasmic disulfide isomerase (DsbC) in the cytoplasm to enhance native disulfide-bond formation (Lobstein et al., 2012). SHuffle® and Rosetta-gami™ (an Origami™ derivative)

strains have been employed in the recombinant production of venom peptides (Li et al., 2006; Sermadiras et al., 2013; Clement et al., 2016). However, they tend to exhibit low growth and yield (Nozach et al., 2013), and successful expression of correctly folded disulfide-rich peptides can require the co-expression of other chaperones (Levy et al., 2001). This is the case for the CyDisCo system (Nguyen et al., 2011; Gaciarz et al., 2016), characterized by the co-expression of two redox enzymes: the mitochondrial oxidase Erv1p from *Saccharomyces cerevisiae* and human protein-disulfide isomerase (hPDI). Erv1p provides the oxidizing equivalents to generate disulfide bonds *de novo*, and hPDI isomerizes non-native disulfides. The CyDisCo co-expression system has been shown to accommodate highly complex disulfide-bonded proteins (Moilanen et al., 2018). In contrast to the SHuffle and Origami strains, where the reducing pathways are disrupted, the CyDisCo system uses an active enzyme system to improve the formation of disulfide bonds and is highly versatile, as it can function in any *E. coli* strain. This system has also been slightly modified to include co-expression of a conotoxin-specific PDI (csPDI) that was found to significantly accelerate folding of conotoxins *in vitro* (Safavi-Hemami et al., 2016; Nielsen et al., 2019). Recently, the CyDisCo system has been modified to create a more stable version of the system (called DisCoTune), which alleviates potential problems with resource competition (Bertelsen et al., 2021).

3.1.2 Purification From Inclusion Bodies

Despite the multiple molecular biology strategies to express proteins in bacteria that have been presented, expression of recombinant toxins might result in protein aggregates packed into inclusion bodies (IBs). IBs protect *E. coli* from the potential toxicity of the expressed protein, leading to an accumulation that could increase the expression yield. Most protocols are developed and optimized to avoid the appearance of IBs, since it is more straightforward to purify the correctly folded and soluble protein from the periplasm or cytosol. Thus, even though IBs may contain a high percentage of active proteins that can be extracted under non-denaturing conditions (Peternel et al., 2008), the toxins present in IBs are often misfolded and inactive. To alleviate this issue, excellent procedures do, however, exist for the solubilization of inclusion bodies and refolding of their protein content, which consist primarily of the protein of interest. Therefore, several toxins have been expressed in IBs and refolded into structurally stable, bioactive molecules with a high yield and purity (Bayrhuber et al., 2006; Shulepko et al., 2017). Many toxins are remarkably stable and can withstand extreme temperature and pH conditions without denaturation. Therefore, to refold toxins, it is necessary to first disrupt their three-dimensional structure by using chaotropic agents (e.g., guanidinium chloride or urea) that disrupt the hydrogen-bonding network between water molecular solvating the toxin in combination with reducing agents (e.g., dithiothreitol or β-mercaptoethanol), which break incorrectly formed disulfide bonds (Rudolph and Lilie, 1996; Saikia et al., 2021). The refolding process consists of eliminating the denaturating agents through dilution, dialysis, or gel filtration, usually at low temperatures for hours to weeks. Even though it is well known that the protein fold is encoded in its amino acid sequence and the folding process is driven by thermodynamically favored intermediates (Anfinsen, 1973), the specific folding pathway is typically unpredictable. In the case of disulfide-rich toxins, the folding process goes through stable intermediates that require partial unfolding to expose buried non-native disulfide bonds to the action of disulfide reshuffling agents. The disulfide pair reorganization task is catalyzed intracellularly by thiol-disulfide oxidoreductases, e.g., protein disulfide isomerases (PDI). *In vitro*, disulfide isomerization can be achieved using enzymes or redox pairs, such as reduced and oxidized glutathione (GSH/GSSG) in a mildly basic pH environment that promotes the nucleophilic attack of the thiolate anion (Hingorani and Gierasch, 2014; Saikia et al., 2021). Refolding conditions, including temperature, pH, ionic strength, and other specific additives, must be defined and selected on a case-by-case basis, as it is difficult to predict *a priori* (Saikia et al., 2021). Favoring the formation of IBs is not the classical approach but can be worth consideration due to the potential high yield and purity (Hoffmann et al., 2019).

3.2 Yeast: *Pichia pastoris*

Prokaryotic expression systems are relatively easy to manipulate and scale up. However, producing proteins from eukaryotic organisms (e.g., animal toxins) in such systems might result in misfolding and lack of PTMs and, as a consequence, result in loss of protein function. Yeast expression systems present an excellent alternative that can address this issue.

Whilst a miscellany of yeast strains exist, *Saccharomyces cerevisiae* and *Pichia pastoris* are the most widely used yeast expression systems. Both can produce disulfide-bonded and glycosylated proteins. However, *P. pastoris* lacks the mannosyl transferase, which yields immunogenic α-1, 3-linked mannosyl terminal linkages in *S. cerevisiae* (Darby et al., 2012) and is, therefore, a preferred system to produce proteins for biotherapeutic purposes.

P. pastoris is a methylotrophic yeast commonly used because of its ability to produce proteins in exceptionally high-density cultures. The shuttle vectors used in *P. pastoris* lack episomal status; they are integrated into the *P. pastoris* genome, generating stable and productive strains (Li et al., 2007). This yeast expression system offers a high yield for proteins that, so far, have not been successfully produced in bacteria, such as zinc-metalloproteases from snakes or hyaluronidases from scorpions (Zhu et al., 2010; Jangprasert and Rojnuckarin, 2014; Amorim et al., 2018). For other toxins that can be produced in bacteria, the yield increases when expressed in *P. pastoris*, as seen in the case of the potent blocker of Acid-Sensing Ion Channel 3, the APETx2 from a sea anemone. This protein has a potential application in the treatment of chronic pain, and it has been produced in both bacteria and yeast, showing a four-fold higher yield when produced in *P. pastoris* (Anangi et al., 2012). Snake venom serine proteases and neurotoxins from funnel-web spiders have also been successfully produced in *P. pastoris* in high yield (Pyati et al., 2014; Boldrini-França et al., 2015).

P. pastoris only produces low levels of endogenous secretory proteins, making purification of recombinant proteins from the culture media straightforward. Therefore, one of the most

popular strategies for protein production in *P. pastoris* involves the fusion of the protein of interest with a signal sequence from *Saccharomyces cerevisiae*: the alpha-mating factor pre-pro signal peptide (α-MF). The α-MF secretion signal consists of two parts: a 19-amino acid N-terminal signal sequence that directs translocation into the ER, followed by a 66-amino acid pro region that mediates receptor-dependent packaging into ER transport vesicles to the extracellular media. However, if the protein of interest that is fused to the α-MF secretion signal folds rapidly in the yeast cytosol, the protein may be unable to cross the ER membrane and enter the secretory pathway. For the expression of toxins in the extracellular media, *P. pastoris* has been used successfully (Anangi et al., 2012; Ahmad et al., 2014; Pyati et al., 2014), although secretion levels have often been variable and dependent on the target protein. There is no golden standard for the secretion of recombinant proteins in *P. pastoris*, and numerous new signal peptides have been found in recent years. As described for periplasmic expression in *E. coli*, signal peptide screening and optimization are necessary to exploit the possibilities for *P. pastoris* expression systems (Aw et al., 2018; Duan et al., 2019).

Although most toxin expression experiments have been performed using the common *P. pastoris* strain X-33 (Guo et al., 2001; Anangi et al., 2010; Zhu et al., 2010; Jangprasert and Rojnuckarin, 2014), some foreign proteins are unstable in *P. pastoris* culture medium due to the action of secreted proteases. To this end, optimizing the culture conditions, such as altering the temperature or pH of the media, or switching to alternative carbon sources, is critical for enhancing yield and reducing toxin degradation. Nevertheless, there are several protease-deficient strains (e.g., SMD1163, SMD1165, and SMD1168) that have been shown effective in reducing degradation (Ahmad et al., 2014; Karbalaei et al., 2020).

As described before, N-glycosylation can have a critical effect on proper protein folding and activity of toxins. However, for therapeutic applications non-human glycosylation patterns are often involved in immunogenic responses that can even lead to anaphylactic shock (Zhou and Qiu, 2019). Therefore, the potential biotherapeutic application of recombinantly expressed toxins necessitates the use of expression systems for which the glycosylation patterns are tolerated by humans. For this purpose, a *P. pastoris* strain, Pichia GlycoSwitch® has been engineered to reproduce "human-like" glycosylation patterns, resulting in reduced immunogenicity of protein products (Karbalaei et al., 2020). Additionally, further modifications, such as PEGylation of toxins produced in yeast, have been demonstrated to lead to toxin products with reduced immunogenicity and extended half-life (Pinheiro-Junior et al., 2021).

P. pastoris is considered an outstanding cell factory for industrial production of recombinant proteins. It is a microbial system relatively easy to scale up in batch/fed-batch systems. Continuous cultivation in bioreactors is also a feasible option with numerous advantages, such as reduction of the running cost and minimization of equipment (Nieto-Taype et al., 2020). Nevertheless, optimization is required to achieve maximum productivity, particularly regarding methanol and sorbitol concentrations, temperature, and incubation times (Karbalaei et al., 2020). Finally, some proteins expressed in *P. pastoris* can be hyper-glycosylated in comparison to their wild-type version, resulting in products with reduced or without biological activity (**Figure 4**).

3.3 Insect Cells: Baculovirus Expression Systems

Insect cell expression systems are an excellent alternative to microbial heterologous expression systems, as these can provide high yield production of functional toxins in a high-throughput format (Hitchman et al., 2012) at a lower cost than mammalian expression systems (López-Vidal et al., 2015). Unlike bacteria, insect cells possess chaperones for correct folding of complex toxins, such as cysteine-rich peptides, and the necessary metabolic routes for complex PTMs, such as glycosylation or acetylation, that do not necessarily exist in microbial systems (Quintero-Hernández et al., 2011). Furthermore, considering the biosynthetic route of toxins produced by arthropods (e.g., arachnids, insects, myriapods), insect cells are often the closest available host system in terms of protein expression processing for animal toxins (Chambers et al., 2018). For example, Pctx1, an inhibitor cystine knot spider toxin, and LALLT, a *Loxosceles* allergen-like toxin have been successfully expressed in insect cells without the need for *in vitro* refolding, unlike bacterial and yeast expression for the same toxin (Escoubas et al., 2003; Justa et al., 2020).

One of the main drawbacks of insect cell expression systems is the complexity of the setup compared to microbial systems in terms of facilities and biochemical tools needed to establish cell lines. However, over the last 30 years, insect cell expression systems have experienced a remarkable evolution, with new versatile and flexible tools and methods being developed (Possee and King, 2016; Chambers et al., 2018).

Insect cell expression systems are presented in various formats that have been extensively used for toxin expression, from whole insect systems, such as silkworm expression systems (Kato et al., 2010), to the most commonly used, cultured insect cell lines, like the baculovirus expression vector system (BEVS) (Deng et al., 2019; Justa et al., 2020; Schemczssen-Graeff et al., 2021). Baculoviruses are a group of viruses that infect insects and are harmless to humans. BEVS is not only a unique system for expressing cysteine-rich toxins, but it has also been useful for testing insecticidal activity of toxin candidates, since the toxicity of an expressed toxin in insect host cells might mean inherent insecticidal activity of the expressed toxin (Justa et al., 2020). BEVS comprises a collection of virus backbones, such as AcMNPV (from *Autograpaha californicata*), OpMNPV (from *Orgyia pseudotsugata*), and BmNVP (from *Bombyx mori*), which can infect various cell lines (Ali et al., 2015). The most commonly used insect cell lines are Sf9 (*Spodoptera frugiperda*) and Hi5 (*Trichoplusia ni*). However, screening other cell lines is recommended since the choice of host cell can impact the expression level, yield, and glycosylation pattern (Geisse, 2007; Wilde et al., 2014).

Toxin expression with BEVS relies on expression promoters for early-stage or late-stage infection (Slack and Arif, 2006). The selection of a promoter impacts the pathology of the baculovirus

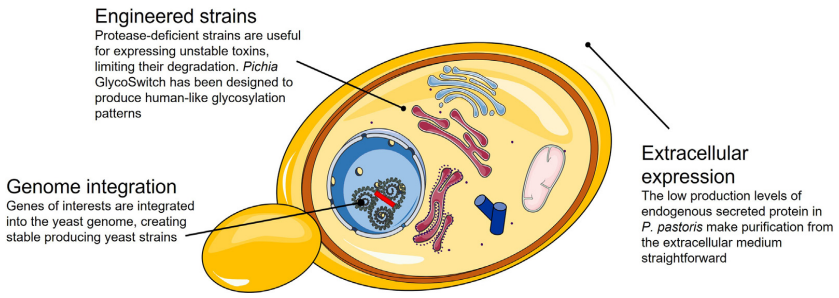


FIGURE 4 | Summary of the strategies available and their advantages for the expression of correctly folded toxins in a *P. pastoris* expression system.

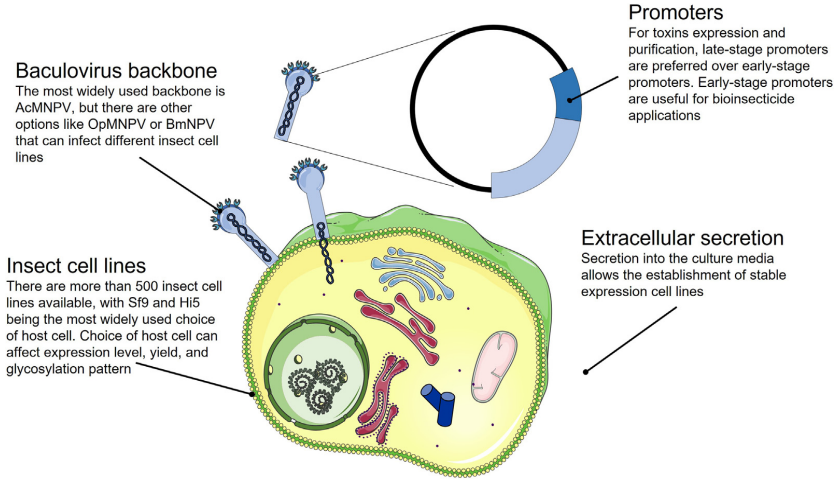


FIGURE 5 | Summary of the strategies available for the expression of correctly folded toxins in a baculovirus insect cell expression system.

and can lead to premature cell death due to the effect of the expressed toxin on cell viability (Ardisson-Araújo et al., 2013). Late-stage infection promoters are preferred to obtain high yields of a toxin that affects host viability, such as Ba3 spider toxin (Ardisson-Araújo et al., 2013), as these allow the insect cells to grow enough before producing the toxin that challenges their viability. In comparison, early-stage promoters are not useful for producing the toxin and purifying it for downstream analysis if the toxin has insecticide activity. Early-stage promoters are typically chosen if the goal is to use the toxin as a bioinsecticide since the main objective is to exert toxicity as soon as possible to kill the insect cells.

One of the most attractive features of BEVS is the possibility of having the toxin secreted into the culture media, which allows the establishment of stable expression cell lines expression cell lines. Stable expression cell lines are easy to maintain and attractive for industrial purposes given their high yield and associated product reproducibility. To express a toxin in the insect cell culture media, the target toxin can be fused to the native signal peptide of melittin, a highly expressed bee venom peptide (Vitale et al., 2010). As an example, this strategy has been successfully applied to the expression of α-latrotoxin, a 130 kDa neurotoxin produced by widow spiders that is extremely difficult to extract from the venom gland in large amounts (Volynski et al., 1999).

Establishing insect cell expression systems usually takes longer than microbial systems. Generating recombinant baculoviruses by conventional methods typically takes up to 6 months. However, new technologies, such as BaculoDirect™ (Thermo Fisher Scientific), can provide faster results, as well as gene editing tools using CRISPR/Cas9 could alleviate some of the cloning difficulties that are often encountered (Pazmiño-Ibarra et al., 2019). The ongoing research on insect cell expression systems

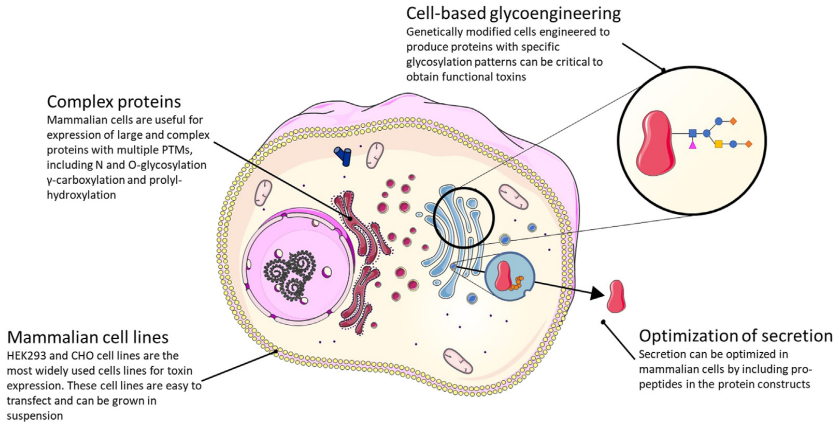


FIGURE 6 | Summary of the strategies available and their main advantages for the expression of functional toxins in a mammalian cell expression system.

focuses on engineering signal peptides and promoters to improve expression, secretion, or folding. For instance, Beek et al. reported an improvement of the lethal activity on insects of the LqhIT2 scorpion toxin by modifying its signal sequence on the AcMNPV virus backbone (van Beek et al., 2003) (**Figure 5**).

Insect cell expression systems have successfully been used to express animal toxins that cannot be produced or fold properly in microbial systems. Nevertheless, the vast diversity of toxin structures and the requirement to achieve specific PTMs may necessitate the use of even more complex systems, such as mammalian cell lines.

3.4 Mammalian Cells

Like yeast and insect cells, mammalian cells offer the possibility of producing disulfide-bonded, correctly folded, and post-translationally modified animal toxins. However, compared to the yeast and insect cell systems, mammalian cells are more native-like for many animal toxins. In general, mammalian cells are also likely better suited to produce larger and more complex animal toxins. Mammalian cells are well-developed for recombinant protein expression and are widely used in academia and for industrial production of biopharmaceuticals, such as monoclonal antibodies and disulfide-rich proteins. Among the wide variety of cell lines available, the expression of animal venom toxins has mostly been performed in human embryonic kidney 293 (HEK293) and Chinese hamster ovary (CHO) cells, which are easily transfectable/transducible and can be grown in suspension (Zhu, 2012; Dumont et al., 2016). The main downside of using mammalian cells for protein production is the relatively low yield and high cost due to slow cell growth, laborious culture conditions, and expensive media (**Figure 6**).

A key area where expression in mammalian cells has a significant potential is for incorporating native(-like) PTMs and disulfide bonds compared to other expression systems. This is desirable in many cases where the addition of specific PTMs influences toxin function and/or stability. Examples include γ-carboxylation of glutamic acid residues and proline hydroxylation. Both modifications are commonly found in conotoxins (Buczek et al., 2005), but cannot be added in native yeast systems since these lack the enzymes necessary to introduce the modifications. Some animal toxins also harbor PTMs that are not, or only rarely, added to mammalian proteins. For instance, different sleep-inducing conotoxins have brominated tryptophan residues (Jimenez et al., 1997, 2004; Buczek et al., 2005). The same modification has been identified in a mammalian brain-specific neuropeptide (Fujii et al., 2002; Tanaka et al., 2003), but the enzyme that performs this modification remains unknown. Thus, recombinant expression of these interesting peptides, which are also γ-carboxylated on several glutamic acid residues, would require further cell line engineering to express the enzyme responsible for the bromotryptophan addition (once identified).

Many animal venom toxins are glycosylated *via* N- and O-glycosylation, which affect folding, trafficking, stability, and function of many, if not most, secretory proteins (Wang et al., 2014b) (**Figure 2**). In the case of Contulakin-G, an O-glycosylated conotoxin with analgesic properties (Craig et al., 1999), glycosylation positively affects analgesic activity most likely by protecting the peptide from metabolic degradation (Lee et al., 2015). In this and similar cases, cell-based glycoengineering (Ma et al., 2020; Schjoldager et al., 2020), which aims to create cells that express proteins with a specific, desired glycosylation pattern, holds the potential to help produce toxins modified with the functionally relevant glycan structure. Especially in the case of O-glycosylation, which is fundamentally different in yeast (Joshi et al., 2018) and insect cells, mammalian cells offer an advantage. Another use of mammalian cells in recombinant production of animal venom toxins that is largely unexplored is the potential for including propeptides in the protein constructs to optimize

secretion. In one known case, the propeptide was demonstrated to be important for efficient secretion of the hydrophobic conotoxin TxVI in the COS7 monkey kidney cell line (Conticello et al., 2003). Of note, secretion of certain animal toxins from mammalian cells may represent a problem in terms of self-intoxication.

Despite the advantages mentioned above, relatively few animal toxins have so far been produced in mammalian cells, compared to the number of animal toxins made in *E. coli*, yeast, and insect cell expression systems. Specific examples include the snake venom proteins rhodocytin (Sasaki et al., 2018) (a heterooctameric C-type lectin with potential as an antiplatelet and antimetastasis biopharmaceutical), acutobin (Wang et al., 2014b) (an α-fibrinogenase with the potential to treat and prevent stroke), ecarin (Jonebring et al., 2012) (a prothrombin activator used, e.g., in diagnostic reagents), and *κ*-bungarotoxin (a neurotoxin used in research on acetylcholine receptors) (Gorman et al., 1997).

The considerable potential of the animal cell expression systems has been convincingly demonstrated by recent work in HEK293 cells, where several hundred "cystine-dense peptides" (CDPs) containing up to 10 cysteines, many of them animal venom toxins, were expressed using a lentivirus transduction system and either displayed on the cell surface or secreted from cells (Correnti et al., 2018; Crook et al., 2018). Moreover, the surface display platform allows for the screening of a large number (tens of thousands) of CDPs (both native sequences and mutagenized variants) for the identification of binding partners of desired targets (Crook et al., 2017, 2018, 2020). This approach has resulted in the identification of one CDP that promotes penetration of the blood-brain barrier by binding the transferrin receptor, and thus shows potential in drug delivery (Crook et al., 2020). The approach has also been utilized to increase accumulation of CDPs in cartilage and was leveraged to deliver a CDP-conjugated steroid, resulting in the alleviation of joint inflammation (Sangar et al., 2020). Taken together, these studies show a large potential for mammalian cells to produce not only native animal venom peptides for characterization, but also to screen mutagenized panels of sequences for identifying interesting novel binding proteins.

While mammalian cells will likely become increasingly important for heterologous expression of specific animal venom proteins that cannot be made by other systems, the examples given above demonstrate that the mammalian cell expression systems may require significant engineering to produce completely native structures. Instead, cultured venom gland cells may, for some purposes, constitute the ideal system for the expression of complex, post-translationally modified venom peptides and proteins, although cultured venom gland cells come with the drawback that it may be difficult to isolate a specific single protein/toxin of interest from the complex cocktail produced by such systems. In this respect, the recent demonstration that isolated venom gland cells can be cultured as organoids that secrete active toxins (Post et al., 2020) is highly encouraging. Isolation and immortalization of venom gland cells would allow for the expression of animal toxins in a native environment. Such expression systems could also find application for transcriptional and proteomic characterization of venom proteins to achieve a better understanding of the complex cellular environment, including chaperones and enzymes involved in PTMs, necessary to produce properly folded and modified animal venom toxins (**Figure 7**). However, while cultured venom gland cells might be beneficial for research applications, it is highly unlikely that they can be used for large-scale manufacture of individual protein products, where monoclonal cell expression systems will be needed.

4 USEFUL TAGS AND FUSION PROTEINS FOR HETEROLOGOUS EXPRESSION AND PURIFICATION

Tags and fusion proteins are useful molecular tools to facilitate proper protein expression (e.g., enhancing solubility or facilitating disulfide-bond formation) and purification, as well as providing unique features to exploit in downstream applications. These tools significantly differ in their size, ranging from small peptide tags (6–15 amino acids) to large fusion proteins. The smaller tags are mainly used for standardized purification protocols or applications involving the use of commercially available antibodies (i.e., immunofluorescence microscopy, immunoprecipitation, Western blotting), and their versatility and small size generally do not interfere with overall protein structure or function (Kimple et al., 2013). Besides the described functions of tags, fusion proteins offer other specific features, such as enhancing solubility or improving disulfide bond formation.

Since numerous reviews provide a detailed overview of the various different tags and fusion proteins that can be used for recombinant protein expression (Terpe, 2003; Young et al., 2012; Kimple et al., 2013), this review will only focus on the ones that have been extensively used for recombinant expression of toxins.

4.1 Tags

The most commonly used affinity tag for protein purification is the poly-His tag, which consists of six to ten consecutive histidine residues. The poly-His tag provides affinity towards divalent metal ions (i.e., Ni^{2+} and Co^{2+}), which can be exploited for purification *via* immobilized-metal affinity chromatography (IMAC) (Yang et al., 2003; Bayrhuber et al., 2006; Clement et al., 2016; Shulepko et al., 2017). IMAC resins have a binding capacity of up to 80 mg/ml and tolerate relatively harsh conditions. Additionally, metal binding is largely independent of protein structure, which enables the purification of toxins from IBs under denaturing conditions (see **Section 3.1.2**). IMAC is also highly suitable for low-cost operation as the resin can be regenerated numerous times. Nevertheless, the poly-His tag charge is critical for binding to the metal ions, therefore restricting the operational pH range for effective purification, which might exclude the utility of the tag for proteins that are not stable at certain pH values (i.e., around the isoelectric point of the protein).

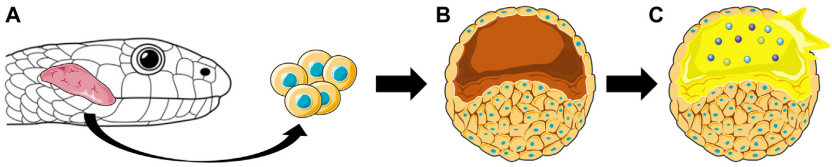


FIGURE 7 | Representation of how venom gland organoids are derived from snake venom gland cells. The cells isolated from the snake venom glands **(A)** are cultured as organoids **(B)** that secrete venom (yellow) containing active toxins (spheres), which can be isolated from the organoids **(C)**.

TABLE 1 | Summary table of the most widely used fusion proteins for recombinant expression of toxins.

Fusion protein	Size (kDa)	Origin	Used in	Usage	Tested toxins
MBP	42	*E. coli*	Bacteria, yeast, and mammalian cells	Increase solubility and expression. Purification	Snake Smith et al. (1997), Giuliani et al. (2001); He et al. (2008), sea anemone (Anangi et al. (2010), cone snail El Hamdaoui et al. (2019), and scorpion Chow et al. (2020).
GST (glutathione-S-transferase)	26	*Schistosoma japonicum*	*E. coli*	Increase solubility and expression. Purification	Snake Gong et al. (1999); Li et al., 2006; Nozach et al. (2013), bee Zhou et al. (2020), and scorpion Chen et al. (2013)
DsbC	23	*E. coli*	*E.coli*	Increase solubility. Promote correct disulfide bond formation	Snakes Nozach et al. (2013); Sequeira et al. (2017); Liu et al. (2021), scorpion, cone snail, and spiders Nozach et al. (2013)
SUMO	11	Yeast	*E. coli* (kits modified to work in prokaryotes)	Increase solubility and expression.	Spider Souza et al. (2012); Wu et al. (2017), snake Shimokawa-Falcão et al. (2017), and centipede Hou et al. (2013)
Ub19	11	Human	*E. coli*	Increase solubility and expression	Cone snails Nielsen et al. (2019)
TrxA	12	*E. coli*	*E. coli*	Increase solubility. Promote correct disulfide bond formation in *E. coli* periplasm.	Snake Yang et al. (2003); Shulepko et al. (2017); Kaur et al. (2019), and sea anemone Kim et al. (2017)
GFP	27	*Aequorea victoria*	Bacteria, yeast, insect, and mammalian cells	Fluorescent detection	Sea anemone Bakrač et al. (2010), and scorpion Kuzmenkov et al. (2016)

The poly-His tag can readily be utilized when recombinant expression is performed with many different host systems, including bacteria, yeast, mammalian, and baculovirus-infected insect cells. However, the poly-His tag is mostly used in bacterial expression systems, where a single-step purification can lead to relatively pure protein (>80%). On the contrary, the background following his-tag purification is often higher in insect and mammalian cells due to the higher percentage of histidine-rich proteins. Therefore, it is typically necessary to conduct subsequent purification steps. For this reason, other epitope tags like c-Myc and FLAG tags are often employed (Bohlen et al., 2010; Shimokawa-Falcão et al., 2017), for which resins functionalized with specific antibodies are commercially available. Using these alternative tags and columns requires milder binding/elution conditions in comparison with IMAC. Nevertheless, antibody-functionalized resins are more expensive and less stable than Ni^{2+}/Co^{2+} functionalized resins used in the purification of His-tagged proteins, making antibody-functionalized resins less attractive from an economic perspective.

4.2 Fusion Proteins

The most popular fusion protein used for heterologous expression of toxins is the maltose-binding protein (MBP). MBP is a 42 kDa protein that originates from *E. coli* K12, and, in combination with its native signal peptide, directs protein expression to the periplasmic space of the host. Since MBP is a native protein in *E. coli*, it folds correctly and is soluble when expressed in bacteria, thereby increasing the expression levels and solubility of the fused toxin. MBP has been successfully fused with a variety of different toxins in both bacterial intracellular or periplasmic expression systems (**Table 1**). Additionally, MBP can be exploited for purification, since columns functionalized with amylose that trap MBP are commercially available. However, amylose resins are gradually degraded by amylase activity present in culture crude extracts, limiting the lifespan of the column.

While fusion proteins are often used to solubilize their toxin partner, they can also assist in protein folding and disulfide bond formation. Thioredoxins are oxidoreductases that, through cysteine thiol-disulfide exchange, facilitate the reduction of disulfides, which has been used for expression of snake toxins, as an example (Yang et al., 2003; Shulepko et al., 2017; Kaur et al., 2019). Like MBP, the native *E. coli* thioredoxin A (TrxA) is a highly soluble protein, and when fused with a toxin, TrxA can increase the solubility of the protein construct. Even though TrxA acts as a reductase in the cytosol, it can exert oxidizing activity

in vitro under the right conditions and thereby promote the formation of disulfide bonds following expression.

Apart from solubility and folding enhancement, fusion proteins can also offer unique characteristics to the toxin. For example, fusion of a toxin with the green fluorescent protein (GFP) makes screening for expression easier and simplifies purification due to its spectroscopic features. Additionally, GFP-tagged toxins can also act as probes for the target of the toxin. This is the case for GFP-equinatoxin, a sea anemone pore-forming protein that recognizes sphingomyelin and is used as a sphingomyelin probe (Bakrač et al., 2010).

Once fusion proteins have fulfilled their mission of improving toxin expression yield, it is often necessary to remove the fusion protein, since it may affect downstream analysis and application. For this purpose, specific protease cleavage sites are often included between the fusion protein and the toxin, such as thrombin and Tobacco etch virus (TEV) cleavage sites (i.e., LVPR|GS and ENLYFQ|G/S, respectively). Both thrombin and TEV cleavage sites leave extra residues after cleavage, which may be undesired depending on the downstream application of the toxin. However, TEV has a broad acceptance of amino acids at position P1, and the TEV cleavage site can be designed so that the residue left after cleavage is the first residue of the native toxin (Sequeira et al., 2017), which makes this cleavage site quite versatile.

Very often, a specific combination of tag and fusion protein is applied during recombinant expression of toxins. For example, as part of the VENOMICS project, Sequiera and others produced thousands of fully oxidized animal venom peptides employing a DsbC fusion partner both for oxidation of disulfides and increased solubility, as well as a His-tag for purification (Sequeira et al., 2017; Turchetto et al., 2017). However, after successful expression and purification, it is important to eliminate both the tag and the fusion protein. This can be a challenging step that reduces the overall process yield dramatically. Therefore, it is necessary to optimize the cleavage conditions to avoid loss of properly folded toxin (e.g., TEV protease is a cysteine protease that requires a specific redox environment to disrupt disulfide bond patterns). After cleavage, it is typically necessary to perform a second purification step based on the molecular weight (i.e., size exclusion chromatography), isoelectric point (i.e., ion-exchange chromatography), hydrophobicity (i.e., reversed-phase HPLC) of the toxin, or even a second affinity purification based on the same tag used for the fusion protein. This second purification often further affects yield.

Given the potential difficulty in eliminating fusion proteins using sequence-dependent cleavage, a clever strategy is to use the small ubiquitin-like modifier (SUMO) as a solubilization tag. SUMO has the advantage of being cleaved by highly specific proteases that recognize the protein structure rather than a particular amino acid sequence (Sermadiras et al., 2013). SUMO originates from yeast, and other eukaryotes have the same conserved family of proteins. Therefore, it is not an optimal tag for expression in these eukaryotic cells due to the presence of intrinsic SUMO proteases. Luckily, commercially available kits for purification of SUMOylated proteins improve the purity of proteins produced in yeast, insects, and mammalian cells.

Finally, another ubiquitin-derived solubility fusion protein widely used in toxin expression is ubiquitin with an internal His-loop (Ub19). Ub19 is an engineered version of the wild-type yeast ubiquitin that presents enhanced solubility and resistance toward nonspecific protease cleavage (Rogov et al., 2012), which takes advantage of the poly-His tag features for purification and detection. Ub19 has been successfully used to express cone snail toxins (Souza et al., 2012; Shimokawa-Falcão et al., 2017; Wu et al., 2017; Nielsen et al., 2019).

The correct selection of fusion proteins in combination with affinity tags is critical for the successful expression and purification of expressed toxins. Unfortunately, given the heterogeneity of structures and physicochemical properties of toxins, there is no winning combination for all cases. It is typically necessary to screen different combinations and potentially explore other expression strategies, such as producing toxins without cells.

5 PRODUCING TOXINS WITHOUT CELLS

Depending on the toxin characteristics and the amount necessary for downstream applications, it is worth considering production systems that do not involve cells. Such strategies are restricted to small proteins or peptides; however, the purification protocols are quite straightforward, and it is sometimes faster to achieve a highly pure toxin than with classic heterologous expression.

5.1 Cell-Free Proteins Synthesis

Cell-free protein synthesis (CFPS) has become increasingly popular for the *in vitro* production of difficult-to-express proteins, such as toxic proteins (Klammt et al., 2006; Zemella et al., 2015). CFPS systems are also suitable for expressing proteins that incorporate non-canonical amino acids or proteins that require tight control of the synthesis in terms of reactant concentrations, which are not easy to regulate in heterologous expression (Zemella et al., 2015; Rolf et al., 2019). These CFPS systems were first developed over 50 years ago to study the genetic code (Ogonah et al., 2017). Briefly, CFPS systems consist of extracts from cultured cells that are treated to reduce the concentration of endogenous RNA and DNA while retaining the minimal machinery for transcription and translation (i.e., RNA polymerases and ribosomes). The culture extracts are supplemented with energy sources (ATP, GTP, etc.) and free amino acids (Sun et al., 2013), and once an enriched extract is in place, expression is initiated by introducing a suitable template, such as linear or circular DNA or mRNA encoding the toxin of interest. Despite the simplicity of CFPS systems, the crude extract source and composition influence the success. Fortunately, many CFPS systems are currently available, originating from Archaea, prokaryotes, fungi, plants, insects, or mammals (Zemella et al., 2015) (**Figure 8**).

CFPS has one significant advantage for toxin production compared to the use of living cells, which is their tolerability to toxic proteins that would otherwise be problematic to produce in living cells. One example is the expression of a phospholipase A_1 from *Serratia* sp., which showed extremely low productivity

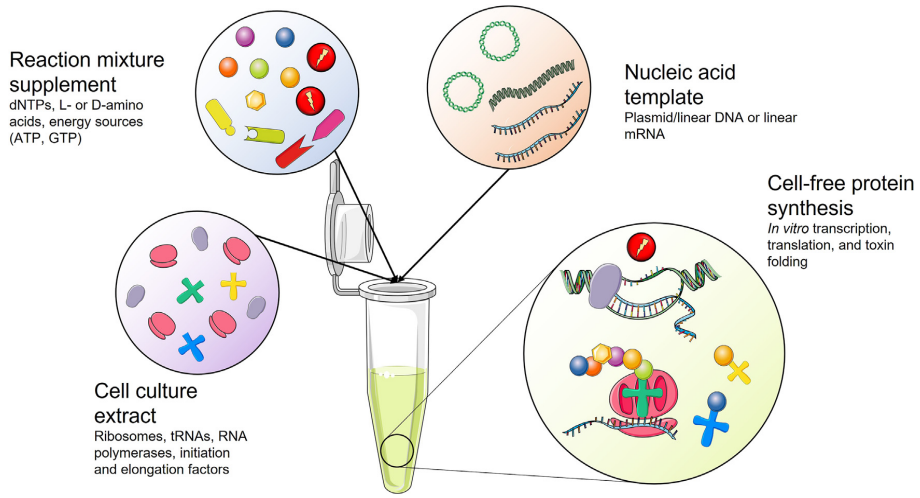


FIGURE 8 | Schematic representation of a cell-free protein synthesis (CFPS) system. Cell culture extract is supplemented with essential reagents for protein synthesis. Upon addition of a nucleic acid template coding for the toxin of interest, the toxin gene is transcribed and translated into a toxin that might need assisted folding.

when produced in living cells, but exhibited a 1,000-fold higher yield in a CFPS setup (Lim et al., 2016). Besides dramatically improving the production yield of toxic proteins, CFPS also allows for the synthesis of modified proteins with embedded non-canonical or unnatural amino acids (Oh et al., 2014; Catherine et al., 2015). Several toxins include non-canonical amino acids, such as defensin-like peptide-2 from *Ornithorhynchus anatinus,* which contains a D-Met and has been successfully produced in CFPS (Torres et al., 2005). Furthermore, CFPS often has a simple liquid-handling process and easy scalability, which has allowed for the development of high-throughput protein production systems (Zemella et al., 2015). Although CFPS has many desirable characteristics as an expression platform, it also has notable disadvantages. CFPS systems have low yield compared to heterologous expression systems, and the vulnerability of the nucleic acids encoding the toxins to nucleases present in the culture extracts (resulting in degradation of the DNA/RNA encoding the toxin) makes the establishment of stable production setups difficult (Rolf et al., 2019). The cost of CFPS used to be high in comparison with microbial systems and comparable to mammalian cell expression. However, recent advances in the field and the CFPS high-throughput production have dramatically reduced the cost of the approach, attracting the attention of pharmaceutical companies (Jérôme et al., 2017; Chiba et al., 2021).

Even though CFPS can be used to synthesize many toxins, the wide variety of structures and physicochemical properties necessitates the identification of alternative production systems for more complicated toxins. One of these alternatives is chemical synthesis.

5.2 Solid-Phase Peptide Synthesis

Solid-phase peptide synthesis (SPPS) consists of coupling α-amino and side-chain-protected amino acids on a solid support one by one from the C- to the N-terminus (Petrou and Sarigiannis, 2018; Camperi et al., 2020). SPPS has been used to produce toxins that are particularly difficult to express in heterologous systems, such as cysteine-rich peptides (de Araujo et al., 2013; Schroeder et al., 2014; Clement et al., 2016; Jaradat, 2018; Zoukimian et al., 2019). After synthesis, the cysteine-rich peptides can be refolded under oxidative conditions to form the disulfide bonds (**Figure 9**). Particularly, to achieve the correct disulfide-bond pattern, the cysteines can be protected/deprotected in pairs to obtain correct cysteine-cysteine pairing and, therefore, the biologically active toxin. Peptides synthesized through SPPS are usually no longer than 50 amino acid residues, as longer peptides are difficult to produce in high yield and purity. However, if the toxin of interest is longer than 50 amino acids, techniques, such as segment condensation, can be utilized to combine several peptides (Nuijens et al., 2016; Zuo et al., 2019). Even though the toxin size limitation means that this system cannot be employed for the production of many toxins, SPPS presents indisputable advantages as it is a relatively fast process, and insertions of non-canonical amino acids and several posttranslational modifications are easily incorporated into the toxin *in vitro* (Nuijens et al., 2016; Petrou and Sarigiannis, 2018). Nevertheless, unlike heterologous expression and CFPS, SPPS is more demanding from a technical perspective and might not be feasible to use for classical biochemistry laboratories.

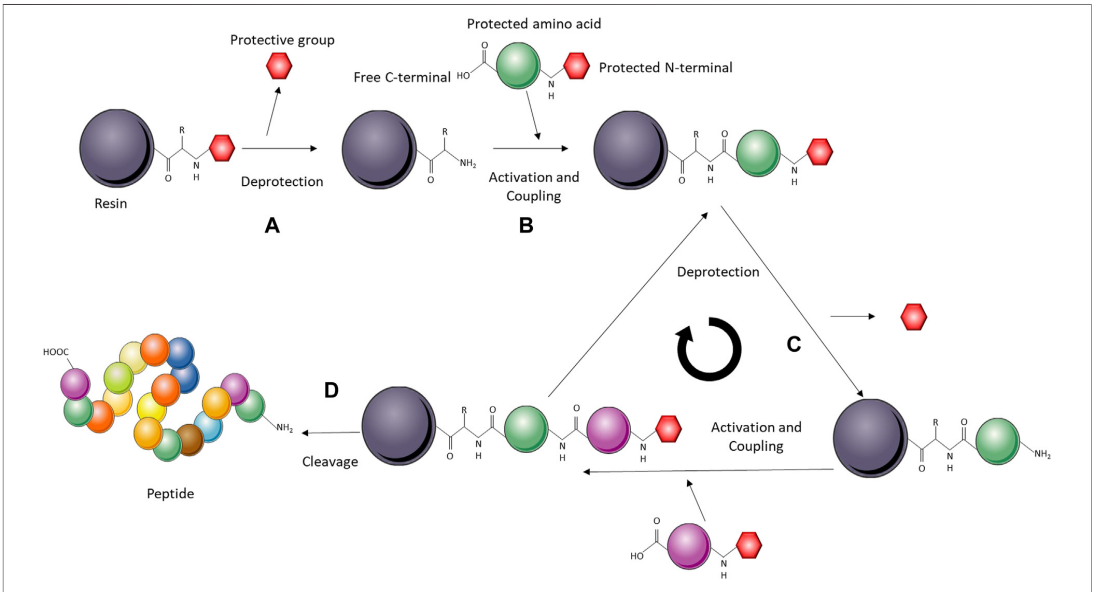


FIGURE 9 | Schematic representation of a solid-phase peptide synthesis (SPPS) system. The solid-phase (resin) is activated for peptide synthesis upon deprotection of the reactive amino group **(A)**. The amino acids are added sequentially on the C-terminal, while remaining protected on the N-terminal. The incoming amino acid forms a peptide bond with the free N-terminal on the resin **(B)**. Deprotection of the amino acid linked to the resin leaves a free amino group ready to react with the next N-protected amino acid **(C)**. The cycle is repeated until all the amino acids are incorporated, upon which the peptide is cleaved from the resin and refolded *in vitro* **(D)**.

6 APPLICATIONS DERIVED FROM RECOMBINANTLY EXPRESSED TOXINS

Due to their inherent bioactive properties, toxins and toxin derivatives can be used for different types of applications within research, medicine, and industry. The toxin scaffolds themselves can be fine-tuned to bind specific targets of therapeutic or industrial relevance. Toxins might also be coupled to other moieties and used as payloads for advanced biotherapeutics. Finally, the scaffolds can be engineered to lack toxicity, thereby providing safer antigens and immunogens for antibody discovery and immunization. This section will present and discuss the current state-of-the-art within the application of recombinant toxins and toxin derivatives.

6.1 Discovery of Broadly-Neutralizing Monoclonal Antibodies Using Designed Consensus Toxins and Cross-Panning on Natural Targets

Recombinant DNA technology allows for expression of toxins that are impossible to obtain from natural sources. It can also prove invaluable when the venomous animal harboring the toxin is rare, does not thrive in captivity, or has very little venom, as exemplified with *Micrurus mipartitus*. This snake produces the lethal toxin mipartoxin, which is not neutralized by existing antivenoms. As this snake venom is challenging to obtain, it is not included in the immunization mixtures used for any existing antivenoms, and the potentially fatal envenomations from this snake cannot be treated (Rey-Suárez et al., 2012). If recombinant mipartoxin could be produced, this toxin could be included as an immunogen together with the venom mixtures used for immunization in traditional antivenom manufacture. In turn, this may lead to a broadening of the neutralization capacity of the antivenom to cover *M. mipartitus* (Bermúdez-Méndez et al., 2018). Recombinant toxins could also be used as antigens for raising monoclonal antibodies or other antibody-like scaffolds (e.g., using phage display technology) (Laustsen, 2018; Jenkins et al., 2019), which likewise could be highly relevant for improving envenomation therapies. Such, monoclonal antibodies could be added to existing antivenoms as fortification agents, or even combined in oligoclonal mixtures to create fully recombinant antivenoms (Kini et al., 2018; Laustsen et al., 2018; Knudsen et al., 2019).

When using heterologous expression, it becomes possible not only to generate the recombinant version of a native toxin but also to generate engineered toxins with special features that improve upon the native toxins for specific purposes, such as consensus toxins that can be used to create polyvalent antivenom. In 2019, de la Rosa et al. demonstrated the utility of a consensus short neurotoxin, which was used to raise a broadly neutralizing serum in both rabbits and horses (de la Rosa et al., 2018, 2019). The

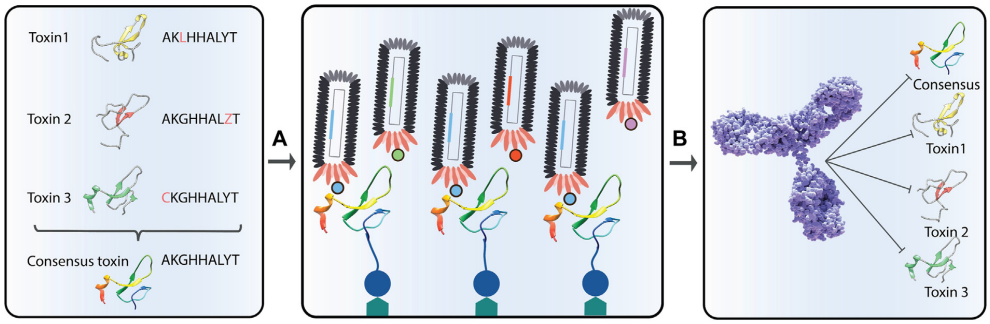


FIGURE 10 | Representation of the discovery of broadly-neutralizing antibodies using consensus toxins. Consensus toxins are designed toxins that represent an average sequence of a collection of homologous toxins **(A)**. By using consensus toxins in phage display selection campaigns **(B)**, selected antibodies might be able to neutralize not only the consensus toxin, but also the natural toxins used in the consensus toxin design.

researchers showed that this antivenom was able to neutralize venoms from a broad range of elapid snakes from several different continents, thereby demonstrating superior broadly neutralizing effects in comparison with the antibodies obtained from immunization with natural toxins. Whether the broadly neutralizing capacity of these antisera was due to polyclonality or the presence of broadly-neutralizing monoclonal antibodies is not known, but both possibilities exist (Ledsgaard et al., 2018). In the future, it could be speculated that other interesting properties, such as increased immunogenicity and reduced toxicity of toxins used as immunogens for immunization, could be investigated. Also, the construction of modified toxins that are better presented to antibodies in different antibody discovery campaigns could potentially be used to drive binding towards a certain epitope.

Finally, the use of heterologous expression systems also allows for expression of toxins without any contaminating toxin isoforms, which can cause trouble in antibody discovery campaigns, as well as create difficulties in the structural and functional characterization of the individual toxins. In both immunization and phage display campaigns, some toxins may dominate and drive the discovery campaign towards antibodies that recognize the contaminating toxin (Lomonte and Calvete, 2017; Laustsen et al., 2018). In immunization processes, this phenomenon is coupled to the immunogenicity of the toxin (Laustsen et al., 2017). In comparison, in phage display experiments, the underlying mechanism for antibody selection is less clear, but speculated to derive from a combination of different toxin properties, such as size and fundamental ability to interact strongly with other proteins through fundamental molecular recognition patterns (Engmark et al., 2016; Krause et al., 2020). For antibody discovery campaigns, where full control of antigen presentation is of high importance, such as when utilizing cross-panning strategies to yield broadly-neutralizing monoclonal antibodies (Ahmadi et al., 2020), recombinant DNA technology for expression of toxins offers great benefits, and it is speculated that many developments and new molecular tools, such as application of tags, consensus toxins, de-immunized toxins, and toxoids, will be brought to life by scientists in the field of toxinology over the next decade (**Figure 10**).

6.2 Bioinsecticides

Control of insect pests is a large concern for agriculture, where pests are reported to cause crop losses in the range of 13–16% (Culliney, 2014). Many insects are also vectors for disease, spreading viruses and parasites among crops, but also to humans and livestock. Unfortunately, such vectors are gaining resistance to traditional chemical insecticides, as has been observed since the 1980s (Brattsten et al., 1986). Due to this unfortunate phenomenon, many traditional insecticides have been de-registered for loss of effectivity or other concerns, such as long-term exposure damage to human and vertebrate health (Windley et al., 2012).

Considering these drawbacks of traditional insecticides, venoms from animals that naturally hunt and feed on insects are a logical source of specific bioinsecticides (Smith et al., 2013). Insecticidal peptides have been discovered in a range of arthropods that prey on insects (Schwartz et al., 2012), including spiders (Bende et al., 2013; King, 2019; Saez and Herzig, 2019), scorpions (Gurevitz et al., 2007; Deng et al., 2019), ants (Touchard et al., 2016; Heep et al., 2019), and centipedes (Yang et al., 2012).

The different requirements for an "ideal" bioinsecticide have been discussed elsewhere (Windley et al., 2012; Saez and Herzig, 2019), but briefly, they should be specific, environmentally benign, have cost-effective high-yield production, and be bioavailable to the insects they target. They need to be specific to insect pest species without being toxic to other animals (e.g., beneficial pollinators) or humans. Consequently, examples of orally active insecticidal toxins are limited but do exist (Mukherjee et al., 2006; Hardy et al., 2013; Guo et al., 2018), and recent studies on whole spider venom reveal that activity upon oral intake by insects is likely to be more common than previously anticipated (Guo et al., 2018).

To improve toxicity upon ingestion of toxin-derived bioinsecticide, delivery strategies to direct toxins to the insect

gut, enhancing the insecticidal effects, have been tested by, e.g., fusing peptides to plant lectins or viral coat proteins (Bonning et al., 2014; Herzig et al., 2014; Nakasu et al., 2014; Yang et al., 2014). Another approach consists of delivering insecticidal toxins through the use of transgenic entomopathogens, such as baculoviruses, the *Bacillus thuringiensis* soil bacterium, or the *Metarhizium* fungus. The latter microbes infect insects while simultaneously expressing the insecticidal toxin, thereby showing a synergistic insecticidal effect (Hughes et al., 1997; Wang and St Leger, 2007; Herzig et al., 2014). Entomopathogens are excellent vectors that narrow down the target pest range because of their insect specificity. Moreover, the inherent entomopathogen lethality in combination with the administered bioinsecticide are less likely to cause resistance (Siegwart et al., 2015).

A bioinsecticide derived from the venom of the Blue Mountains funnel-web spider, *Hadronyche versuta*, has already been commercialized as "SPEAR® bioinsecticides" by the company Vestaron by exploiting the broad-spectrum insecticidal activity of the toxin, GS-ω/κ-Hexatoxin-Hv1a (Hv1a). While Hv1a shows insecticidal effect against a range of crop pests, including aphids, spider mites, thrips, whiteflies, and caterpillars, it is safe against honey bees, birds, fish, and humans. Hv1a has also been trialed for malaria control (Bilgo et al., 2017; Lovett et al., 2019), where it was transgenically expressed by a *Metarhizium* entomopathogen with a narrow host range for *Anopheles* mosquitos. A semi-field trial in an endemic malaria region showed that Hv1a-expressing *Metarhizium* outperformed unmodified *Metarhizium* for mosquito eradication (Lovett et al., 2019). Furthermore, the toxic activity of Hv1a in combination with the innate lethality of the entomopathogenic *Metarhizium* acted synergistically, increasing the mosquito susceptibility (Bilgo et al., 2017). While the release of a transgenic *Metarhizium* for malaria control would require further testing and approval, it is a promising biotechnological application of a venom peptide.

Taking advantage of natural toxicity and specificity is an obvious application to exploit animal toxins' biotechnological potential. Nevertheless, the potential of toxins as tools is not restricted to defeat natural preys. The high binding affinity and target specificity of toxins make them excellent starting points for the development of toxin-inspired biotherapeutics.

6.3 Toxin-Inspired Drugs

Toxins whose toxicity relies on modulating mammalian biochemical targets (e.g., blood coagulation cascades, signaling receptors, or ion channels) can be used as valuable leads to develop biotherapeutics. Many toxins have high selectivity and binding affinity to their molecular targets, which can be exploited to develop drugs causing less adverse reactions compared to traditional small molecule drugs. Some limitations of using venom toxins and peptides as drug leads exist, such as limited membrane permeability and therefore reduced bioavailability for humans, as well as poor *in vivo* stability and fast clearance (Otvos and Wade, 2014; Lau and Dunn, 2018). Where native wild-type toxins fall short of the strict activity or selectivity requirements for a drug, "toxineering" approaches (rational engineering of the toxin sequence) may be employed to improve drug properties and minimize off-target activity (Gui et al., 2014; Klint et al., 2015; Neff and Wickenden, 2021), which has already led to several animal-toxin-derived drugs on the market (**Table 2**).

At first glance, snakes appear to be the most promising source for mammalian-active toxins since many snake species (primarily) prey on mammals. However, early research into therapeutic use of toxins was biased towards snakes due to their large size and the large volumes of venom they produced. Nowadays, mammalian-active toxins with therapeutic potential or toxins active against human pathogens have been found in a range of different animals (Herzig et al., 2020), including, but not limited to, spiders (Saez et al., 2010; Saez and Herzig, 2019), scorpions (Ghosh et al., 2019), centipedes (Hakim et al., 2015; Undheim et al., 2016), and cone snails (Veiseh et al., 2007). In particular, neurotoxins that selectively target the transmitter release machinery, and especially those that affect presynaptic mechanisms by targeting ion channels and receptors, have attracted significant interest from the pharmaceutical fields. These toxins can be used to modulate fundamental processes, such as neurotransmitter release, and may have potential as carriers of molecular cargo and probes (Vetter, 2018; Ovsepian et al., 2019). For example, α-latrotoxin produced by widow spiders and agatoxins from funnel-web spiders are potent neurotoxins affecting presynaptic neurons and are used as molecular probes for studying neurotransmission in mammals and humans (Kaczorowski et al., 2008; Ovsepian et al., 2019).

In recent years, toxins from animal venom have been utilized for novel medical applications. Most notably, chlorotoxin from the venom of the deathstalker scorpion has been engineered as a tool known as "Tumor Paint". Conjugating the toxin to a fluorescent dye enables high resolution and real-time visualization of solid tumor cancers during surgery (Veiseh et al., 2007). Tumor Paint has shown efficacy in Phase 1 clinical trials in brain, breast, and skin cancers, and is currently undergoing Phase 2/3 clinical trials for pediatric central nervous system tumors (Blaze Bioscience). Chlorotoxin-conjugated graphene oxide has also been used for the selective delivery of doxorubicin, a chemotherapeutic agent, to glioblastoma cells and showed higher efficacy and accumulation of the agent than doxorubicin or graphene oxide-conjugated doxorubicin alone (Wang et al., 2014a). Toxins are also useful for diagnostics, as has been proved for the snake toxin batroxobin (Reptilase®), which has been used for decades as a laboratory reagent to measure fibrinogen levels and blood coagulation capability or, RVV-V (Pefakit®), derived from a viper toxin capable of activating factor-V of the coagulation cascade, used to diagnose coagulation pathologies (Funk et al., 1971; Schöni et al., 2007; Bordon et al., 2020). Finally, conjugating cytotoxins to tumor-specific antibodies (called immunotoxins) has also enabled specific targeting of the toxins to cancer cells, where the toxins can exert their cytotoxic effects (Russell et al., 2004; Allahyari et al., 2017).

In the last years, several other noteworthy toxin-derived drugs with novel medical applications have entered clinical trials (Bordon et al., 2020). Dalazatide, a synthetic peptide derivative

TABLE 2 | Examples of approved toxin-derived drugs (Bordon et al., 2020; Herzig et al., 2020).

Drug	Source	Year approved (US FDA)	Indication	Production method	Ref
Captopril[a]	*Bothrops jararaca* (snake)	1981	Antihypertensive	Synthesis	Ondetti et al. (1977)
Batroxobin	*Bothrops sp.* (snake)	Not approved in United States (China: Defibrase, Japan: Reptilase, Korea: Batroxobin; first clinical use early 1990s)	Antithrombotic	Purified from venom and recombinant production	Choi et al. (2018)
Cobratide (cobrotoxin)	*Naja naja atra* (snake)	1998	Painkiller for moderate to severe pain	Purified from venom	Chen and Robinson, (1990)
Eptifibatide	*Sistrurus miliarius* (snake)	1998	Antiplatelet	Synthesis	Ohman et al. (1995)
Tirofiban[a]	*Echis carinatus* (snake)	1998	Antiplatelet	Synthesis	Hartman et al. (1992)
Bivalirudin	*Hirudo medicinalis* (leech)	2000	Anticoagulant	Synthesis	Bates and Weitz (1998)
Enalapril[a]	*Bothrops jararaca* (snake)	2000	Antihypertensive, treatment of diabetic kidney disease, and heart failure	Synthesis	Ferguson et al. (1982)
Desirudin	*Hirudo medicinalis* (leech)	2003	Antithrombotic	Recombinant production	Eriksson et al. (1997)
Ziconotide	*Conus magus* (cone snail)	2004	Painkiller for chronic pain	Synthesis	Sanford, (2013)
Exenatide	*Heloderma suspectum* (lizard)	2005	Treatment of type 2 diabetes	Synthesis	Giannoukakis (2003), Nielsen and Baron (2003)
Lixisenatide	*Heloderma suspectum* (lizard)	2016	Treatment of type 2 diabetes	Synthesis	Christensen et al. (2009), Werner et al. (2010)

[a]*Small molecule.*

of a toxin from the sun sea anemone (*Stichodactyla helianthus*), which toxicity relies on inhibiting voltage-gated potassium channel Kv1.3, has demonstrated efficacy in the treatment of autoimmune disorders, including psoriasis, arthritis, multiple sclerosis, lupus, and rheumatoid arthritis (Liu et al., 2020). Desmoteplase, a recombinant toxin derivative from vampire bat venom, with a function similar to tissue plasminogen activator, has applications in acute ischemic stroke (Reddrop et al., 2005). Soricidin, a synthetic peptide derived from the venomous saliva of the Northern short-tailed shrew, inhibits transient receptor potential channel TRPV6 and causes selective apoptosis of ovarian and prostate cancer cells (Bowen et al., 2013). Two other molecules, Receptin (RPI-78M) and Pepteron (RPI-MN), are modified toxins (cobratoxin and cobrotoxin, respectively) from cobra venoms, which are active on nicotinic acetylcholine receptors. These are being investigated for efficacy against multiple sclerosis and other neurological disorders (Receptin), human immunodeficiency virus, and herpes simplex virus (Pepteron). Notably, the ability of cobrotoxin, the basis of Pepteron, to inhibit viral replication has also been hypothesized to be useful in the treatment of COVID-19 (Lin et al., 2020). Many other animal venom toxins have shown efficacy in *in vivo* models for a range of important human diseases (Chassagnon et al., 2017; Richards et al., 2018; Anand et al., 2019), suggesting that the future of venom-derived therapeutics may be bright.

7 BIOSAFETY CONSIDERATIONS

Working with concentrated solutions or lyophilized preparations of toxins always requires careful handling according to a biosafety plan that follows local regulations. However, with the (low) amounts most often utilized in research labs, working with animal venom toxins presents only a minimal risk to laboratory personnel as well as the public. While their recombinant production allows for the generation of large amounts of single animal toxins, this does not *a priori* present special biosafety issues. In fact, it is worth remembering that working with crude venom can constitute a larger risk due to the combined effect of the individual toxin components in the complex mixture that crude venom represents. In this relation, it is worth mentioning that hypersensitivity reactions have been reported for researchers that have been exposed to lyophilyzed venoms. Therefore, venom powder should be handled under fume hoods, and protective clothing should include face masks to reduce the risk of exposure (Prescott and Potter, 2005; de Medeiros et al., 2008; Chippaux, 2010).

While the high toxicity of certain animal venom toxins, in principle, would allow their use for nefarious purposes, we are not aware of any such reported instances. Still, the European Union [Europe Council Regulation (EC) No. 428/2009] and Australia (Australian Government Federal Register of Legislation Defence and Strategic Goods List 2019) include (all) conotoxins on their lists of regulated toxins, imposing restrictions on their use and

export. Apparently, no other animal venom toxins are regulated by any other country (Bjørn-Yoshimoto et al., 2020). Although the United States (CDC, Federal Select Agent Program) and Denmark (Center for Biosikring og Bioberedskab) have removed all conotoxins, except for a small group of paralytic α-conotoxins from their lists of regulated toxins, we strongly support the argument recently put forward that limiting the use of even the most potent animal venom toxins will have little consequence for their possible use as bioweapons (Bjørn-Yoshimoto et al., 2020). Firstly, the misuse of animal venom toxins in bioterrorism seems unrealistic given the fact that much deadlier and more easily available compounds exist. Secondly, strict regulatory measures on the production and use of animal venom toxins in research labs come with the risk of setting back efforts to deliver on the many promises held by these toxins as biopharmaceuticals and research tools. However, we naturally still advice that researchers working with toxins ensure proper safety measures to protect both themselves and the environment, and that proper safety assessments are done on a case-by-case basis.

8 OUTLOOK

Animal toxins constitute an excellent source of bioactive compounds with promising biotechnological potentials. However, to be of utility, they must be producible in sufficient quantities not only for research and development efforts but also for later industrial application. In this regard, heterologous expression opens the door to a wide variety of applications, allowing for the development of novel biotechnological tools, such as bioinsecticides or biotherapeutics. As an example, heterologous expression allows for the production of thousands of toxins, which can be screened for interesting bioactivity in a high-throughput setup. Having such expression and purification workflows in place, ideally in an automated fashion, can also allow for the identification of new targets for previously uncharacterized toxins (Sequeira et al., 2017; Duhoo et al., 2019; Reynaud et al., 2020).

There is no single expression and purification strategy that can be applied to all animal toxins, and the chosen workflow will depend on the physicochemical features of the toxin, as well as the desired yield, scale, and downstream application. The production approaches discussed in this review not only enable researchers to produce larger quantities of toxins than can be extracted from natural sources, but also make it possible to work with toxins that are unavailable when the natural source cannot be held in captivity, when the toxin of interest only exists in trace amount within the venom, or when the toxin is not even present in the venom (i.e., dormant genes). Here, heterologous expression makes it possible to exploit the increasing amount of proteomic and transcriptomic data from venomous animals. The heterologous expression and synthetic approaches discussed in this review differ in their capabilities to produce correctly folded and functional toxins with correct PTMs, which should be taken into consideration when selecting the method of toxin production. From the industrial perspective, expression systems present differences in the cost of manufacture and scalability, with the microbial expression systems often being cheaper and easier to scale in comparison with insect or mammalian cell culture setups, although this should always be evaluated on a case by case basis. Expanding our knowledge and toolbox in the field of heterologous toxin expression will hopefully boost the biotechnological applications derived from different subfields in the important area of research that is toxinology.

AUTHOR CONTRIBUTIONS

AL and ER-T conceptualized the review and wrote the first outline. ER-T coordinated the project. AL, TJ, and LE supervised the writing. ER-T and TJ prepared the figures. All authors contributed to writing and editing the manuscript.

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Chapter 3 – Manuscript I

A comparative study of the performance of *E. coli* and *K. phaffii* for expressing α-cobratoxin

In this study, we aimed to investigate and compare the expression of α-cobratoxin using different recombinant expression systems, namely *Escherichia coli* and *Komagataella phaffii* (formerly known as *Pichia pastoris*). The choice of α-cobratoxin was based on the availability of native α-cobratoxin, enabling a comparison between the behavior of recombinantly expressed toxins and the native counterparts. We employed two different *E. coli* systems: SHuffle cells and the csCyDisCo plasmid. SHuffle cells are a cell strain designed to promote disulfide bond formation. On the other hand, the CyDisCo plasmid utilizes a co-expression system involving sulfhydryl oxidase and protein disulfide isomerases. For the expression *in E. coli*, three different tags were employed: His_6, $UbHis_{10}$, and His_6-SUMO. Additionally, we explored the potential of *K. phaffii*, a yeast expression system known for its ability to perform post-translational modifications similar to higher organisms. By utilizing these expression systems, we aimed to evaluate the yield, purity, and functional activity of the recombinantly expressed α-cobratoxin.

The results provided valuable insights into toxin expression. All three systems and various tags showed potential for α-cobratoxin production, but none fully matched the native variant. Circular dichroism (CD) spectra analysis revealed some structural differences, indicating that recombinant toxins may not fully mimic native α-cobratoxin.

To assess the binding capability to the α7 subunit of the nAChR, the recombinantly expressed α-cbratoxin was compared to the native toxin. Here, only the His_6-tagged α-Cobratoxin expressed in *E. coli* exhibited binding affinity towards α7 similar to that of the native α-Cobratoxin.

Our findings highlight the importance of choosing the appropriate expression system for the production of α-cobratoxin and other 3FTx. The study serves as a proof-of-concept for the recombinant expression of α-cobratoxin, shedding light on the challenges and possibilities in utilizing alternative production methods for snake toxins.

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A comparative study of the performance of *E. coli* and *K. phaffii* for expressing α-cobratoxin

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Abstract:

Three-finger toxins (3FTxs) have traditionally been obtained via venom fractionation of whole venoms from snakes. This often yields functional toxins, but it can be difficult to obtain pure isoforms, as it is challenging to separate the many different toxins with similar physicochemical properties that often exist in venom. This issue is circumvented by recombinant expression. However, here, achieving the correct disulfide bond formation in recombinant toxins is challenging and requires extensive optimization of expression and purification methods to enhance stability and functionality. In this study, we investigated the expression of α-cobratoxin, a well-characterized 3FTx from the monocled cobra (*Naja kaouthia*), in three different expression systems, namely *Escherichia coli* with SHuffle cells and BL21(DE3) cells with the csCyDisCo plasmid, and *Komagataella phaffii* (formerly known as *Pichia pastoris*). While none of the tested systems yielded α-cobratoxin identical to the variant isolated from whole venom, a His_6-tagged

α-cobratoxin expressed in *E. coli* exhibited comparable binding properties to the α7 subunit of the nicotinic acetylcholine receptor, despite having a different CD spectrum compared to the native toxin. The findings presented here illustrate the advantages and limitations of the different expression systems and can help guide researchers that wish to express 3FTxs.

Keywords: (5 words max)

α-cobratoxin; recombinant toxin expression; snake venom; yeast expression; *E. coli* expression

Abbreviations:

3FTx, Three-finger toxin; nAChR, Muscle-type nicotinic acetylcholine receptor; PDI, Protein Disulfide Isomerase; SUMO, Small Ubiquitin-like Modifier; Ub, Ubiquitin; TEV, Tobacco etch virus; Ulp1, Ubiquitin-like-specific protease 1; CD, Circular dichroism; DELFIA, Dissociation-Enhanced Lanthanide Fluorescence Immunoassay.

Introduction

Three-finger toxins (3FTxs) are a highly diverse group of toxins primarily present in the venom of snakes belonging to the *Elapidae* and *Colubridae* families.[1] These toxins are characterized by a unique three-fingered fold consisting of three loops or fingers protruding from a central β-sheet core held together by four conserved disulfide bonds.[2] While 3FTxs are typically known for their neurotoxicity and paralysis-inducing effects, this family of toxins has evolved to encompass a wide range of functions, including cytotoxic, anticoagulant, and cardiotoxic effects.[3]

The α-neurotoxins are a functional subgroup in the 3FTx family, which target postsynaptic nicotinic acetylcholine receptors (nAChRs), preventing acetylcholine from binding and activating them, which in turn may cause flaccid paralysis and possibly death by asphyxiation in victims envenomed with these toxins.[4–7] One well-studied member of this subgroup is α-cobratoxin, which is the medically most important toxin in the venom of the monocled cobra (*Naja kaouthia*)[8–10], and which is a widely utilized toxin in toxinology and antivenom research. α-cobratoxin is currently obtained through a labor-intensive process that involves capturing or breeding *N. kaouthia* snakes, followed by the potentially hazardous task of venom extraction.[11,12] The purified toxin is then obtained using High-performance liquid chromatography. This method presents significant challenges, including the dangers associated with snake handling and the potentially inconsistent quality of the purified toxin in different batches, as venom varies with age, sex, diet, and other environmental factors.[13,14] To address these drawbacks, recombinant expression may be an alternative solution for obtaining a steady supply of toxins without needing to catch or keep snakes and extract their venom.[15–18]

To express a correctly folded and functional recombinant toxin, it is necessary to find a recombinant expression system that can accommodate all the necessary features of the native toxin, including correct disulfide bond

formation and other post-translational modifications.[19] Common expression hosts, such as *Escherichia coli* and the yeast *Komagataella phaffii* (formerly known as *Pichia pastoris*), each have their own advantages and disadvantages, making it relevant to compare their performance in expressing recombinant toxins.[19] *E. coli* is a widely used expression system due to the fast growth rate of this bacterium, the low cost of media and other materials needed for its growth, and the ease of genetically manipulating this host. In this study, two different *E. coli*-based systems were used for the expression of α-cobratoxin, namely SHuffle cells and BL21(DE3) with the csCyDisCo plasmid.[20] SHuffle cells are designed to promote the formation of disulfide bonds, which are critical for proper folding and activity of 3FTxs, such as α-cobratoxin, while the csCyDisCo plasmid utilizes a co-expression of a sulfhydryl oxidase and two protein disulfide isomerases (PDIs) to improve the solubility of and formation of disulfide bonds in recombinant proteins.[21,22] In addition to *E. coli*-based system, the yeast *K. phaffii*, is also a popular choice for protein expression due to its ability to secrete recombinant proteins, resulting in easier downstream processing. *K. phaffii* also can perform post-translational modifications similar to those in higher organisms, including correct folding and disulfide bond formation.[23]

Here, we compare the expression of α-cobratoxin in *E. coli*, using both SHuffle cells and BL21(DE3) with csCyDisCo plasmid, and *K. phaffii*, with a focus on yield, purity, and functional activity of the recombinant toxin. By exploring the utility of each of these expression systems, we aim to facilitate the production of recombinant α-cobratoxin and other 3FTxs for research within toxinology, and thereby benefit antivenom development and potentially unlock the investigation of potential therapeutic applications of proteins based on this toxin scaffold.

Material and Methods

Plasmid construction

Recombinant α-cobratoxin was produced in *E. coli* using the plasmid pET39-UbHis$_{10}$-α-cobratoxin, as previously described.[24] Briefly, the gene encoding α-cobratoxin (Uniprot: P01391) was codon optimized for expression in bacteria and inserted into the pET39 vector with an N-terminal ubiquitin (Ub) solubility tag, which contained an internal His-loop (Ub-His$_{10}$), and a recognition site for the Tobacco Etch Virus (TEV) protease located between the Ub-His10 and α-cobratoxin. Other plasmids were created by substituting Ub-His$_{10}$ with either a His$_6$-Small Ubiquitin-like Modifier (SUMO) or a His$_6$ tag using the NEBuilder HiFi DNA Assembly method (New England Biolabs), following the manufacturer's instructions.

For expression in *K. phaffii*, the gene encoding His$_6$-α-cobratoxin was inserted into a pPICZα A vector (Invitrogen) using the NEBuilder HiFi DNA Assembly method.

The design of all primers for plasmid construction was performed using the NEBuilder Assembly Tool (New England Biolabs). The coding sequences were cloned in phase with the α-mating factor of *S. cerevisiae* for secretion of the recombinant protein to the culture media and contained a His$_6$ tag on the N-terminal to streamline the purification. The resulting plasmids were verified by DNA sequencing to confirm the correct insertion of the α-cobratoxin gene and the respective tags (Eurofins Genomics).

The construction of the protease SuperTEV is described elsewhere.[24]

Expression of α-cobratoxin in E. coli

Three variations of α-cobratoxin constructs in the pET39 vector, namely His$_6$-SUMO-α-cobratoxin, Ub-His$_{10}$-α-cobratoxin, and His$_6$-α-cobratoxin, were transformed into chemically competent *E. coli* SHuffle cells (New England Biolabs). Similarly, BL21(DE3) cells (Invitrogen) were transformed with the α-cobratoxins along with the csCyDisCo plasmid. The transformed SHuffle

cells were plated on kanamycin-containing 2xYT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) agar plates, while the BL21(DE3) cells were plated on 2xYT agar plates supplemented with both kanamycin (50 µg/mL) and chloramphenicol (20 µg/mL). The plates for SHuffle cells were incubated at 30 °C according to the manufacturer's protocol, while the plates for BL21(DE3) cells were incubated at 37 °C. For the overnight preculture, a 50 mL volume of 2xYT medium supplemented with kanamycin (50 µg/mL) was inoculated with the transformed SHuffle cells and grown at 30 °C with continuous shaking at 220 rpm. In the case of BL21(DE3), the medium was additionally supplemented with chloramphenicol (20 µg/mL) and grown at 37 °C. The following day, a 1 L volume of 2xYTGK medium was inoculated with the overnight preculture at an initial optical density at 600 nm (OD600) of 0.1 and grown at 30 °C or 37 °C with continuous shaking at 220 rpm. The culture was allowed to reach an OD_{600} of 0.8, at which point it was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). In the case of BL21(DE3), the medium was supplemented with chloramphenicol (20 µg/mL).

Subsequently, the temperature was lowered to 16 °C, and the toxins were expressed for approximately 20 hours. The next day, the cells were harvested by centrifugation at 6,000 x *g* for 15 minutes at 4 °C and the cell pellets were subsequently resuspended in 30 mL lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 20 mM imidazole) supplemented with lysozyme (1 mg/mL) and benzonase (1 µL/50 mL). The cells were lysed on ice by sonication using a Fisherbrand FB120 sonicator, operated in pulsed mode with 90 cycles of 2-second intervals at 40% amplitude of vibration, with a 2-second pause in between pulses to avoid overheating. The soluble fraction was separated from the unsoluble debris by centrifugation at 15,000 x *g* for 30 minutes at 4 °C. The resulting clarified supernatants were collected and stored at 4 °C for subsequent purification steps.

Expression of α-cobratoxin in K. phaffii

The generation of electrocompetent *K. phaffii* cells followed a previously described protocol.[25] Briefly, plasmid DNA (10 µg) was linearized using SacI digestion and then electroporated into the KM71H strain using the Bio-Rad Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA). Cells containing the integrated sequences were selected on YPDS plates (20 g/L Peptone, 10 g/L Yeast Extract, 100 mL/L Dextrose 20% (w/v), 182.2 g/L Sorbitol, 20 g/L Agar) supplemented with increasing concentrations of Zeocin (100, 500, or 1000 µg/mL).

Each individual clone was separately inoculated into 50 mL BMGY medium (10 g/L Yeast Extract, 20 g/L Peptone, 0.1M Potassium phosphate pH 6.0, 1.34% (w/v) YNB, 0.04 µg/mL Biotin, 1% (v/v) Glycerol) and incubated overnight at 30 °C with shaking at 220 rpm to assess the expression levels of different clones. The overnight cultures were centrifuged at 4000 x *g* for 10 minutes, and the resulting cell pellets were resuspended in 5 mL BMMY medium (10 g/L Yeast Extract, 20 g/L Peptone, 0.1M Potassium phosphate pH 6.0, 1.34% (w/v) YNB, 0.04 µg/mL Biotin, 0.5% (v/v) Methanol). The cultures were further incubated at 25 °C for 4 days with continuous shaking. Methanol was added to the cultures every 24 hours at a final concentration of 0.5% (v/v) to maintain protein expression. To evaluate protein expression, 1000 µL samples were taken from the cultures every 24 hours. The samples were centrifuged at 5000 x *g* for 1 minute, and the supernatants were subjected to SDS-PAGE analysis to assess expression levels. Based on the expression analysis, the clone with the highest expression level was selected for large-scale expression.

For large-scale expression, the selected clone was inoculated into 5 mL YPD medium (20 g/L Peptone, 10 g/L Yeast Extract, 20% (w/v) Dextrose) and incubated overnight at 30 °C with shaking at 200 rpm. The following day, 2.5 mL of the saturated culture was transferred to 1 L of BMGY medium and grown for 24 hours at 30 °C with shaking at 200 rpm. The culture was then

centrifuged at 5000 x *g* for 10 minutes, and the cell pellet was resuspended in 100 mL of BMMY. Similar to the test expression, the cells were further cultured at 25 °C for 4 days with the addition of methanol to a final concentration of 0.5% (v/v) every 24 hours. After 96 hours, the cells were harvested by centrifugation at 17,000 x *g* for 30 minutes at 4 °C, and the supernatant was collected. The supernatant was sterilized by filtration through a 0.2 µM membrane filter (Milipore). The filtered supernatant was then stored at 4 °C for subsequent purification steps.

Expression and purification of SuperTEV

The SuperTEV protease was expressed and purified as previously described.[24] Briefly, chemically competent *E. coli* BL21(DE3) cells were transformed and plated on 2xYT agar plates supplemented with kanamycin (50 µg/mL). Starter cultures were prepared and then inoculated into ZYM-5052 autoinducing medium.[26] The cultures were grown at 37 °C with agitation and then shifted to 16 °C for 20 hours for protein expression. After the overnight expression, the cells were harvested by centrifugation at 6,000 x *g* for 15 minutes. The subsequent steps for cell lysis and purification of SuperTEV were performed using sonication and HIS-Select® Nickel Affinity Gel (Millipore, Burlington, USA) according to the method described in the aforementioned reference.

The eluted protein was analyzed by SDS-PAGE and subsequently dialyzed against PBS. Following dialysis, the protein was concentrated using centrifugal filtration units and stored at -80 °C.

Preparation of the SUMO protease Ulp1 protease

The Ubiquitin-like-specific protease 1 (Ulp1) was purchased from Sigma-Aldrich (SAE0067), and the lyophilized protease was reconstituted in 100 µL water supplemented with 1 mM DTT.

Purification of α-cobratoxin and protease cleavage

The cleared cell lysate from *E. coli* and the filtered supernatant from *K. phaffii* were subjected to His-purification using gravity flow purification. First, 5 mL of equilibrated HIS-Select® Nickel Affinity Gel resin (Millipore, Burlington, USA) washed and equilibrated with wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 20 mM imidazole), was mixed with the supernatant and incubated at 4 °C for 1 hour with end-over-end rotation. The resin was subsequently transferred into chromatography columns, and the flow-through fractions were collected. The columns were washed with a wash buffer until the A_{280} of the eluent was >0.05. Then, the toxins were eluted using 5 column volumes (CV) of elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 400 mM imidazole). The fractions containing the eluted consensus toxins were dialyzed twice against dialysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl) at 4 °C and subsequently concentrated using Amicon® Ultra-15 Centrifugal Filters (Millipore, Burlington, USA).

The removal of Ub-His_{10} and His_6-SUMO tags from α-cobratoxin was carried out using TEV and Ulp1 protease, respectively. For TEV cleavage, the protease was added at a molar ratio of 1:20 (TEV:toxin), while Upl1 was added at a concentration of 10 U per mg of toxin. To create a reducing environment necessary for the protease activity, 1 mM of DTT was included in the reaction mixture. The reactions were incubated at 30 °C for 1 hour.

Subsequently, the cleaved toxins were subjected to a second purification step using HIS-Select Nickel Affinity Gel resin. Prior to binding the toxin-protease reactions, 2 mL of the resin was washed and equilibrated with wash buffer 2 (50 mM Tris-HCl, pH 8.0, 300 mM NaCl). The resin was then mixed with the toxin-protease reactions and incubated for 1 hour with end-to-end rotation. The flow-through, containing the cleaved toxin without any His-tags, was collected. The resin was further washed with 2 CV of wash buffer 2 followed by 4 CV wash with wash buffer. The removed tags and

proteases were eluted using 4 CV of elution buffer. Finally, the purified toxins were stored at -20 °C for subsequent use.

Preparation of native α-cobratoxin

Native α-cobratoxin from *N. kaouthia* purified to homogeneity by chromatographic methods was purchased from Latoxan SAS (Portes-lès-Valence, France). The toxin was shipped in lyophilized form and used as a comparative control for the recombinantly expressed α-cobratoxin. The native α-cobratoxin was reconstituted in 50 mM Tris-HCl, pH 8, 300 mM NaCl, and either used directly or biotinylated as described below.

Biotinylation of α-cobratoxin

Native and recombinantly expressed α-cobratoxins were biotinylated using a 1:1 (toxin:biotinylation reagent) molar ratio of as previously described[27], using EZ-Link™ NHS-PEG4-Biotin, No-Weigh™ (21329, Thermo Scientific) according to the manufacturer indications. The biotinylation ratio was analyzed using MALDI-TOF in a Proteomics Analyzer 4800 Plus mass spectrometer (Applied Biosystems).

Circular dichroism (CD) spectroscopy

To compare the secondary structures of the recombinant α-cobratoxins with the native counterpart, CD spectroscopy was performed following a previously described protocol.[24] In brief, the toxins were dialyzed against a 10 mM potassium phosphate buffer (pH 7.0). Far-UV CD measurements were conducted using a JASCO J-1500 spectrophotometer (Easton, MD, USA) equipped with a 0.1 mm quartz cuvette. The spectrum was recorded by performing 10 measurements between 250 nm and 190 nm, with a bandwidth of 0.1 nm and intervals of 1 nm. The scan speed was set at 50 nm/sec.

The acquired spectra were processed and smoothed using SpectraManager software (JASCO), which also facilitated the estimation of

the secondary structure content using Yang's reference predictions[28]. Graphs depicting the CD spectra were generated using GraphPad Prism software (GraphPad Software).

In vitro binding of nAChR

A blocking dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) was performed to assess the binding ability of the different purified α-cobratoxins to the α7-acetylcholine receptor chimera, following a protocol described elsewhere.[29] Briefly, Black MaxiSorp plates (Nunc A/S, Roskilde, Denmark) were coated with 4 µg/mL α7-AChR in PBS (200 ng/well). The plates were blocked using PBS + 1% BSA and washed thoroughly with PBS-T (PBS with 0.1% Tween-20) and PBS before the toxins were added in 3-fold dilutions ranging from 15 µg/mL to 0.76 ng/mL. The measurement of recombinant α-cobratoxin expressed in BL21(DE3) with the $UbHis_{10}$-tag was conducted within a concentration range of 10 µg/mL to 0.51 ng/mL, limited by the available amounts. The signals were detected using europium-labelled streptavidin at a concentration of 0.2 µg/mL and detected using a VICTOR Nivo Multimode Microplate. Measurements were performed in duplicates.

Results

Expression and purification of recombinant α-cobratoxin

Recombinant α-cobratoxin was expressed using three different systems: 1) csCyDisCo in *E. coli* BL21(DE3), 2) genetically modified *E. coli* SHuffle, and 3) the yeast *K. phaffii*. Plasmids were constructed and verified by Sanger sequencing to confirm the correct constructs (Figure 1A). In addition to expressing His_6-α-cobratoxin, α-cobratoxin was also expressed in *E. coli* fused with either a $UbHis_{10}$- or a SUMO-tag, with the aim of enhancing protein solubility during expression. The cleared cell lysates obtained from the *E. coli* expressions and the filtered supernatant from the *K. phaffii* expression were subjected to purification using HIS-Select® Nickel Affinity

Gel resin (Figure 1B and C). Those α-cobratoxin constructs harboring a solubility fusion protein were further processed by removing the solubility tag using TEV or Upl1 for $UbHis_{10}$- or His_6-SUMO-tag, respectively (Figure 1D). Subsequent experiments were conducted using the toxins after the removal of the tags. However, for clarity and to distinguish between the different constructs, the toxins are still referred to by their respective tag names.

The formation of disulfides was evaluated by preparing samples with and without DTT and assessing the electrophoretic migration pattern of α-cobratoxins (Figure 1E). No observable migration shifts were detected in the samples expressing α-cobratoxin fused to either the $UbHis_{10}$- or the SUMO-tag. This lack of shift is likely attributed to the addition of 1 mM DTT during the protease cleavage process. The reason for including DTT is that both TEV and Ulp1 proteases are Cys proteases, and they require the Cys on their active site to be reduced for them to become catalytically active. The presence of DTT facilitates the reduction of these essential Cys residues, enabling the proteases to effectively cleave the tags from the toxins. However, noticeable migration shifts were observed in α-cobratoxin expressed solely with a His_6-tag in both *E. coli* and *K. phaffii*, indicating the formation of disulfides.

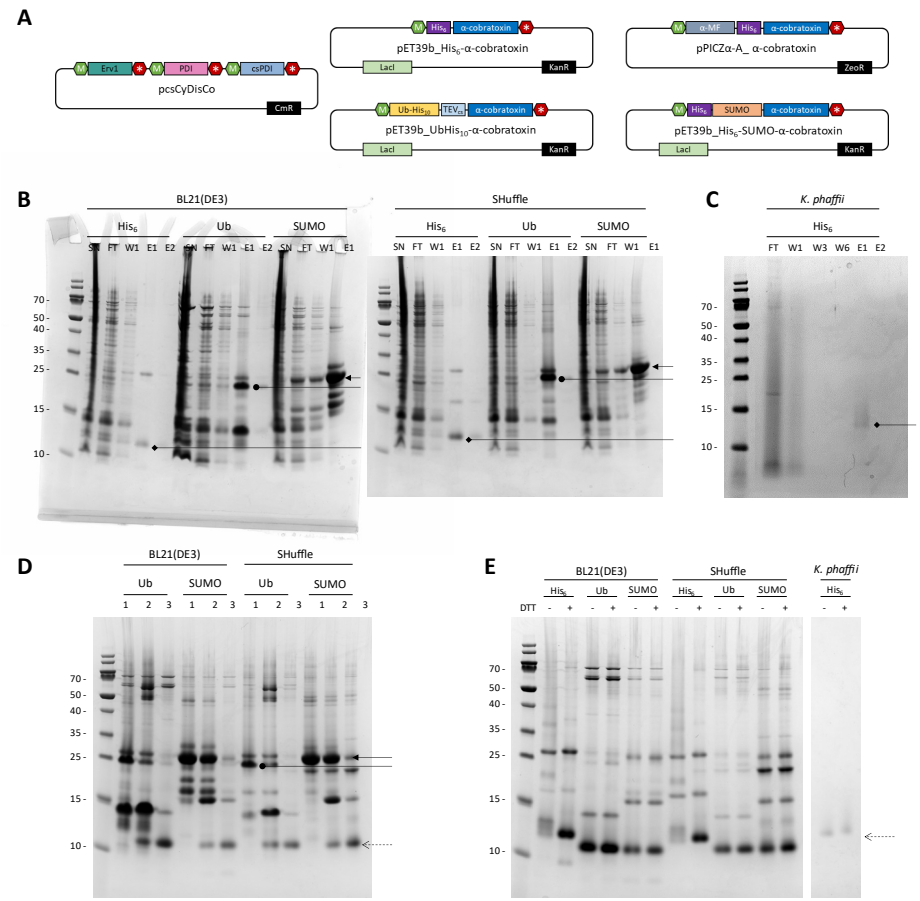


Figure 1. Vector maps and gel pictures from the expression and purification of α-cobratoxin from E. coli and K. phaffii. *A) The helper plasmid csCyDisCo containing the sulfhydryl oxidase Erv1 and the protein disulfide isomerases PDI and csPDI is shown, along with the four expression plasmids used for α-cobratoxin expression in different host systems. In E. coli expression, three plasmids were used: His_6-α-cobratoxin, $UbHis_{10}$-α-cobratoxin, and His_6-SUMO-α-cobratoxin. In K. phaffii expression, α-cobratoxin was fused with α-mating factor (α-MF) and a His_6-tag. B) The SDS-PAGE analysis of the first purification step for E. coli expression, including the supernatant after lysis (SN), flowthrough (FT), first wash (W1), and two elution fractions (E1 and E2). The migration of His_6-α-cobratoxin is indicated by a diamond-shaped arrow, $UbHis_{10}$-α-cobratoxin by a circle, and His_6-SUMO-α-cobratoxin by an arrowhead. C) The purification of His_6-α-cobratoxin in K. phaffii is presented, with*

the flowthrough (FT), three wash fractions (W1, W3, and W6), and two elution fractions (E1 and E2). The migration of His_6-α-cobratoxin is indicated by a diamond-shaped arrow. D) The SDS-PAGE analysis demonstrates the protease cleavage of toxins containing the $UbHis_{10}$ and His_6-SUMO tags using TEV and Ulp1. The gel shows the samples before protease addition (1), after 1 hour of cleavage (2), and after the second purification step (3). The circle indicates the migration of $UbHis_{10}$-α-cobratoxin, the arrowhead represents His_6-SUMO-α-cobratoxin, and the dashed arrow indicates α-cobratoxin after tag removal. D) shows the SDS-PAGE analysis of the purified protein with 50 mM DTT or without DTT. Note that the tag-names are kept to separate the different expressions, even though the tags are now removed. The observed migration shift after the addition of DTT suggests that disulfide bonds are present in the purified protein. The dashed arrow indicates the migration of α-cobratoxin.

Table 1 summarizes the yield obtained from different expression systems and plasmids used in this study for the expression of α-cobratoxin. The use of the SHuffle system in *E. coli* resulted in a higher yield compared to the csCyDisCo system in BL21(DE3) cells. Moreover, when comparing the two fusion tags, the His_6-SUMO showed better performance compared to the $UbHis_{10}$ in terms of yield. However, it is noteworthy that the α-cobratoxin fused only to a His_6 tag expressed well in comparison with fusion protein versions in both *E. coli* systems, even though it was not coupled to a solubility tag.

Notably, the expression of His_6-α-cobratoxin in *K. phaffii* resulted in a similar yield compared to His_6-α-cobratoxin expressed in *E. coli*.

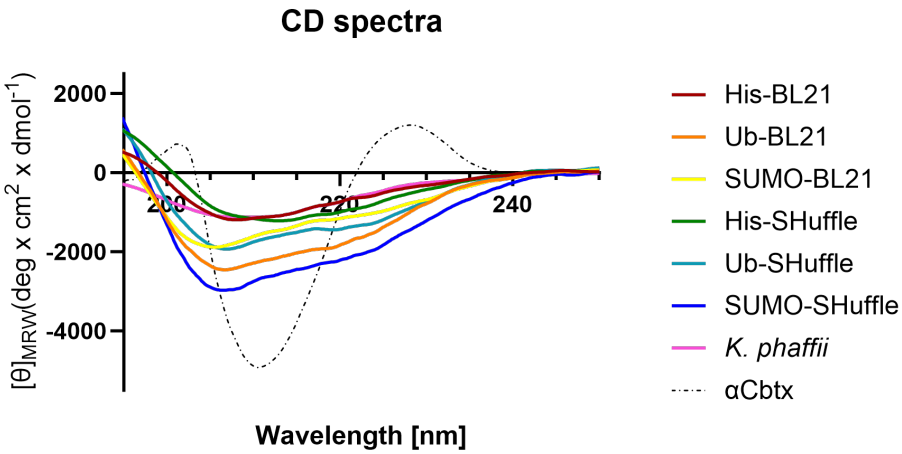
Expression system	α-cobratoxin construct	Yield (mg per 1 L culture)	
		After the first purification	After protease cleavage
***E. coli* BL21(DE3) with csCyDisCo**	His_6-α-cobratoxin	1.0	-
	$UbHis_{10}$-α-cobratoxin	0.8	0.3
	His_6-SUMO-α-cobratoxin	7.2	0.6
***E. coli* SHuffle**	His_6-α-cobratoxin	1.5	-
	$UbHis_{10}$-α-cobratoxin	2.0	0.1
	His_6-SUMO-α-cobratoxin	10.0	0.8
K. phaffii	His_6-α-cobratoxin	1.0	-

Table 1. Comparison of α-cobratoxin yields (mg per 1 L culture) using different expression systems. *The table presents the yield of α-cobratoxin obtained from the use of different expression systems. The expression systems compared include E. coli SHuffle cells, E. coli with the csCyDisCo plasmid, and K. phaffii. The yield is reported in milligrams per liter (mg/L) of culture volume.*

Characterization of the secondary structure in recombinant α-cobratoxins

Assessment of the folding and secondary structure of the recombinant α-cobratoxin samples was performed using CD spectroscopy, comparing them with the structure of the native α-cobratoxin.

The CD spectra revealed important insights into the secondary structure of the proteins. It is known that the ellipticity peak centered around 228 nm in CD spectra is pH sensitive and is believed to involve residues His18 and Tyr21, as well as the disulfide bonds within the hydrophobic core.[30]



Toxin	Helix	Beta	Turn	Random
BL21(DE3) His$_6$-α-Cobratoxin	0.6%	80.8%	0.0%	18.6%
BL21(DE3) UbHis$_{10}$-α-Cobratoxin	1.6%	76.7%	0.0%	21.6%
BL21(DE3) His$_6$-SUMO-α-Cobratoxin	1.1%	77.9%	0.0%	21.0%
SHuffle His$_6$-α-Cobratoxin	1.4%	80.5%	0.0%	18.2%
SHuffle UbHis$_{10}$-α-Cobratoxin	1.9%	77.7%	0.0%	20.4%
SHuffle His$_6$-SUMO-α-Cobratoxin	3.0%	74.4%	0.0%	22.6%
***K. phaffii* His$_6$-α-Cobratoxin**	0.0%	80.6%	0.0%	19.4%
Native αCbtx	0.6%	82.0%	0.0%	17.4%

Figure 2. CD spectra of the different α-cobratoxin constructs*. The CD spectra of the various α-cobratoxin constructs were analyzed and compared to the native α-cobratoxin (αCbtx). The predicted secondary structure composition of each construct is presented in the table below. However, none of the recombinant toxins were obtained in a pure fraction (Figure 1E), and the estimations are based on all present components. It is important to note that despite the tag being used as an identifier, the CD analysis was performed after tag removal by protease cleavage.*

In Figure 1E, it is observed that the recombinant α-cobratoxin samples were not pure, which had an impact on the accuracy of the CD spectra, as the spectra were the result of all the components present in the mixture. This makes it impossible to draw firm conclusions about the secondary structure of the α-cobratoxin variants.

Furthermore, Figure 1E indicated that the previously tagged α-cobratoxin variants lacked disulfide bonds. This, combined with the presence of impurities, may explain why the CD spectra of the recombinant α-cobratoxins did not match those of the native α-cobratoxin. Figure 1E also indicated the presence of disulfide bonds in the His_6-α-cobratoxin constructs. However, the CD spectra for these constructs also differed from the native α-cobratoxin. These differences may suggest that the His_6-α-cobratoxins might not have the correct disulfide pattern. Nevertheless, further experiments are needed to draw definitive conclusions in this regard.

Despite the limitations imposed by impurities, the secondary structure prediction suggested that the recombinant α-cobratoxin samples exhibited a β-sheet conformation, which aligns with the predominant β-sheet structure characteristic of 3FTxs. However, the spectra also hinted at a potential difference in the environment of the hydrophobic core, suggesting that the disulfide bonds may not have formed correctly.

It is worth noting that the concentration of the α-cobratoxin expressed in *K. phaffii* was relatively low (50 µg/mL), posing challenges in obtaining accurate spectra for this particular sample.

Assessment of the activity of the toxins through binding to α7-acetylcholine receptor chimera

The binding capacity of the recombinant α-cobratoxins to the α7-acetylcholine receptor chimera was evaluated using DELFIA binding assays. The purpose of this assessment was to determine if the recombinant toxins retained the ability to recognize and bind to the receptor subunit. Binding experiments were conducted using α-cobratoxin variants obtained using the different expression systems to compare their binding profiles (Figure 3).

Interestingly, only the His_6-α-cobratoxins expressed in *E. coli* demonstrated binding to the α7 receptor in a manner similar to the native α-cobratoxin control. This indicates that the *E. coli*-expressed His_6-α-cobratoxin

maintained its recognition and interaction capabilities with the receptor. In contrast, both the previously tagged α-cobratoxins and the His_6-α-cobratoxins expressed in *K. phaffii* showed significantly reduced binding to the α7 receptor compared to the native α-cobratoxin. This diminished binding is likely attributed to the absence of disulfide bonds in the previously tagged α-cobratoxins.

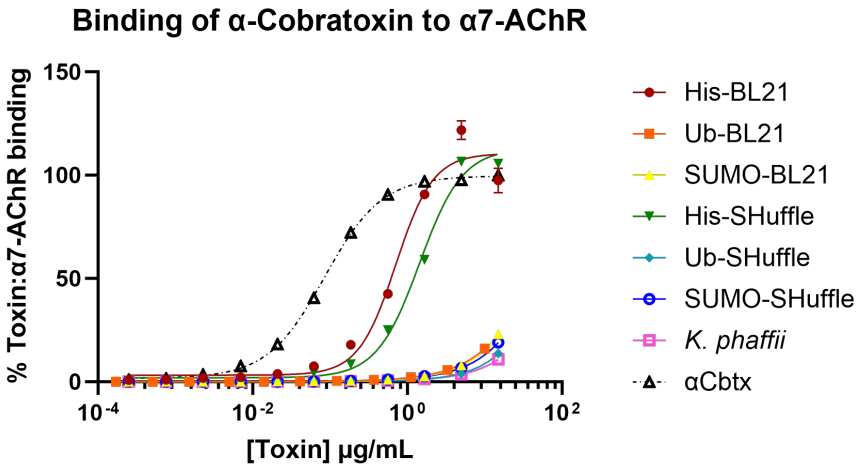


***Figure 3. Binding of recombinant α-cobratoxins to the α7-subunit of nAChR**. The α-cobratoxins expressed in the different expression systems were evaluated for their ability to bind to the α7-subunit. Even though the tag is written as the identifier, this DELFIA was conducted after tag removal by protease cleavage. The native α-cobratoxin (αCbtx) was used as a positive control.*

Despite the observed differences in the CD spectra between the His_6-α-cobratoxins and the native toxin, the His_6-α-cobratoxins still exhibited the ability to bind to the α7 receptor. This suggests that although the conformation of the His_6-α-cobratoxins may differ from that of the native toxin, it does not significantly impair the binding capability of the toxin.

Taken together, these findings highlight the importance of choosing the most optimal expression system for generating functional recombinant α-cobratoxins. The *E. coli*-expressed His_6-α-cobratoxins retained their binding

affinity, while the previously tagged toxins and the *K. phaffii*-expressed His_6-α-cobratoxins exhibited diminished binding. These results provide valuable insights into the functional integrity of the recombinant toxins and their suitability for further investigations and potential applications.

Discussion

In this study, the expression of α-cobratoxin, a representative member of the 3FTx family, was achieved using three different systems: *E. coli* SHuffle cells, *E. coli* with csCyDisCo, and *K. phaffii*. Each of these systems offers distinct advantages and limitations, making the choice of expression system dependent on the specific requirements of the protein.

In terms of post-translational modifications, disulfide formation is a critical aspect of the proper folding and function of 3FTxs, such as α-cobratoxin. However, despite the successful expression in all three systems, none of them yielded α-cobratoxin that was identical to the native available variant. Nevertheless, the comparison of the different expression systems yielded valuable insights. In *E. coli* SHuffle cells, the use of the SUMO tag resulted in the highest titer, but inadequate cleavage during purification led to a lower final yield. Moreover, the addition of DTT, necessary for protease cleavage using both Ulp1 and TEV, might have reduced the formation of disulfide bonds in the recombinant toxin. Conversely, incorporating the $UbHis_{10}$-solubility tag in *E. coli* did not significantly improve the expression of α-cobratoxin compared to using only the His_6-tag.

The observed differences in CD spectra between the native α-cobratoxin and the recombinantly expressed toxins revealed that none of the recombinantly expressed α-cobratoxin variants had a structural conformation identical to the native protein purified from the natural source. However, SDS-PAGE analysis with and without DTT suggested that the removal of tags using Cys-proteases than need a reductive environment to be catalytically active could potentially disrupt the disulfide bonds in the recombinant α-cobratoxin

variants, leading to a slightly different fold compared to the native toxin. This variation could explain the deviation in CD spectra. However, due to the limitations of the secondary purification process, drawing definitive conclusions about the folding proved challenging. The CD spectra may have also been affected by the presence of free fusion proteins and other impurities that were not entirely removed from the toxin samples. To establish a more conclusive assessment, further purification and validation through e.g., size-exclusion chromatography or ion-exchange chromatography would have been necessary.

In contrast, the proteins expressed in *E. coli* without fusion proteins exhibited a folded structure, displaying a β-sheet conformation consistent with the theoretical folding of α-cobratoxin. Similarly, the proteins expressed in *K. phaffii* also showed a folded structure comparable to the *E. coli*-expressed toxins without fusion proteins. However, the lower concentration of protein in the *K. phaffii* samples might have affected the quality of the CD spectra to some extent.

When considering the CD spectrum of native α-cobratoxin, obtained through purification from the natural source using chromatographic methods, it is worth noting that the purified native α-cobratoxin exists as roughly 50% monomers and 50% dimers stabilized by intramolecular disulfide bonds, which may further complicate the interpretation of CD spectra and structural analyses of the recombinant toxins.[9,31] This complexity makes it challenging to attribute the curvature of the CD spectrum solely to monomeric α-cobratoxin. Therefore, interpreting the native α-cobratoxin spectrum requires caution, considering the likelihood that it represents a mixture of at least two isoforms. It is also possible that the recombinantly expressed proteins contain multiple populations of α-cobratoxin with different disulfide bond patterns, causing the actual correctly folded α-cobratoxin to be only a fraction of the total polypeptides present. Therefore, further optimization of the expression system and purification methods should be explored to enhance the formation

of correct disulfide bonds and ensure the structural stability of the expressed toxins.

Interestingly, α-cobratoxin expressed solely with a His_6-tag displayed similar binding behavior to the α7 subunit of the nicotinic acetylcholine receptor (nAChR) as the native α-cobratoxin. However, according to the CD spectra, the secondary structure of the His_6-tagged recombinant toxin differed from that of the native α-cobratoxin. This discrepancy could arise from the presence of the His_6-tag, indicating that while the His_6-tagged toxin maintains its binding capability, it may adopt a slightly altered conformation.

In contrast to previous studies on the expression of 3FTxs, the findings of this study highlight the challenges encountered in producing correctly folded α-cobratoxin. Glanville *et al.* utilized HEK cells to produce various 3FTxs and demonstrated their utility as antigens in phage display experiments.[32] Similarly, Liu *et al.* employed *E. coli* BL21(DE3) and Rosetta(DE3) to express 3FTxs fused to DsbC and demonstrated that they could successfully be used as immunogens to generate polyclonal sera that could neutralize whole venoms from three different cobras.[16] While the correct folding of the toxin might be less crucial for immunizations, where the antigen undergoes processing by the immune system, in antibody phage display selection experiments, there is no such processing involved. The successful selection of high-affinity antibodies depends almost entirely on molecular recognition and therefore the correct folding of both toxin and antibody fragment is critical. For *in vitro* display-based discovery approaches, structural integrity, correct folding, and post-translational modifications are therefore essential.

It is relevant to consider that not all disulfide bonds may be correctly formed in the recombinant toxins, and that this could potentially impact their structural stability.[33] The proper formation of disulfide bonds is crucial for maintaining the native conformation and functional properties of 3FTx.

Disrupted disulfide bonds or incorrect bonding patterns could result in altered structural dynamics and compromised functionality of the recombinant toxins.

Recombinant toxins have been utilized to advance immunization strategies [34], offering a means to obtain pure and well-characterized toxins essential for antivenom development against snake venom.[15,16,32,35] Furthermore, the genetic engineering of snake toxins has opened new therapeutic possibilities, exemplified by engineered toxin variants like modified α-cobratoxin. These variants enable experimentation with toxin scaffolds to explore the potential design of non-toxic or less toxic versions with exploitable bioactive properties for therapeutic purposes.[36,37]

In conclusion, this study highlights the potential of recombinant toxin expression as a reliable and scalable source of toxin proteins, offering insights into venomous organisms and facilitating research and therapeutic development. The choice of expression system proved crucial, and further optimization is needed to achieve correct folding and ensure proper recognition and binding to the nAChR. The ability to express toxins recombinantly paves the way for innovative approaches in antivenom development and the design of toxin-derived therapeutics with potential applications beyond snakebite envenoming therapy.

Author contributions:

ADJ, CH, ERdT, and AHL conceptualized the project. ADJ, CH, and ERdT designed the experiments, ADJ, AV, and IB performed the experiments. ADJ, ERdT, and AHL wrote the manuscript. All authors revised and approved the manuscript.

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Chapter 4 – Article II

A single-chain variable fragment selected against a conformational epitope of a recombinantly produced snake toxin using phage display, *New Biotechnology*, 2023, Volume 76

The objective of this study was to explore the feasibility of using recombinantly expressed α-neurotoxins for monoclonal antibody discovery through phage display selection, similar to the native toxin. The combination of phage display technology and recombinant expression systems allowed for the selection of monoclonal antibodies and the production of recombinant toxins, respectively, using neither animal nor human material in the process.

To assess the folding and stability of the recombinant toxin compared to the native toxin, CD analysis was performed. This allowed us to evaluate the structural properties of the toxins and ensure that the recombinant toxin maintained its native-like structure and functionality. The functional properties of the toxins were evaluated by utilizing patch-clamp assays. This technique enabled direct investigation of the toxins' impact on ion channels, specifically their interaction with the nAChR subunit. By measuring the electrophysiological responses, we gained insights into the toxins' functionality and potency.

Phage display technology was employed to selectively isolate monoclonal antibodies against α-cobratoxin, utilizing both recombinant α-cobratoxin expressed in *E. coli* and the native α-cobratoxin as antigens. Through this selection process, an scFv antibody capable of recognizing α-cobratoxin was identified, leading to the blocking of α-cobratoxin's binding to a nAChR subunit *in vitro*. The outcomes of the selections using either α-cobratoxins demonstrated similar results.

The main objective of this research was to demonstrate the viability of using recombinant toxins as antigens for antibody discovery through phage display.

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A single-chain variable fragment selected against a conformational epitope of a recombinantly produced snake toxin using phage display

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ABSTRACT

Phage display technology is a powerful tool for selecting monoclonal antibodies against a diverse set of antigens. Within toxinology, however, it remains challenging to generate monoclonal antibodies against many animal toxins, as they are difficult to obtain from venom. Recombinant toxins have been proposed as a solution to overcome this challenge, but so far, few have been used as antigens to generate neutralizing antibodies. Here, we describe the recombinant expression of α-cobratoxin in *E. coli* and its successful application as an antigen in a phage display selection campaign. From this campaign, an scFv (single-chain variable fragment) was isolated with similar binding affinity to a control scFv generated against the native toxin. The selected scFv recognizes a structural epitope, enabling it to inhibit the interaction between the acetylcholine receptor and the native toxin *in vitro*. This approach represents the first entirely *in vitro* antibody selection strategy for generating neutralizing monoclonal antibodies against a snake toxin.

Introduction

Every year, snakebite envenoming causes a large number of deaths and amputations when victims do not receive timely administration of antivenom [1]. Existing antivenoms on the market consist of antibodies or antibody fragments derived from the plasma of immunized animals [2]. However, new treatment modalities are being investigated, including the use of monoclonal antibodies obtained using *in vitro* display technologies such as phage display, which can potentially deliver therapeutic antibodies that are highly specific, possess high neutralizing capacities, and benefit from improved safety profiles [3,4]. A number of such monoclonal antibodies and single domain nanobodies that can neutralize snake toxins *in vivo* have already been reported [5-9]. However, all these efforts have relied on toxins from native sources. Of the estimated 19–25,000 snake toxins predicted to exist [10], only very few are commercially available, thereby imposing a major bottleneck on the development of monoclonal antibodies against most snake toxins. To circumvent this challenge, the use of recombinant toxins may seem obvious. However, while several studies involving immunization using recombinant snake toxins have been reported [11-14], there are so far no reports on the use of recombinant snake toxins as antigens for the discovery of monoclonal antibodies using *in vitro* display technologies and naïve libraries. This observation could reflect that the structural integrity of snake toxins produced recombinantly thus far is potentially inadequate for such toxins to be used as antigens, *i.e.* incorrectly folded toxins will only select for suboptimal binders that do not sufficiently recognize the toxins in their native conformation. Therefore, new systems for recombinant protein expression could help unlock access to the myriads of snake toxins that are currently unavailable to researchers. Finally, the ability to manipulate and carefully alter toxins using recombinant DNA technology may also open up further applications, as it may allow easier study of toxin biochemistry and venom evolution *via* the creation of toxin mutants, as well as facilitating the development of new molecular tools, such as toxoids, tagged toxins, or fusion proteins [15].

Abbreviations: BLI, Biolayer interferometry; scFv, Single-chain variable fragment; MALDI-TOF, Matrix-Assisted Laser Desorption Ionization - Time of Flight; HPLC, High-performance liquid chromatography; TEV, Tobacco etch virus; nAChR, Muscle-type nicotinic acetylcholine receptor; ACh, Acetylcholine; DELFIA, Dissociation-Enhanced Lanthanide Fluorescence Immunoassay; CDR, Complementarity-Determining Region.

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To address the above challenges and opportunities, we used the csCyDisCo expression system (cytoplasmic disulfide bond formation in *E. coli*) [16] for the generation of a model toxin (α-cobratoxin) from the monocled cobra (*Naja kaouthia*), which was then further employed as an antigen in a phage display-based antibody selection campaign. The CyDisCo system is based on co-expression of one catalyst of disulfide bond formation, the mitochondrial oxidase Erv1p from *Saccharomyces cerevisiae*, and another catalyst of disulfide bond isomerization, hPDI (human protein-disulfide isomerase). Erv1p provides the oxidizing equivalents to generate disulfide bonds *de novo*, and the hPDI isomerizes non-native disulfide bonds. The CyDisCo co-expression system has been shown to accommodate highly complex disulfide-bonded proteins, including Fab antibody fragments [17] (five disulfide bonds), the vtPA [18] (a tissue plasminogen activator fragment with nine intra-molecular disulfide bonds), Resistin [18] (five intra-molecular disulfide bonds and an inter-molecular disulfide bond), and a SARS-CoV-2 spike protein receptor binding domain [19] (five disulfide bonds). In the present study, the modified csCyDisCo system was used, which includes an additional protein-disulfide isomerase expressed in the venom gland of the cone snail species *Conus geographus* and has been successfully used to produce conotoxins with up to five disulfide bonds [20]. It is shown that a toxin recombinantly expressed in the csCyDisCo system can be used to select binders to the native toxins *in vitro*, and that the antigen-antibody interactions rely on structural epitopes. Thereby, a fully *in vitro* pipeline is presented for the selection of monoclonal antibodies against snake toxins, which could find utility for the development of recombinant antivenoms even against snake venoms which cannot be procured, as long as toxin sequence information is available. With the relatively recent rise of snake genomics [21], such entirely *in vitro* methodologies and pipelines may become increasingly important in the field of toxinology.

Material and methods

Toxin preparation

α-cobratoxin and *N. nivea* venom were obtained in lyophilized form from Latoxan SAS (Portes-lès-Valence, France), and prepared as previously described [7]. The α-cobratoxin was reconstituted in

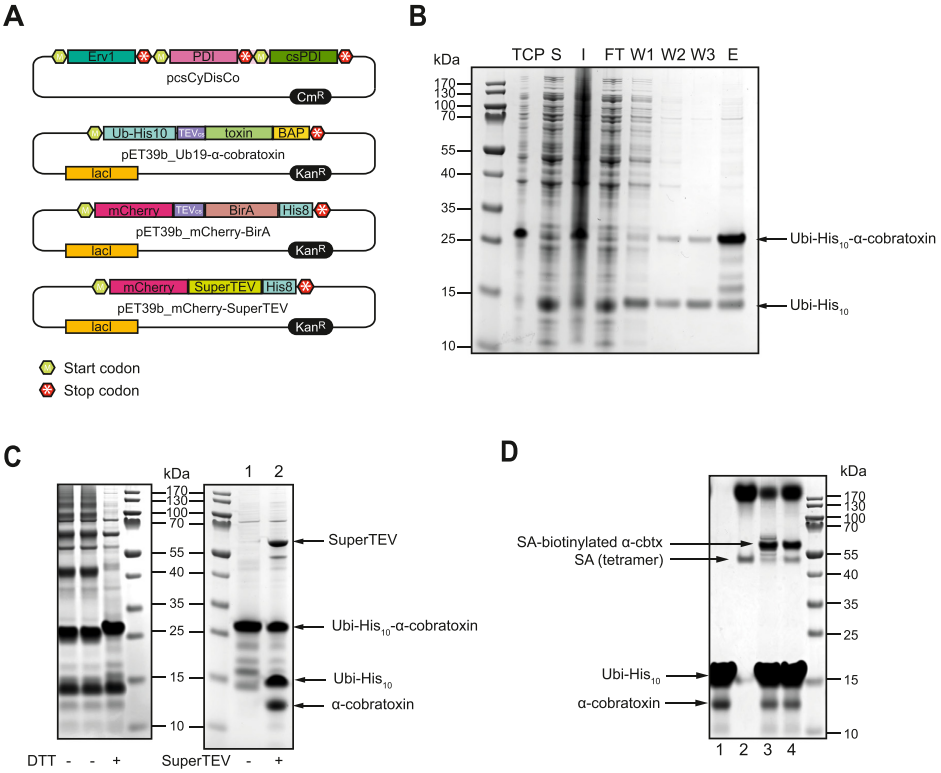

Fig. 1. *E. coli* expression, purification, and biotinylation of α-cobratoxin. A) Overview of the different plasmids used in this study. pcsCyDisCo encodes Erv1p, hPDI, and csPDI under the control of a tac promoter and confers chloramphenicol resistance. pET39b_Ub19-α-cobratoxin encodes Ub-His10-α-cobratoxin under the control of a T7 promoter and confers kanamycin resistance. This vector contains the *lac*I gene, which encodes the lac repressor, as well as a C-terminal biotin acceptor peptide (BAP). pET39b_mCherry-BirA and pET39b_mCherry-SuperTEV encode mCherry-BirA-His8 and mCherry-SuperTEV-His8, respectively, under the control of a T7 promoter. B) Colloidal blue-stained reducing SDS-PAGE analysis of *E. coli* extracts and IMAC purification steps after Ub-His10-α-cobratoxin expression. TCP, Total cell protein fraction; S, Soluble fraction; I, Insoluble fraction; FT, Flow-through from IMAC; W, wash; E, elution from the nickel resin. Premature termination of protein translation or *in vivo* cleavage after the ubiquitin tag was observed, as reported by others [1]. C) Colloidal blue-stained SDS-PAGE analysis of IMAC purified α-cobratoxin under reducing and non-reducing conditions (gel on the left) and reducing SDS-PAGE analysis of IMAC purified α-cobratoxin before (lane 1) and after TEV cleavage (20 h) (lane 2) with the SuperTEV endoprotease (gel on the right). D) Colloidal blue-stained reducing SDS-PAGE analysis of α-cobratoxin *in vitro* biotinylation. Lane 1, α-cobratoxin fraction after Size Exclusion Chromatography; lane 2, Streptavidin (ThermoFisher Scientific, #21135); lane 3, α-cobratoxin incubated with 1 μL of Streptavidin; lane 4, α-cobratoxin incubated with 2 μL of Streptavidin. The streptavidin tetramer (53 kDa) and the streptavidin:α-cobratoxin complex (~63 kDa) are indicated on the gel. Gel analysis has been performed with the BioRad Image Lab Software.

phosphate-buffered saline (PBS, 10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and biotinylated using a 1:1 (toxin:biotinylation reagent) molar ratio as previously described [6]. Following biotinylation, Amicon® Ultra-4 Centrifugal Filter Units (Millipore, Burlington, USA) with a 3 kDa membrane cut-off were used for purification of the biotinylated toxin. The protein concentration was measured by the absorbance at 280 nm using a NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted using the molar extinction coefficient predicted by ProtParam (web.expasy.org). The degree of biotinylation was analyzed using MALDI-TOF in an Ultraflex II TOF/TOF spectrometer (Bruker Daltonics, Billerica, USA). *N. nivea* venom was fractionated by reverse-phase HPLC as previously described [22].

Plasmid construction

To produce the recombinant α-cobratoxin, a modified version of the pET39_Ub19 vector [23] was used that allowed the production of toxins with an N-terminal ubiquitin (Ub) solubility tag with an internal His_{10}-loop (Ub-His_{10}), followed by a Tobacco Etch Virus (TEV) protease cleavage site. The gene encoding the α-cobratoxin was inserted between the *Kpn*I and *Hin*dIII restriction sites into pLE879 [16] (Fig. 1A). The toxin sequence, codon-optimized for expression in *E. coli*, was synthesized by Eurofins Genomics GmbH (Ebersberg, Germany) and encoded a C-terminal biotin acceptor peptide (AviTag) preceded by a flexible linker (-GSGGS-). The construction of the helper plasmid, pcsCyDisCo (pLE577), containing Erv1p, hPDI, and $csPDI_{GH/GH}$ [24], has previously been described [16]. For the expression of the mCherry-tagged *E. coli* biotin ligase (BirA), the sequence encoding mCherry-BirA was amplified from the pACYC-mCh-BirA (kindly donated by Dr Matthieu Sainlos, Bordeaux University, France) [25] and inserted into pLE879 between *Nde*I and *Xho*I, thus creating an mCherry-BirA construct, fused to a C-terminal His_8-tag. A plasmid encoding the TEV protease was designed by fusing a codon-optimized mCherry gene with a codon-optimized version of the SuperTEV protease [26]. The mCherry-SuperTEV gene was synthesized by Eurofins Genomics GmbH (Ebersberg, Germany) and PCR amplified. Like the mCherry-BirA construct, the mCherry-SuperTEV was inserted between *Nde*I and *Xho*I in pLE879, hence fusing a C-terminal His_8-tag to the mCherry-SuperTEV construct.

Recombinant expression and purification of the SuperTEV protease and the BirA ligase

Chemically competent *E. coli* BL21(DE3) cells (New England Biolabs, Ipswich, USA) were transformed with pET39-mCherry-SuperTEV or pET39-mCherry-BirA and plated on an LB agar plate containing kanamycin (50 μg/mL). A total of 5 mL of LB starter cultures with appropriate antibiotics were inoculated into 500 mL of ZYM-5052 auto-inducing medium as described in [27]. Cultures were grown for 5 h at 37 °C with 200 rpm agitation, and thereafter the temperature was reduced to 16 °C for ~20 h, allowing for expression of the target protein. Cultures were then centrifuged at 6000*g* at 4 °C, for 15 min. The supernatant was discarded, and the bacterial pellet was resuspended in 40 mL of lysis buffer composed of TA buffer (50 mM Tris.OAc pH 8.0, 1 mg/mL lysozyme, and, for the BirA ligase only, 1 μL/mL protease inhibitor cocktail set III (Calbiochem, Merck KGaA, Darmstadt, Germany)) followed by sonication on ice (Qsonica Q500, Thermo Fisher Scientific) for 2 min at 40% amplitude with 2 s on cycle and 1 s off cycle. The cells were then centrifuged at 14,000*g*, 4 °C for 30 min and 4 mL of HIS-Select® Nickel Affinity Gel (Millipore, Burlington, USA) was added to the supernatant. After 1 h of incubation at 4 °C with end-over-end rotation, the resin was centrifuged for 15 min at 3000*g* and the supernatant discarded. The resin was then resuspended in 40 mL of equilibration buffer (TA buffer with 20 mM imidazole), centrifuged for 10 min at 3000*g*, and the supernatant was discarded. The resin was then resuspended into 10 mL of equilibration buffer and transferred into a gravity flow chromatography column. The protein was then eluted with 10 mL of elution buffer (TA buffer with 400 mM imidazole). *E. coli* extracts and IMAC purification steps samples were run on an sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) gel, stained with colloidal Coomassie blue. The purified protein was dialyzed three times against 1 × PBS at 4 °C. Following dialysis, the protein was concentrated using centrifugal filtration units and flash-frozen with liquid nitrogen for conservation at − 80 °C.

Expression of recombinant α-cobratoxin, TEV cleavage, biotinylation, and purification

Recombinant α-cobratoxin (Rα-cobratoxin) was generated as follows. Chemically competent *E. coli* BL21(DE3) cells were co-transformed with pET39_Ub19-α-cobratoxin and pcsCyDisCo and plated on an LB agar plate containing kanamycin (50 μg/mL) and chloramphenicol (30 μg/mL). The toxin was then expressed as described above for the SuperTEV endoprotease. Following dialysis into PBS, the protein was concentrated to 40 μM using centrifugal filtration units. The SuperTEV endoprotease was added at a molar ratio of 1:20 (SuperTEV: Rα-cobratoxin), and cleavage was carried out at 30 °C for 1 h, whereafter it was moved to 4 °C overnight. TEV cleavage efficiency was checked by running samples before and after TEV cleavage on an SDS-PAGE gel and stained with colloidal Coomassie blue. Quantification of the intensity of Coomassie-stained bands was performed with ImageLab Software. For *in vitro* biotinylation, BirA was added at a molar ratio of 1:100 (BirA:Rα-cobratoxin) along with 5 mM ATP and 300 μM biotin. The solution was left at room temperature for 1 h, after which a new batch of 300 μM biotin and 5 mM ATP was added for another hour of incubation at 30 °C. Biotinylation of the toxin was analyzed by a Streptavidin Gel-Shift assay evaluated by SDS-PAGE analysis [28]. After confirmation of tag cleavage and biotinylation status, the Rα-cobratoxin was filtered through a 0.22-μm filter and purified further on a size exclusion chromatography column (HiLoad 16/600 Superdex 75 pg, Cytiva, Marlborough, USA) using a BioRad NGC Quest 10 Plus chromatography system and 1 x PBS as running buffer.

Circular dichroism (CD)

Rα-cobratoxin (20 μg/mL) was dialyzed against 10 mM potassium phosphate buffer (pH 7.0). The Far-UV CD was recorded using a JASCO J-1500 spectrophotometer (Easton, MD, USA) using a 0.1 mm quartz cuvette. The spectrum was acquired by conducting 10 measurements between 250 nm and 190 nm with a bandwidth of 1 nm and intervals of 1 nm, with a scan speed of 50 nm/sec. The measurements were carried out at a temperature of 15 °C, and the spectra were processed and smoothened using SpectraManager software (JASCO) and GraphPad Prism (GraphPad Software).

Electrophysiology

The toxin's ability to inhibit the muscle-type nicotinic acetylcholine receptor (nAChR) was assessed using a Qube 384 automated electrophysiology platform (Sophion Bioscience A/S, Ballerup, Denmark) as described elsewhere [7]. In brief, the human-derived rhabdomyosarcoma RD cell line (ATCC cat. #CCL-136) was used, which endogenously expresses the muscle-type nicotinic acetylcholine receptor (consisting of the α1, β1, δ, γ, and ε subunits). The cells were patched with an extracellular solution containing 145 mM NaCl, 2 mM $CaCl_2$, 4 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH adjusted to 7.4 and osmolality adjusted to 296 mOsm and an intracellular solution containing 140 mM CsF, 10 mM EGTA, 10 mM NaCl, 10 mM HEPES, pH adjusted to 7.3 and osmolality adjusted to 290 mOsm. The toxins were prepared in the extracellular solution supplemented with 0.1% human serum albumin. Current mediated by nAChR was elicited by the addition of 70 μM acetylcholine (ACh), and a wash with 2 U acetylcholinesterase was used to ensure complete ACh removal. The cells were preincubated with the toxins before the addition of ACh in combination with the

toxins. Rα-cobratoxin was tested at two different concentrations, 150 nM and 15 nM, while α-cobratoxin also was tested at 1.5 nM.

A previously isolated IgG antibody against α-cobratoxin, 2554_01_D11 [8], was used to neutralize the effect of both Rα-cobratoxin and α-cobratoxin as described elsewhere [7]. To test the neutralization, 100 nM IgG was preincubated with the toxins at various concentrations for at least 30 min at room temperature prior to the application. The analysis of the data was performed using the Sophion Analyzer (Sophion Bioscience) and GraphPad Prism (GraphPad Software).

Phage display selection

Selection of the scFvs was performed by panning the IONTAS phage display library [29] (diversity of 4×10^{10} human scFv clones, kindly donated by IONTAS Ltd., Pampisford, UK) against biotinylated native and recombinant α-cobratoxin. Panning was carried out as described elsewhere [29], except that the biotinylated toxins were captured on streptavidin-coated beads (Dynabeads M-280, Invitrogen, Waltham, MA, USA) instead of direct coating to a 96-well microtiter plate. The concentration of the target toxins was decreased stepwise through the three rounds of selections, starting at 100 nM in the first round and ending at 25 nM in the third round.

Subcloning and primary screening of scFvs

Subcloning of the α-cobratoxin-binding selection output into the pSANG10–3 F expression vector and primary screening of candidates was performed as described elsewhere [6]. In brief, scFv genes from the selection outputs were subcloned from the pSANG4 phagemid vector to the pSANG10–3 F expression vector using *Nco*I and *Not*I restriction endonuclease sites and transformed into *E. coli* strain BL21(DE3). This expression vector allows for the production of scFvs with a C-terminal His6 tag followed by a 3xFLAG tag. From the two subcloned selection outputs, colonies from the Rα-cobratoxin and the α-cobratoxin selections were picked and expressed in 96-well plates. The scFvs were assessed for their binding to biotinylated α-cobratoxin and Rα-cobratoxin (5 μg/mL) indirectly immobilized on black MaxiSorp plates (Nunc A/S, Roskilde, Denmark) coated with streptavidin (10 μg/mL) using a DELFIA assay [7] (Perkin Elmer, Waltham, MA, USA). After thorough washing with PBS-T (PBS, 0.1% Tween-20) and PBS, scFv binding to the immobilized toxins was detected using a 1 in 1500 dilution of anti-FLAG M2 (F1804, Sigma, Saint-Louis, USA) previously conjugated with Europium (DELFIA Eu-N1 ITC chelate, 1244–301, Perkin Elmer).

Secondary screening and sequencing of scFvs

Clones from the α-cobratoxin (24 clones) and Rα-cobratoxin (51 clones) selection were cherry-picked and assessed for their binding to α-cobratoxin, Rα-cobratoxin, streptavidin, and the ubiquitin tag using a DELFIA assay as described above. From those, 20 clones from each selection were sequenced (Eurofins Genomics Sanger sequencing service, Ebersberg, Germany). The antibody framework and CDR regions were annotated and analyzed to identify 11 unique clones from the α-cobratoxin selection and 10 unique clones from the Rα-cobratoxin selection.

Expression-normalized capture DELFIA on native and denatured toxins

A DELFIA sandwich immunoassay was carried out on the unique clones as described elsewhere [6] using a biotinylated antigen concentration of 100 nM. Briefly, black MaxiSorp plates (Nunc) were coated overnight with anti-FLAG M2 antibody (Sigma, 2.5 μg/mL in PBS, 50 μL per well). After blocking with 3% M-PBS (skim milk in PBS), washing with PBS, and addition of 25 μL of 6% M-PBS to each well, 25 μL of individual auto-induction culture supernatants containing expressed scFv was added for each scFv to the assay plate and incubated for 1 h. Plates were washed three times with PBS-T and three times with PBS. Binding of biotinylated antigen (100 nM of each antigen in 3% M-PBS, 50 μL per well) was allowed to occur for 1 h, which was followed by a detection step using Europium-labeled streptavidin (Perkin Elmer, 1244–360, 1 μg/mL in M-PBS, 50 μL per well) for 30 min. For the DELFIA using denatured toxins, the necessary amount of snake toxins (α-cobratoxin and long-neurotoxin 1 from *N.nivea*) and Rα-cobratoxin were boiled for 15 min in presence of 4 mM DTT before being diluted into PBS to their final concentration (leading up to a final DTT concentration of 0.1 mM) and added to the wells.

scFv expression and purification

The top 3 binders were expressed and purified for further characterization as described elsewhere [30]. The scFvs were purified using HisTrap FF 1 mL columns (17531901, Cytiva, Marlborough, MA, USA) on an NGC Quest 10 system (Bio-Rad, Hercules, CA, USA). Protein concentration was determined by absorbance at 280 nm and the molar extinction coefficient predicted by ProtParam (web.expasy.org).

Determination of binding affinities with BLI (biolayer interferometry)

The binding affinities of the selected clones were measured using the Octet K2 system (FortéBio, Fremont, CA, USA). Measurements were performed at 30 °C in 96-well, black microplates (655209, Greiner Bio-One, Kremsmünster, Upper Austria) that were agitated at 1000 rpm. The biotinylated α-cobratoxin (ligand, 50 nM) was captured on a Streptavidin (SA) Biosensor (18–5019, Sartorius, Göttingen, Germany). After a brief acid conditioning with glycine buffer (10 nM, pH 2.0), the toxin-coupled biosensor was neutralized in kinetic buffer (18–1105, Sartorius). Steady-state measurements consisted of equilibration of the toxin-coupled biosensor and the reference biosensor in kinetic buffer for 600 s, followed by a baseline reading for an additional 120 s. The sensors were then dipped into wells containing the scFvs at concentrations ranging from 2 μM to 4 nM in kinetic buffer for 600 s. The sensors were then dipped into wells containing kinetic buffer for 150 s to see if there was a dissociation rate before being regenerated with glycine buffer. To determine affinity, steady-state analysis was performed plotting the binding response at "equilibrium" (599 s) against the scFv concentration. Equilibrium dissociation constant (K_D) values were determined as the scFv concentration, at which half of the toxin sites are occupied at equilibrium using the One-site binding equation from GraphPad Prism 9.

In vitro blocking DELFIA

In vitro neutralization of the α-cobratoxin interaction with the α7 nicotinic acetylcholine receptor (α7-nAChR), recombinantly expressed as in [31], by the selected clones was performed using a similar DELFIA protocol to that described above, but with some modifications as described elsewhere [31]. Briefly, Maxisorp 96-well plates were coated overnight at 4 °C with 500 ng of α7-AChR/well. Mixtures of serially diluted anti-α-cobratoxin clones and a fixed amount of biotinylated α-cobratoxin (0.1 μg/mL) were pre-incubated at room temperature for 30 min prior to being added to the coated plates. Wells containing only the biotinylated α-cobratoxin with no added anti-α-cobratoxin scFv or wells containing blocking buffer only (1 x PBS, 1% BSA) were used as controls to determine the percentage of inhibition of the binding between α-cobratoxin and α7-nAChR. Biotinylated α-cobratoxin bound to α7-nAChR was detected using Europium-labeled Streptavidin (Perkin Elmer, 1244–360, 1 μg/mL in assay buffer, 100 μL per well for 30 min). Each concentration was run in duplicate and presented as mean ± SEM values. The IC_{50} value of each scFv was determined by fitting dose-response curves to the data with GraphPad Prism 9 ([Inhibitor] *vs.* response - Variable slope equation).

Results and discussion

Patch-clamp-based characterization of the recombinant toxin demonstrates functionality

The recombinant α-cobratoxin, a 7.8 kDa three-finger toxin from *N. kaouthia* (71 amino acid residues, five disulfide bridges), was expressed in *E. coli* using the csCyDisCo system designed for the production of disulfide-rich peptides and proteins in the cytosol of *E. coli* [16]. After expression using auto-inducing medium, the recombinant α-cobratoxin-AviTag (Rα-cobratoxin-AviTag) fusion protein was purified from crude lysate by metal-affinity chromatography (Fig. 1B). Premature termination of protein translation or *in vivo* cleavage after the ubiquitin tag was observed, as reported by others [32], leading to two purified proteins; one corresponding to the full-length Ubi--His_{10}-α-cobratoxin and the other corresponding to the N-terminal fragment (Ubi-His_{10}). Before undergoing a TEV protease cleavage with the SuperTEV endoprotease, an SDS-PAGE analysis of the eluted protein under reducing and non-reducing conditions was performed to verify disulfide-bond formation (Fig. 1C). The species migrating with a molecular weight corresponding to that of the full-length protein (Ubi--His_{10}-α-cobratoxin; theoretical mass: 22.3 kDa) shifted up when using a reducing SDS-PAGE loading buffer, confirming the presence of disulfide bridges. Since all molecules present in the band representing Ubi--His_{10}-α-cobratoxin shifted upon reduction, it was concluded that disulfide bond formation had occurred. As observed in many other cases [33-36], TEV protease cleavage was incomplete and estimated to be around 50% based on SDS-PAGE analysis. To confirm the proper folding of the recombinant Rα-cobratoxin, its secondary structure was evaluated through circular dichroism analysis, demonstrating that the protein had a structural fold (suppl. Fig. S1). The *in vitro* biotinylated Rα-cobratoxin was further purified by size exclusion chromatography (SEC). As judged by a streptavidin gel-shift assay (Fig. 1D), the biotinylation level was estimated to be above 30%.

To assess whether the protein preparation contained properly folded Rα-cobratoxin, thereby allowing its use as antigen in a phage display selection campaign, the ability of the recombinant toxin to inhibit the nicotinic acetylcholine receptor (nAChR) was investigated using planar patch-clamp. The Rα-cobratoxin was tested at two different concentrations in parallel with native α-cobratoxin to test their ability to inhibit acetylcholine-induced current in a human cell line endogenously expressing the nAChR. At 150 nM, both Rα-cobratoxin and α-cobratoxin were able to fully inhibit the current, while more than 90% of the response was inhibited by 15 nM of Rα-cobratoxin (Fig. 2A). Complete inhibition was still observed using 15 nM of native α-cobratoxin, and even at 1.5 nM of native α-cobratoxin, significant inhibition of the receptor was measured. In these experiments, both native α-cobratoxin and Rα-cobratoxin could be neutralized by a human monoclonal IgG previously reported against native α-cobratoxin [8] (Fig. 2B). The IgG could, however, neutralize a higher concentration of Rα-cobratoxin than native α-cobratoxin. This observation could potentially be explained by Rα-cobratoxin being a mixture of correctly and incorrectly folded species. Nevertheless, based on its clear ability to inhibit nAChR, it was deemed that Rα-cobratoxin was of sufficient quality to be used as antigen in further phage display selection experiments.

Phage display selection on recombinantly expressed α-cobratoxin yields scFv binders

A naïve human single-chain variable fragment (scFv) phage display library containing 4×10^{10} clones was used for phage display selection [29]. Three rounds of selection were performed on streptavidin-coated magnetic beads functionalized with the biotinylated recombinant α-cobratoxin (TPL0442 selection) or the biotinylated α-cobratoxin from *N. kaouthia* (TPL0441 selection). The antigen concentration was decreased between each round to increase the stringency (from 100, 50–25 nM). After the third round, antibody-encoding genes (scFv format) from both selections were isolated and subcloned into a bacterial expression vector [37]. In total, 128 clones from the α-cobratoxin selection and 92 clones from the Rα-cobratoxin selection were picked and analyzed by a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) as previously described [6] (Fig. 3A and B). DELFIA is an alternative to traditional Enzyme-linked Immunosorbent Assay (ELISA), where HRP-labeled immunoreagents are replaced by lanthanide-labeled immunoreagents. Immunoreagent binding is measured by time-resolved fluorometry (TRF) instead of absorbance

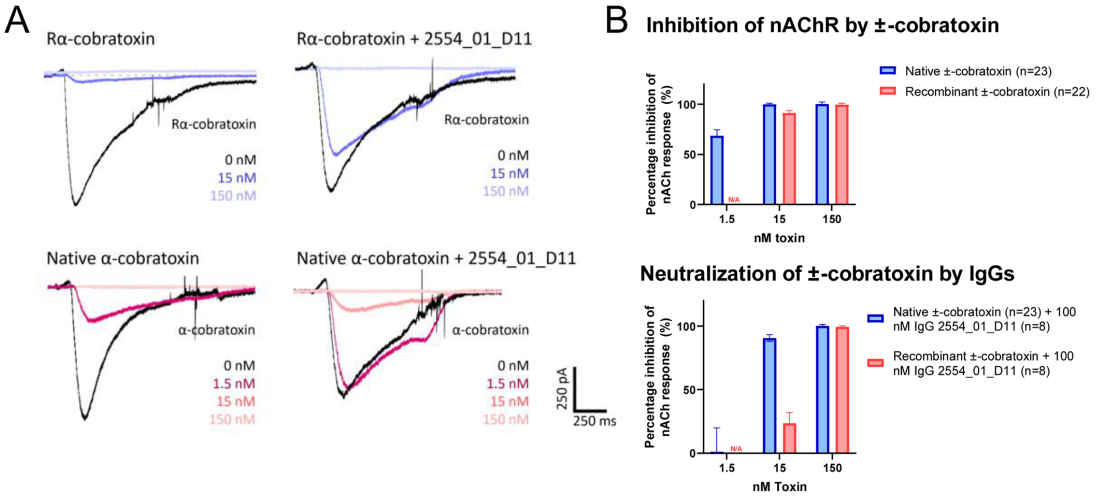

Fig. 2. *In vitro* inhibition of nAChR by recombinant and native α-cobratoxin. A) Representative current traces showing the nAChR-mediated current inhibited by increasing concentrations of native and recombinant α-cobratoxin. At 150 nM and 15 nM α-cobratoxin, the current is completely inhibited, and the current traces are therefore superimposed. B) Top: Inhibition of the nACh response by increasing concentrations of native and recombinant α-cobratoxin. Bottom: IgG 2554_01_D11 (against native α-cobratoxin) can neutralize the effect of both the native and recombinant α-cobratoxin.

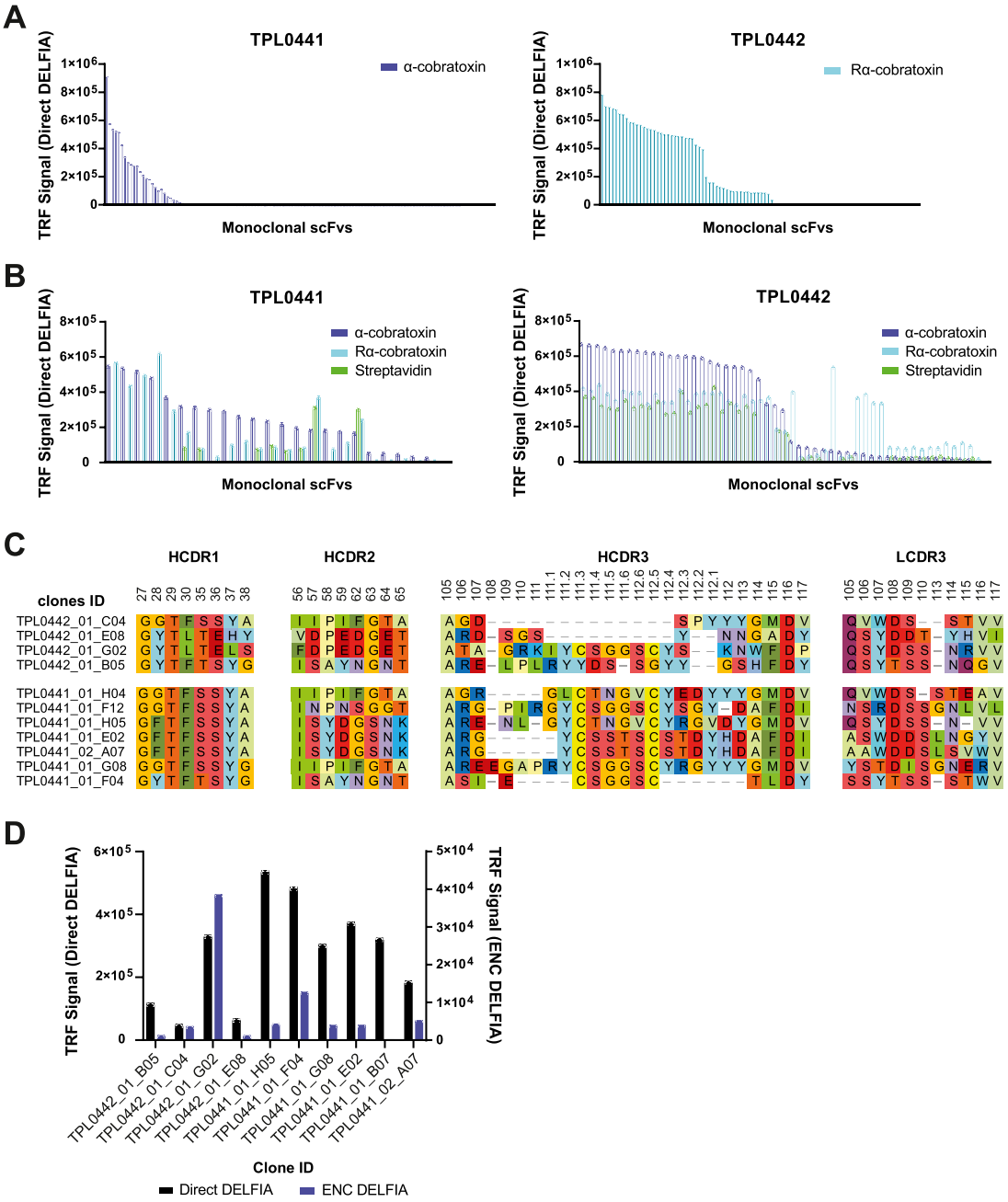

Fig. 3. Affinity ranking of scFvs and their sequences. A) Direct DELFIA against native α-cobratoxin and Rα-cobratoxin of monoclonal scFvs from selections TPL0441 and TPL0442. B) Direct DELFIA against native α-cobratoxin (dark blue), Rα-cobratoxin (light blue), and streptavidin (green) of cherry-picked monoclonal scFvs from selections TPL0441 and TPL0442. C) CDR sequences of the best binders selected from selections TPL0441 and TPL0442 (IMGT numbering). D) Direct and ENC DELFIA of the top ten monoclonal scFv-containing supernatants from both selections.

(ELISA). Of these first screened clones, 10 clones displaying a specific binding signal against α-cobratoxin from TPL0441 selection and 10 clones from TPL0442 selection were picked for DNA sequencing and further characterization (Fig. 3C and supplementary file 1). Interestingly, a high proportion of these clones (>60% for TPL0441 and 10% for TPL0442) showed a six amino acid disulfide loop, C-X_4-C, in the V_H CDR3 sequences, as noticed previously [7]. These scFvs were then evaluated in an expression-normalized capture (ENC) DELFIA assay, which reduces the influence of the clone expression levels on the signal (Fig. 3D). The three α-cobratoxin-binding scFvs that yielded the highest binding signals (two from TPL0441 selection and one from TPL0442 selection) were expressed for further characterization.

Isolated scFvs have similar affinity to a positive control antibody

Binding affinities of TPL0441_01_F04, TPL0441_01_H05, and TPL0442_01_G02 to α-cobratoxin were evaluated by steady-state analysis of biolayer interferometry binding curves alongside a positive control antibody 368_01_C05 (Fig. 4). The positive control antibody was previously selected against the native α-cobratoxin from *N. kaouthia* and was characterized *in vitro* and demonstrated to have partially neutralizing activity *in vivo* [7]. The affinities of TPL0441_01_H05 and TPL0442_01_G02 were similar to each other (around 10 nM) and very close to the affinity of the positive control antibody (8.4 nM). The weakest binder, TPL0441_01_F04, showed a submicromolar affinity resulting from a fast dissociation from the α-cobratoxin coated tip in the biolayer interferometry setup.

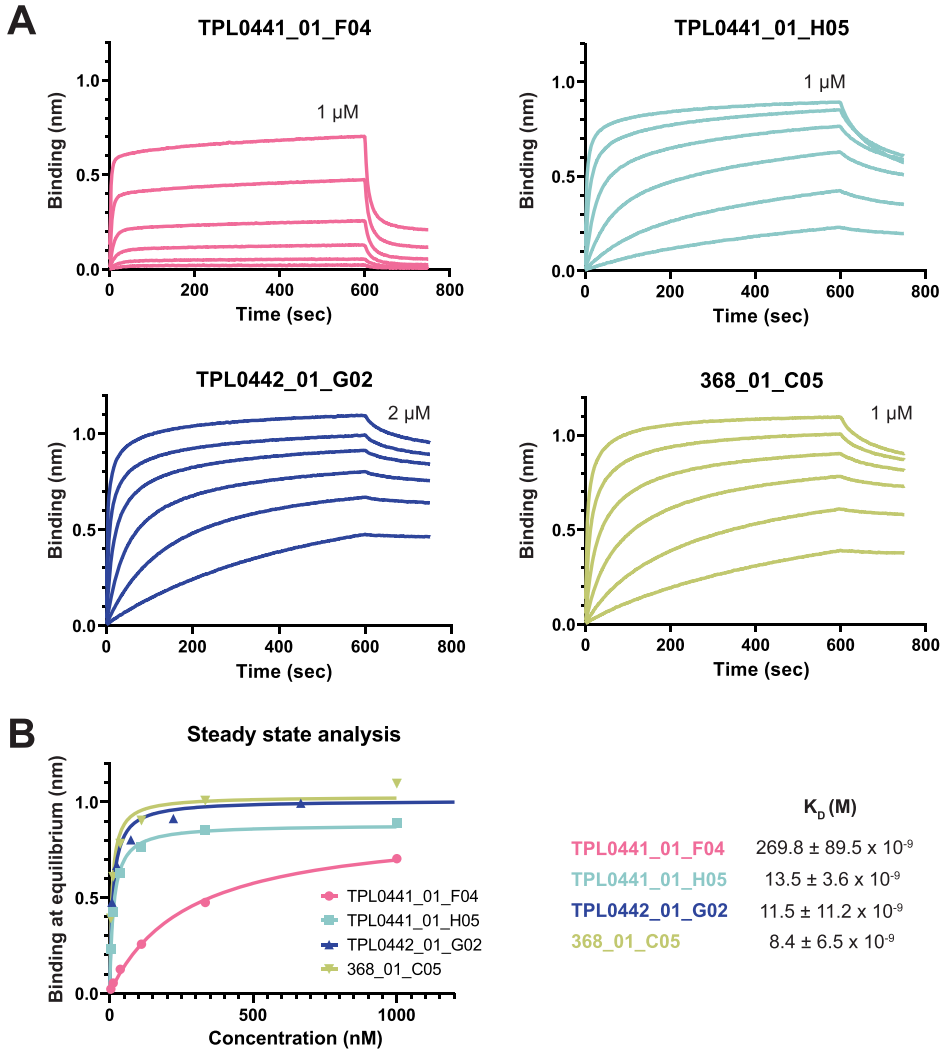

Fig. 4. Estimation of dissociation constants with biolayer interferometry. A) Affinity of three selected scFvs to α-cobratoxin was measured using streptavidin-coated biosensors to capture the biotinylated α-cobratoxin. The reported concentrations (above the highest positioned curve) represent the highest analyte concentration used in the threefold dilution series. B) Steady-state analysis of the binding curves presented in A).

The selected scFvs block the α-cobratoxin:receptor binding interaction

To assess if the scFvs selected against the recombinant α-cobratoxin prevented the interaction between the nAChR and the native toxin, a receptor-blocking assay was performed with the three selected clones and our positive control scFv. Results showed that the three selected scFvs were able to prevent binding between the receptor subunit and α-cobratoxin (Fig. 5). The IC_{50} values were determined to be in the nM to sub-μM range, with TPL0441_01_H05 being the most potent inhibitor (IC_{50} of 18.9 nM) and TPL0442_01_G02 being the least potent (IC_{50} of 240 nM). Interestingly, TPL0441_01_F04, the weakest binder, which has a sub-μM affinity for α-cobratoxin (32-fold weaker than the positive control antibody), showed a better blocking potency than TPL0442_01_G02, which has a nM affinity, indicating that affinity does not always directly correlate with neutralization capacity, as previously observed [7,8].

The toxin-antibody binding interaction relies on a structural epitope

Upon determination of the binding affinities and the IC_{50} values of the selected clones, a DELFIA-based assay was employed to investigate the binding mode(s) of the scFvs to the native and denatured toxin (Fig. 6). In addition to α-cobratoxin from *N. kaouthia*, another three-finger toxin from the *Naja* genus with 81% sequence identity, the long neurotoxin 1 from *N. nivea*, was also employed. Here, TPL0442_01_G02, TPL0441_01_F04, and 368_01_C05 lost their ability to bind to α-cobratoxin when the toxin was denatured, demonstrating that those scFvs interact with one or more structural epitopes on the toxin. Notably, the two clones selected against the native α-cobratoxin also showed binding to the denatured long neurotoxin 1 from *N. nivea,* but not to its native form. This might indicate that a linear epitopic element may exist in this toxin, which has been unfolded upon denaturation, but which is not accessible to the scFv when the toxin is folded in its three-dimensional structure. In regard to clone TPL0441_01_H05, binding to α-cobratoxin was not detected using the capture DELFIA, even though biolayer interferometry showed measurable binding. This could be due to the scFv orientation in the capture DELFIA assay, where the scFv is immobilized in the well as opposed to the biolayer interferometry assay, where the toxin is immobilized to the streptavidin coated tip.

Conclusion

In this study, it is demonstrated that α-cobratoxin from *N. kaouthia* can be recombinantly expressed in a form that allows for the selection of monoclonal scFv antibodies with similar binding affinities and functional neutralization potency as those scFvs selected against the native toxin. Thereby, it is exemplified that neutralizing monoclonal antibodies against snake toxins can be obtained entirely *in vitro* and without the need of snakes and animals for immunization. Importantly, the methods presented here not only remove a potential procurement bottleneck for snake venoms but may also enable the expression and study of non-natural toxins, such as toxin mutants, toxoids, tagged toxins, or fusion proteins. While such other toxins might find utility as molecular tools for research, it is also envisaged that they can help unravel new biology in the field of toxin and venom evolution. However, it is also noted that the recombinant expression of three-finger toxin is complicated by the presence of many cysteine residues in these toxins and that methods that could improve correct protein folding are warranted.

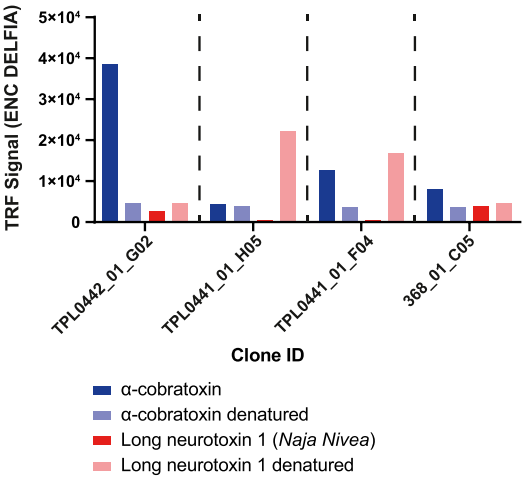

Fig. 6. Binding to structural epitope(s). ENC DELFIA of the selected clones against native (dark shade) and denatured toxins (light shade): α-cobratoxin from *N. kaouthia* in blue and long neurotoxin 1 from *N. nivea* in red.

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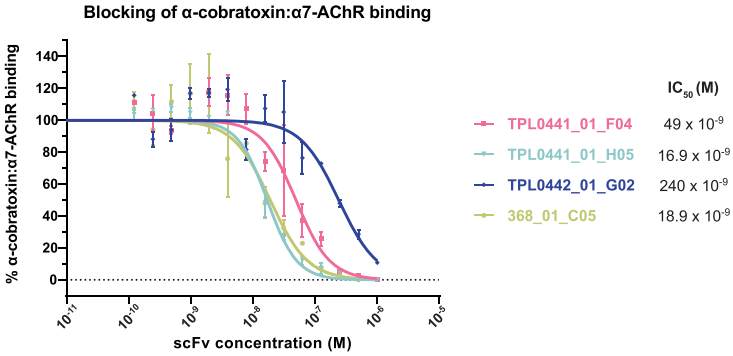

Fig. 5. Inhibition of the binding interaction between the α7-AChR and native α-cobratoxin. scFvs block α-cobratoxin binding to its receptor (α7-AChR) in a concentration-dependent manner. As a negative control, an scFv specific to *Tityus serrulatus* toxin Ts1 was used, which showed no blocking (data not shown). Y-axis is the signal relative to that of the positive control.

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CRediT authorship contribution statement

A.H.L. and C.R. designed the research and wrote the article; A.D.J., C.R., and C.M.H. performed the cloning; A.D.J. performed electrophysiology experiments with the help of K.B.; C.R. and P.D.K. expressed and purified the α-cobratoxin and the enzymes; A.D.J. performed the circular dichroism spectroscopy experiments; P.D.K. performed phage display selections and DELFIAs ; H.A. expressed the α7-nAChR; C.R. fractionated *N. nivea* venom, expressed scFvs, and performed biophysical and biochemical experiments; A.H.L., M.F.B., and L.E. participated in project supervision and reviewed and edited the manuscript. A.H.L. and L.E. secured funding for the research. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.04.002.

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Chapter 5 – Manuscript II

Discovery of neutralizing nanobodies with Consensus α-Neurotoxins via Phage Display

This manuscript focuses on the expression of two consensus toxins, SC-α-neurotoxin (SCC) and LC-α-neurotoxin (LCC), in *P. pastoris*. These toxins were employed as antigens in a phage display selection campaign aimed at discovering cross-binding nanobodies with broad neutralization capabilities.

Both consensus toxins demonstrated successful expression in *K. phaffii*, and analysis using CD spectroscopy revealed that the secondary structure of SCC closely resembled that of native toxins, thus retaining a secondary structure similar to the native toxin.

For the phage display selection campaign, a nanobody library derived from immunized camelids was utilized. Two separate campaigns were conducted, utilizing either the consensus SCC or the LCC as antigens. These campaigns led to the discovery of several unique nanobodies that exhibited the ability to bind to a range of either SC- or LC-α-neurotoxins originating from both cobra and mamba species.

Following the identification of nanobodies displaying cross-binding properties, their characterization was conducted using Bio-Layer Interferometry (BLI) and patch-clamp techniques. The evaluated clones exhibited remarkable binding affinities towards the tested toxins, with dissociation constant (K_D) values ranging from 200 nM to below 1 pM, predominantly falling within the low nanomolar to picomolar range. Moreover, all the tested clones demonstrated the capability to neutralize at least three out of five α-neurotoxins tested *in vitro*.

In conclusion, this manuscript provides evidence that consensus toxins can effectively serve as antigens in phage display experiments, facilitating the discovery of nanobodies capable of cross-binding and neutralizing toxins from diverse species.

Discovery of broadly neutralizing nanobodies using designed consensus antigens

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Abstract:

Broadly neutralizing monoclonal antibodies hold a huge potential for treating infectious diseases and various pathologies, including snakebite envenoming, which currently relies on polyclonal antibodies from immunized animals. To develop improved envenoming therapies based on monoclonal antibodies, we present a methodology using consensus toxins and phage display technology to select cross-reactive nanobodies that can broadly neutralize snake venom toxins. The discovered nanobodies exhibit high affinity towards native toxins from different snake species, with some showing sub-picomolar affinity and the ability to neutralize toxins from diverse elapid species. Our study presents a methodology using consensus toxins and phage display technology to select cross-reactive nanobodies capable of broadly neutralizing snake venom toxins, showcasing the potential for broadly neutralizing antibody discovery against entire snake toxin families. Beyond snakebite envenoming, this

versatile approach opens new avenues for the discovery of broadly neutralizing antibodies and nanobodies against various disease targets, where cross-reactivity is essential.

Keywords:

Phage display technology; neurotoxins; snakebite envenoming; broadly neutralizing antibodies; consensus toxins; antibody discovery

Abbreviations:

nAChR, nicotinic acetylcholine receptors; SC, short chain; LC, long-chain; SCC, short-chain consensus; LCC, long-chain consensus; ACh, acetylcholine; DELFIA, dissociation-enhanced lanthanide fluorescence immunoassay; BLI, biolayer interferometry.

Introduction

Broadly neutralizing antibodies have become important therapeutic tools against diseases with multiple similar target antigens, such as infectious diseases with high antigenic variation, rapidly mutating cancers, and envenomings by venomous animals.[1] Snakebite envenoming is an extreme example of such a disease due to the enormous complexity of snake venoms.[2] As multiple toxins within a single snake venom must be neutralized to properly treat a snakebite victim, the only specific treatment for snakebite envenoming relies on polyvalent antibodies derived from the plasma of immunized animals, *i.e.*, antivenoms. Unfortunately, these animal-derived antivenoms suffer from several drawbacks, such as low content of therapeutically active antibodies, batch-to-batch variation, high cost, and risks of causing adverse reactions related to their immunogenicity in human recipients.[3,4] Therefore, there is a need to develop more effective and affordable treatments for snakebite envenomings.[5,6] One of the suggested solutions entails the development of recombinant antivenoms based on defined mixtures of oligoclonal antibodies.[7,8] However, to develop such complicated products, it is essential that broadly neutralizing agents can be developed rationally and efficiently, as snake venoms are among the therapeutically most complex targets known to man as they consist of dozens of similar and dissimilar toxins that must be neutralized.[1,2] Therefore, snake venoms may also serve as an optimal model system to explore new approaches for the discovery of broadly neutralizing antibodies and fragments thereof.

Here, we report a new approach within the discovery of broadly neutralizing monoclonal antibodies involving the use of consensus antigens, which are designed antigens representing an 'average sequence' of an entire group of related antigens, thereby capturing their common epitopes and other shared characteristics. We furthermore showcase how this approach can specifically be used to discover broadly neutralizing nanobodies against two

subfamilies of proteins, namely the short-chain (SC) and long-chain (LC) α-neurotoxins from the three-finger toxin superfamily.

Beyond their utility as a molecular biology tool to investigate new antibody discovery approaches, the α-neurotoxins are also prevalent across elapid snake venoms and medically very important.[6,7] SC- and LC-α-neurotoxins are therefore highly relevant targets to develop broadly neutralizing nanobodies against. SC- and LC-α-neurotoxins share a common protein fold with minor differences in length (between 60 and 62 or 66 and 75 residues, respectively), and the number and position of disulfide bonds.[9,10] They both bind to and inhibit nicotinic acetylcholine receptors (nAChRs) with varying selectivity against different members of this family of receptors.[11,12]

Several monoclonal antibodies, including both single-chain variable fragments (scFvs), fragments antigen binding (Fabs), immunoglobulin Gs (IgGs), and nanobodies (also known as variable domain of heavy chain of heavy chain antibodies or V_HHs) have previously been discovered against α-neurotoxins from snake venoms.[8,13–17] However, most of these antibodies were discovered using native toxins, and all of the antibodies show limited broadly-neutralizing effects.

In the following, we show how rational design and expression of consensus SC- and LC-α-neurotoxins can be combined with phage display-based nanobody discovery techniques to yield monoclonal nanobodies that are both broadly neutralizing and exhibit very high affinities across different target α-neurotoxins.

Material and methods

Construction of consensus toxins

Amino acid sequences of SC- and LC-α-neurotoxin from African snakes were obtained from UniProt (http://www.uniprot.org). SnapGene® software (from Insightful Science; available at snapgene.com) was used to perform multiple

alignments of either 20 or 14 sequences of SC-α-neurotoxin or LC-α-neurotoxin, respectively, and from the two alignments, two consensus amino acid sequences were determined: short-chain consensus (SCC) and long-chain consensus (LCC) (Table S1-S2). The consensus sequence was built by taking decisions for each position depending on the amino acid abundance. For each position, the most repeated amino acid was kept, and in case of a tie, the most abundant amino acid within the most present physicochemical property (acid – (E, D), basic (K, R), polar (S, T, Y, N, N), non-polar (G, A, V, L, I, M, W, F). Proline (P) and cysteine (C) formed a group themselves.

Plasmid construction for toxin expression

The genes for the consensus toxins were purchased from Eurofins and cloned into a plasmid as described elsewhere (Manuscript I). Briefly, the genes encoding the 6xHis-consensus toxin were inserted into a pPICZα A vector (Invitrogen) using the NEBuilder HiFi DNA Assembly method. The resulting plasmids were verified by DNA sequencing to confirm the correct insertion (Eurofins Genomics Sanger sequencing service, Ebersberg, Germany).

Expression and purification of consensus toxins

The expression and purification of the two consensus toxins followed a previously described protocol (Manuscript I). Briefly, *Komagataella phaffii* (previously known as *Pichia pastoris*) KM71H were electroporated with the Sanger-sequenced confirmed plasmid, and positive transformants were identified by plating on YPDS plates (20 g/L Peptone, 10 g/L Yeast Extract, 100 mL/L Dextrose 20% (w/v), 182.2 g/L Sorbitol, 20g/L Agar) containing 1000 ug/mL of Zeocin. Following a preliminary assessment of small-scale expression, colonies exhibiting higher toxin production, as determined by SDS-PAGE analysis of culture supernatants, were selected. Subsequently, the selected recombinant toxins were cultured in YPD medium overnight at 30

°C. To ensure long-term preservation, 15% (v/v) glycerol was added to the cultures, which were then aliquoted and stored at -80 °C.

A 5 mL overnight culture of the selected clone in YPD medium (20 g/L Peptone, 10 g/L Yeast Extract, 20% (w/v) Dextrose) was inoculated with a single colony of transformed *P. pastoris* and incubated overnight at 30 °C. The culture was then scaled up to 1 L of BMGY medium (10 g/L Yeast Extract, 20 g/L Peptone, 0.1 M Potassium phosphate pH 6.0, 1.34% (w/v) YNB, 0.04 µg/mL Biotin, 1% (v/v) Glycerol) and grown for 24 hours at 30 °C. The cells were harvested, resuspended in BMMY medium (10 g/L Yeast Extract, 20 g/L Peptone, 0.1 M Potassium phosphate pH 6.0, 1.34% (w/v) YNB, 0.04 µg/mL Biotin, 0.5% (v/v) Methanol), and cultured at 25 °C for 4 days with methanol induction. After 96 hours, the cells were harvested, and the supernatant was collected and sterilized by filtration. The filtered supernatant was dialyzed against wash buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole) and purified using HIS-Select® Nickel Affinity Gel resin. The consensus toxins were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 400 mM imidazole), dialyzed against a dialysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl), and concentrated using Amicon® Ultra-15 Centrifugal Filters (Millipore, Burlington, USA).

The supernatant and purification steps samples were run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and stained with colloidal Coomassie blue.

Native toxin preparation

Lyophilized forms of short neurotoxin 1 (L8101), α-cobratoxin (L8114), α-Bungarotoxin (L8115) and whole venom derived from *Dendroaspis jamesoni* (L1308), *D. polylepis* (L1309), *D. viridis* (L1310) *Hemachatus haemachatus* (L1311), *Naja annulifera* (L1314), *N. haje* (L1315*), N. melanoleuca* (L1318), *N. nivea* (L1328) were obtained from Latoxan SAS (Portes-lès-Valence,

France) and prepared following established procedures.[13] The whole venom underwent fractionation using RP-HPLC, as detailed in a separate publication,[18] to isolate fractions enriched with either SC- or LC-α-neurotoxins.

In vitro biotinylation of toxins

The toxins, both consensus and native, were subjected to biotinylation, following a previously established protocol,[19] using a molar ratio of 1:1 (toxin:biotinylation reagent). To purify and concentrate the biotinylated toxins, Amicon® Ultra-4 Centrifugal Filter Units (Millipore, Burlington, USA) equipped with a 3 kDa membrane cut-off were employed. The degree of biotinylation was assessed using MALDI-TOF analysis performed on an Ultraflex II TOF/TOF spectrometer (Bruker Daltonics, Billerica, USA).

Phage display selection campaigns

Phage display selection was conducted using an immune nanobody library. To generate the nanobody-displaying phage libraries, two camelids were immunized with a mixture of 18 elapid venoms (*D. angusticeps*, *D. jamesoni*, *D. polylepis*, *D. viridis*, *N. anchietae*, *N. annulifera*, *N. ashei*, *N. haje*, *N. katiensis*, *N. melanoleuca*, *N. mossambica*, *N. nigricincta*, *N. nigricollis*, *N. nubiae*, *N. pallida*, *N. senegalensis*, and *H. haemachatus*) over a 16-week period, followed by three booster injections within a 6-week period after a year to enhance the immune response. The libraries were constructed as described previously by Pardon *et al*.[20] The resulting libraries exhibited a diversity of $5 \cdot 10^8$ and $1.6 \cdot 10^8$ individual clones, respectively. The two libraries were mixed before usage.

The selection process followed a previously described methodology,[21] with a modification in the technique for capturing biotinylated toxins. Instead of direct coating onto a 96-well microtiter plate, the biotinylated toxins were

captured on streptavidin-coated beads (Dynabeads M-280, Invitrogen, Waltham, MA, USA).

Three consecutive rounds of selections were performed using 50 nM of the consensus toxins in the two first rounds, while in the third round, the concentration was reduced to 5 nM. The enrichment of phages achieved with two rounds with LCC as the antigen was considered sufficient, leading to the decision to perform only two selection rounds with this antigen. The selection using LCC was designated as TPL1158, while the selection using SCC was named TPL1163, referring to the respective phage display campaigns.

Subcloning and initial screening of the nanobodies

The nanobody encoding genes obtained from TPL1158 and TPL1163 were subcloned into the pBDS100 expression vector for nanobodies. This subcloning process involved the use of *Eco91I* and *PstI* restriction enzymes, followed by transformation into the *E. coli* BL21(DE3) strain. The pBDS100 vector enables the production of nanobodies with a C-terminal 3xFLAG tag and a 6xHis tag.

From the two subcloned selection outputs, colonies were picked, and clones were expressed individually in 96-well plates in 150 µL autoinduction media at 30 °C overnight. 50 µL of the supernatant from the expression was left to bind on black MaxiSorp plates (Nunc A/S, Roskilde, Denmark) coated with anti-FLAG M2 at a concentration of 2.5 µg/mL. Following extensive washing with PBS-T (PBS with 0.1% Tween-20) and PBS, the binding of the nanobodies towards 20 nM SCC or LCC was evaluated using a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA).[13] Detection was accomplished using streptavidin conjugated with Europium (Eu-Streptavidin) at a concentration of 0.2 µg/mL.

Screening and sequencing of the nanobodies

A subset of clones having a signal greater than 10 times the background was cherry-picked and subjected to a DELFIA assay to determine their capability to recognize native toxins. In total 88 clones from TPL1158 and 76 from TPL1158 were selected. The clones from the TPL1158 selection were screened against α-cobratoxin (*N. kaouthia*), venom fractions containing primary LC-α-neurotoxins (fraction 8 from *N. nivea* (Nn8) and fraction 4 from *D. viridis* (Dv4)), short neurotoxin 1 (*N. pallida*), and streptavidin. Conversely, the clones from the TPL1163 selection were screened against short neurotoxin 1 (*N. pallida*), venom fractions enriched with primary SC-α-neurotoxins (fraction 4 from *N. nivea* (Nn4), fraction 4 from *D. polylepis* (Dp4), and fraction 1 from *D. viridis* (Dv1)), α-cobratoxin (*N. kaouthia*), and streptavidin.

The chosen clones were subjected to expression in a 96-deep well plate using a 1 mL autoinduction media,[22] allowing for overnight incubation at 30 °C. The following day, the plates were centrifuged at 4,000 × *g* for 15 minutes at 4 °C, and subsequently frozen for 24 hours. After thawing on ice, the cell pellets were reconstituted in 110 µL of PBS supplemented with complete EDTA-free Protease Inhibitor (Roche). Another centrifugation step at 4,000 × *g* at 4 °C for 30 minutes was performed to collect the supernatant, which contained the periplasmic fraction along with the nanobodies. 60 µL of the periplasmic fraction containing nanobodies was diluted 1/100 in 3% Milk-PBS. The diluted periplasmic fraction was then added to black MaxiSorp plates (Nunc A/S, Roskilde, Denmark) coated with anti-FLAG M2 at a concentration of 2.5 µg/mL. The plates were blocked with 3% milk-PBS and thoroughly washed with PBS-T and PBS before 25 nM of the indicated biotinylated toxins were added and detected using Eu-Streptavidin at a concentration of 0.2 µg/mL.

The selected clones were subsequently sent for sequencing (Eurofins Genomics Sanger sequencing service, Ebersberg, Germany).

Purification of nanobodies

Twenty-eight nanobodies were selected and expressed in 5 mL autoinduction media at 30 °C overnight. After centrifugation at 4,000 × *g* for 10 minutes at 4 °C, the cell pellets were frozen at -20 °C overnight. The pellets were thawed on ice and resuspended in 1 mL ice-cold PBS supplemented with complete EDTA-free Protease Inhibitor (Roche) and 10 mM imidazole. Following a centrifugation step at 20,000 × *g* for 45 minutes at 4 °C, the supernatant containing nanobodies was collected.

Nickel affinity chromatography was performed by incubating the supernatant with HIS-Select® Nickel Affinity Gel resin (Millipore) pre-equilibrated with wash buffer (PBS, 20 mM imidazole, pH 8.0). After 1 hour incubation at 4 °C with end-over-end rotation, the resin was transferred to chromatography columns, and the flow-through fractions were collected. The column was washed with 6 column volumes of wash buffer, and nanobodies were eluted using 500 µL elution buffer (PBS, 250 mM imidazole, pH 8.0).

Elution fractions containing the nanobodies were dialyzed twice against PBS at 4 °C to remove the imidazole. The purified nanobodies were then ready for downstream applications and further characterization.

The purified nanobodies were run on an SDS-PAGE gel and stained with colloidal Coomassie blue to confirm their purity.

Measurement of binding affinities of nanobodies using BLI (biolayer interferometry)

The binding affinities of the selected clones were determined using the Octet K2 system (FortéBio, Fremont, CA, USA). The measurements were conducted in 96-well black microplates (655209, Greiner Bio-One,

Kremsmünster, Upper Austria) at 25 °C with agitation at 1000 rpm. 1×kinetics buffer (1×KB, 18–1105, Sartorius) was prepared in PBS and used as the running buffer in the experiment. Prior to the experiment streptavidin Biosensors (18–5019, Sartorius, Göttingen, Germany) were dipped in 1×KB for at least 10 minutes, followed by loading of the biosensors with the biotinylated toxins (ligand) at a concentration of 1 µg/mL. A brief acid conditioning step with glycine buffer (10 nM, pH 2.0) followed by neutralization in 1×KB was carried out for 5 seconds x 5 cycles.

Next, a baseline was established in 1×KB for 120 seconds and the biosensors were immersed in wells containing nanobodies from 9.4–300 nM in a 2-fold dilution in 1×KB for 600 seconds, followed by a dissociation step of 900 seconds in 1×KB. The biosensors were regenerated using glycine buffer pH 2.0 for 10 seconds x 7 cycles between rounds. ForteBio's data analysis software was used to fit the curves to a 1:1 binding model to derive the kinetic constants (global fitting model).

Cross-binding assessment of nanobodies using DELFIA

To assess the cross-binding capabilities of the top 9 nanobodies, we conducted binding experiments against a range of α-neurotoxins. The nanobodies discovered against SCC were assessed for binding to seven different SC-α-neurotoxins and venom fractions enriched for SC-α-neurotoxins. The nanobodies discovered against LCC were evaluated for binding to six different LC-α-neurotoxins and venom fractions enriched for LC-α-neurotoxins. Additionally, one cytotoxin with a three-finger fold was included in the testing panel against all nanobodies. A detailed compilation of the toxins employed in this analysis can be found in Table S4. The evaluation of nanobody binding was conducted using a DELFIA assay, as described above.

In the DELFIA assays, 30 nM of each nanobody was captured using anti-FLAG antibodies. To evaluate the binding of the nanobodies to different

toxins, varying concentrations of the toxins were tested, ranging from 16 pM to 250 nM.

Each nanobody was tested in triplicate for each toxin concentration. The results were analyzed and plotted using GraphPad Prism 9 (log(agonist) vs. response – variable slope).

Assessment of neutralization using automated patch-clamp

To evaluate the potential of the nanobodies to neutralize the blocking effects of different α-neurotoxins on the muscle-type nAChR, we employed the Qube 384 automated patch clamp platform (Sophion Bioscience), following a previously established method.[13] Human-derived rhabdomyosarcoma RD cells, endogenously expressing the α1, β1, δ, γ, and ε subunits of nAChR, were patch-clamped for the experiments.

To determine the concentration of the toxins, inhibiting 80% of a nAChR-mediated current (IC_{80}), we elicited a current using 70 μM acetylcholine (ACh), followed by toxin administration and measurement of their inhibitory effects. To ensure full toxin effect, we preincubated with toxin for at least 5 minutes before the addition of ACh. For testing nanobody neutralization, various concentrations of the nanobodies were preincubated with toxins. The chosen toxin concentrations ranged between IC_{10} and IC_{90}, allowing for a toxin:nanobody molar ratio between 1:3 and 3:1. The inhibitory effect was quantified by normalizing the elicited current to that of the initial ACh addition.

Data analysis was performed using the Sophion Analyzer (Sophion Bioscience) and GraphPad Prism (GraphPad Software).

Results

The consensus α-neurotoxins can be successfully expressed in K. phaffii

Successful expression of the consensus toxins in *K. phaffii* was confirmed, checking for a close replication of the structural attributes of native α-neurotoxins purified from snake venoms. The genes responsible for encoding the SCC and LCC consensus toxins were cloned into the pPICZα A expression vector (Figure 1A). A secretion signal sequence was utilized to secrete the toxins into the culture media. To assess the success of the expression and purification process, SDS-PAGE (Figure 1B) and MALDI-TOF (data not shown) output were used, confirming the expected size of the toxins and successful purification. The SDS-PAGE analysis was performed under reducing and non-reducing conditions to verify the successful formation of disulfide bonds in the consensus toxins. A shift in the migration pattern on the gel was observed when treated with the reducing agent DTT, suggesting the presence of disulfide bonds (Figure 1B).

The consensus toxins α-neurotoxins resemble native structure

The secondary structure of the consensus toxins SCC and LCC was evaluated using CD spectroscopy (Figure 1C). This analysis showed that the secondary structure of the SCC closely resembled that of the native α-neurotoxins from snake venom, specifically α-cobratoxin and short neurotoxin 1. However, the low concentration of the LCC hindered the reliability of the CD spectra, hence no definitive conclusions were drawn about its secondary structural similarity to native toxins.

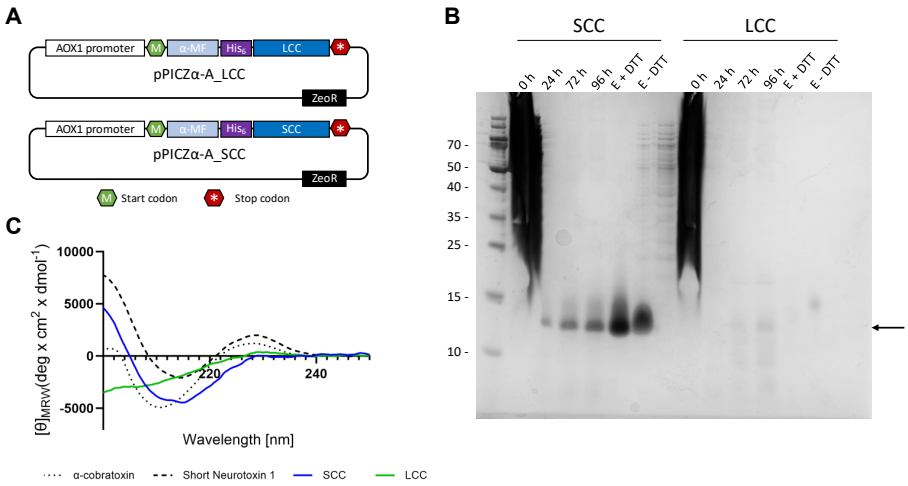


Figure 1 Consensus toxins expression, purification, and structural analysis. *A) Schematic representation of the two vectors employed for electroporation of the consensus toxins into K. phaffi. The consensus toxins, in phase with the AOX1 promoter, are preceded by the α-mating factor secretion signal (α-MF), enabling the secretion of the consensus toxins into the media. A 6xHis-tag is included for purification. B) Analysis of the supernatant samples collected at 24 hours, 72 hours, and 96 hours during the expression of SCC and LCC. Additionally, the elution fractions from the Ni-NTA purification, both with and without reduction using DTT, were examined. The consensus toxins are indicated by the arrow. C) Circular dichroism (CD) spectra comparing the secondary structure of the consensus toxins SCC (blue) and LCC (green) to two native toxins: the LC-α-neurotoxin α-cobratoxin from N. kaouthia (black dotted line) and short Neurotoxin 1 from N. pallida (black dashed tine).*

Effective nanobody selection from immunized camelid libraries

Prior to selection, the successful biotinylation of both LCC and SCC consensus toxins was confirmed through mass spectrometry analysis, showing one biotin molecule per toxin (data not shown). Phage display selection was performed using mixed nanobody libraries derived from two immunized llamas. Two separate selection campaigns were carried out; one with two rounds of selection using LCC as the antigen (TPL1158), and the other with

three rounds of selection using SCC as the antigen (TPL1163). The enrichment of the rounds was evaluated using a polyclonal phage ELISA (data not shown).

Identification of potential nanobody candidates

Following the selection rounds, the nanobody-encoding genes from both campaigns were isolated and subcloned into a bacterial expression vector for further characterization. A total of 186 clones from each selection campaign were randomly chosen for evaluation using a DELFIA. Biotinylated SCC or LCC was used to determine if the nanobodies could recognize and bind to the respective consensus toxins (Figure S1). The output of this screening process was then narrowed down through subsequent screens and characterization, leading to the identification of the top clones.

The progression from the initial pool of clones to the final selected clones was achieved as the selection criteria became more stringent through various screens and characterization steps (Figure 2).

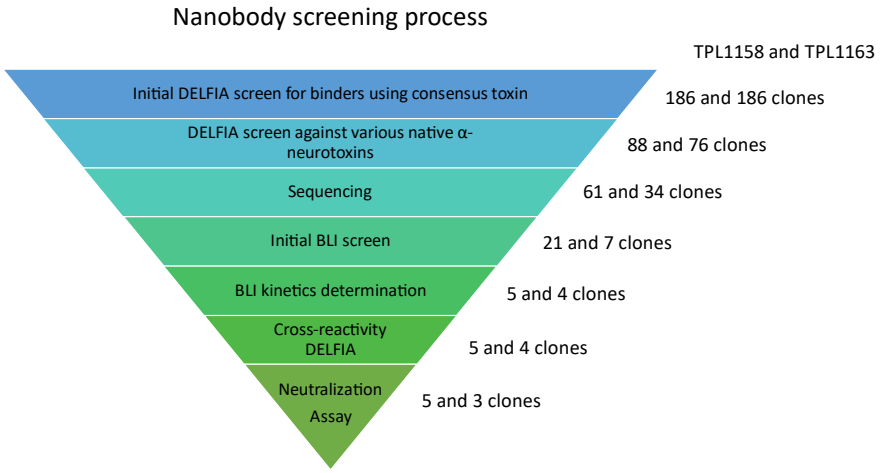


Figure 2. Graphical illustration of the nanobody screening process. *The initial 186 clones from both selections round, TPL1158 and TPL1163, were screened and characterized using different DELFIA and BLI experiments, to narrow the selection down to the top 4 clones from each selection.*

The nanobodies that demonstrated a positive signal in the primary screening were selected for further analysis. A total of 88 clones from TPL1158 and 76 clones from TPL1163, all exhibiting binding against their respective consensus toxin with a signal higher than 10 times the background, underwent a second DELFIA screening (Figure S1). During this screening, the clones were assessed for their binding affinity to various biotinylated native toxins.

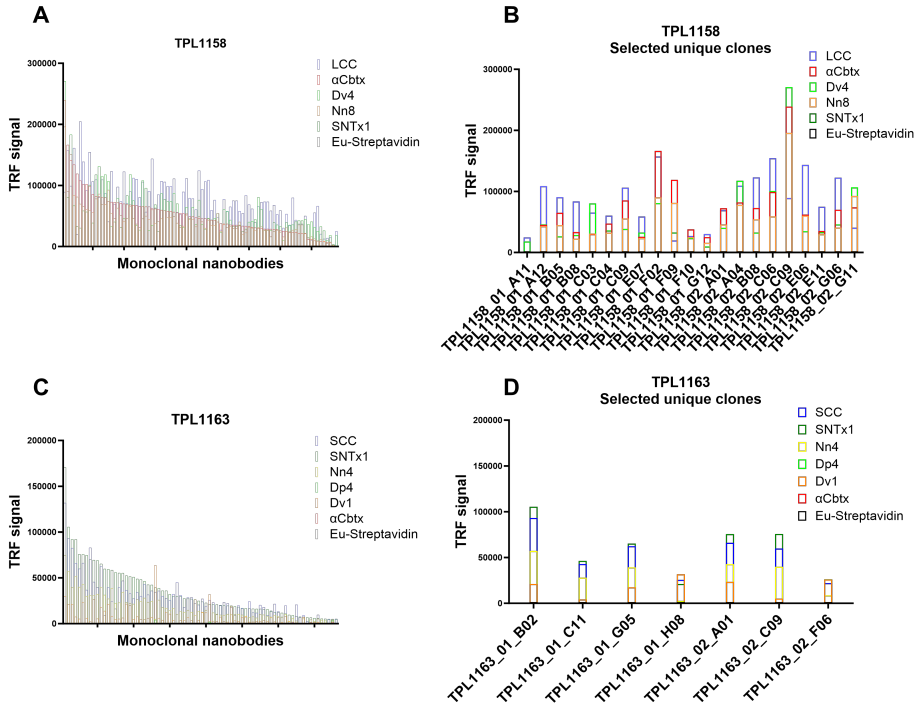
Clones from TPL1158 were tested against the LC-α-neurotoxin α-cobratoxin (*N. kaouthia*), venom fractions containing primary LC-α-neurotoxins (fraction 8 from *N. nivea* (Nn8) and fraction 4 from *D. viridis* (Dv4)), and short neurotoxin 1 (*N. pallida*). TPL1163 clones were tested against short neurotoxin 1 (*N. pallida*), venom fractions enriched with primary SC-α-neurotoxins (fraction 4 from *N. nivea* (Nn4), fraction 4 from *D. polylepis* (Dp4), and fraction 1 from *D. viridis* (Dv1)), and the LC-α-neurotoxin α-cobratoxin (*N. kaouthia*) (Figure 3). Additionally, the binding of the nanobodies to Eu-Streptavidin alone was evaluated in samples without added toxins.

The majority of the tested nanobodies showed the capability to bind to a diverse range of native α-neurotoxins purified from venom produced by different snake species, suggesting their cross-binding ability. Clones from TPL1158 were also evaluated for their binding affinity to SC-α-neurotoxins, whereas clones from TPL1163 were tested for their recognition of LC-α-neurotoxins. As expected, TPL1158 clones showed no binding to LC-α-neurotoxins, and TPL1163 clones showed no binding to SC-α-neurotoxins. Signals comparable to the background were detected in the negative control, suggesting that the observed signals are due to nanobody binding to the toxins, not to non-specific binding to streptavidin.

Selection of unique nanobody clones

After the second DELFIA screen, a subset of 61 clones from TPL1158 and 34 clones from TPL1163, all demonstrating cross-binding to native toxins, were chosen for DNA sequencing. The sequencing aimed to ensure diversity, with unique sequences selected from groups of clones with similar DELFIA signals. As a result, 26 unique clones from TPL1158 and 9 unique clones from TPL1163 were identified and the antibody framework and complementarity-determining regions (CDRs) of the clones were annotated and analyzed.

A subset of these clones was selected for further characterization due to the limitations of conducting extensive BLI and DELFIA experiments. Ultimately, 21 clones from TPL1158 and 7 clones from TPL1163 were selected for further analysis. (Figure 3E and Table S4).



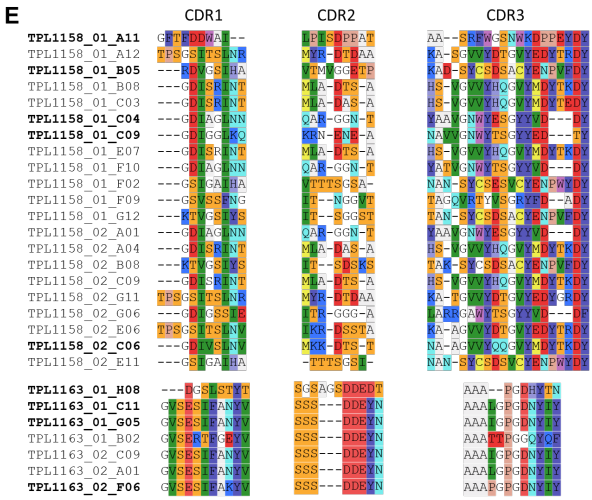


Figure 3 Monoclonal DELFIA screen against various toxins and CDRs of unique clones. *A) The binding of the 88 nanobody clones from TPL1158 was assessed against LCC, α-cobratoxin (αCbtx), and two venom fractions enriched in LC-α-neurotoxins (Dv4 and Nn8). Additionally, the nanobodies were screened against Short Neurotoxin 1 (SNTx1). The negative control included no toxin to ensure the Eu-labeled streptavidin did not bind to the nanobodies. B) DELFIA results depicting the binding of the selected 21 nanobodies from TPL1158 to various toxins. C) Screening of the 76 clones from TPL1163 against SCC, short neurotoxin 1 (SNTx1), and three venom fractions enriched in SC-α-neurotoxins (Nn4, Dp4, and Dv1). Similar to the TPL1158 screen, the nanobodies from TPL1163 were also tested for binding to α-cobratoxin (αCbtx), with Eu-Streptavidin used as a negative control. D) DELFIA results displaying the binding of the selected 7 nanobodies from TPL1163 to various toxins. E) Sequence alignment of the complementarity-determining regions (CDRs) from the selected 21 clones from TPL1158 and 7 clones from TPL1163. The clones chosen for kinetics experiments are highlighted in bold. The TPL1163 clones with identical CDRs had variations within the framework.*

The selected nanobodies bind to several different α-neurotoxin toxins with high affinity

To investigate the binding capability of the selected nanobody clones from TPL1158 and TPL1163, BLI analysis was employed against a panel of native

α-neurotoxins. Specifically, the screening was performed against three LC-α-neurotoxins (α-cobratoxin, Dv4, and Nn8) and three SC-α-neurotoxins (short neurotoxin 1, Dv1, and Dp4) (data not shown).

After the initial BLI screening, the clones that exhibited the most promising binding curves with a fast on-rate and slow off-rate were selected. In total five clones from TPL1158 and four clones from TPL1163 were further assessed in kinetics experiments. All of these clones showed high binding affinity towards the tested toxins, with dissociation constant (K_D) values ranging from 200 nM to below 1 pM, mostly in the low nanomolar to picomolar range (Table 1). A more detailed table with the kinetic constants and error values can be found in Table S3.

Nanobody ID	**α-cobratoxin** (***N. kaouthia***)	**Dv4** (***D. viridis***)	**Nn8** (***N. nivea***)
TPL1158_01_A11	$2.03 \cdot 10^{-7}$	$4.07 \cdot 10^{-10}$	$4.56 \cdot 10^{-8}$
TPL1158_01_B05	$<1.0 \cdot 10^{-12}$	$<1.0 \cdot 10^{-12}$	$<1.0 \cdot 10^{-12}$
TPL1158_01_C04	$1.54 \cdot 10^{-9}$	$9.33 \cdot 10^{-11}$	$5.15 \cdot 10^{-10}$
TPL1158_01_C09	$1.20 \cdot 10^{-9}$	$1.20 \cdot 10^{-9}$	$6.30 \cdot 10^{-10}$
TPL1158_02_C06	$<1.0 \cdot 10^{-12}$	$<1.0 \cdot 10^{-12}$	$<1.0 \cdot 10^{-12}$
	Short neurotoxin 1 (***N. pallida***)	**Dv1** (***D. viridis***)	**Dp4** (***D. polylepis***)
TPL1163_01_H08	$9.69 \cdot 10^{-10}$	$<1.0 \cdot 10^{-12}$	$3.92 \cdot 10^{-10}$
TPL1163_01_C11	$<1.0 \cdot 10^{-12}$	$6.78 \cdot 10^{-11}$	NB
TPL1163_01_G05	$1.88 \cdot 10^{-12}$	$1.45 \cdot 10^{-10}$	NB
TPL1163_02_A01	$1.0 \cdot 10^{-9}$	$1.34 \cdot 10^{-11}$	NB

Table 1 K_D values obtained from BLI experiment. *The K_D values for the selected nanobodies were obtained through the binding of the nanobodies to three different α-neurotoxins. The K_D values are given in M. NB, denotes no detected binding. A more detailed table with the kinetic constants and error values can be found in Table S3.*

The observed low K_D values for most of the clones can be attributed to the exceptionally slow off-rates below $1.0 \cdot 10^{-7}$ s^{-1} measured during the kinetics experiments, highlighting the strong and stable interactions between the nanobodies and the respective toxins (Table S3, Figure S2 and S3).

Notably, clone TPL1158_01_A11 behaves differently compared to the other four nanobodies targeting LC-α-neurotoxins. This nanobody possesses high affinity and a slow off-rate specifically towards Dv4. In contrast, the K_Ds towards α-cobratoxin and Nn8 is approximately 100 and 1000 times higher, respectively. This could indicate that this nanobody is able to differentiate between α-neurotoxin originating from different elapid genera.

The selected nanobodies bind to a range of α-neurotoxins from different snake species

To evaluate the binding capabilities of the nanobodies and their recognition of various α-neurotoxins, a comprehensive DEFLIA assay was conducted, covering multiple toxin fractions as outlined in Table S4. The nanobodies from TPL1158, specific to LC-α-neurotoxins, were tested against six different LC-α-neurotoxins, while the nanobodies from TPL1163, targeting SC-α-neurotoxins, were tested against seven different SC-α-neurotoxins (Figure 4). Additionally, all nanobodies were examined for their binding affinity towards a cytotoxic 3FTx. A sequence alignment and percentage identity of the tested toxins can be found in Table S5 and S6, providing insights into the similarities and differences between the consensus toxins and the toxins used in this experiment.

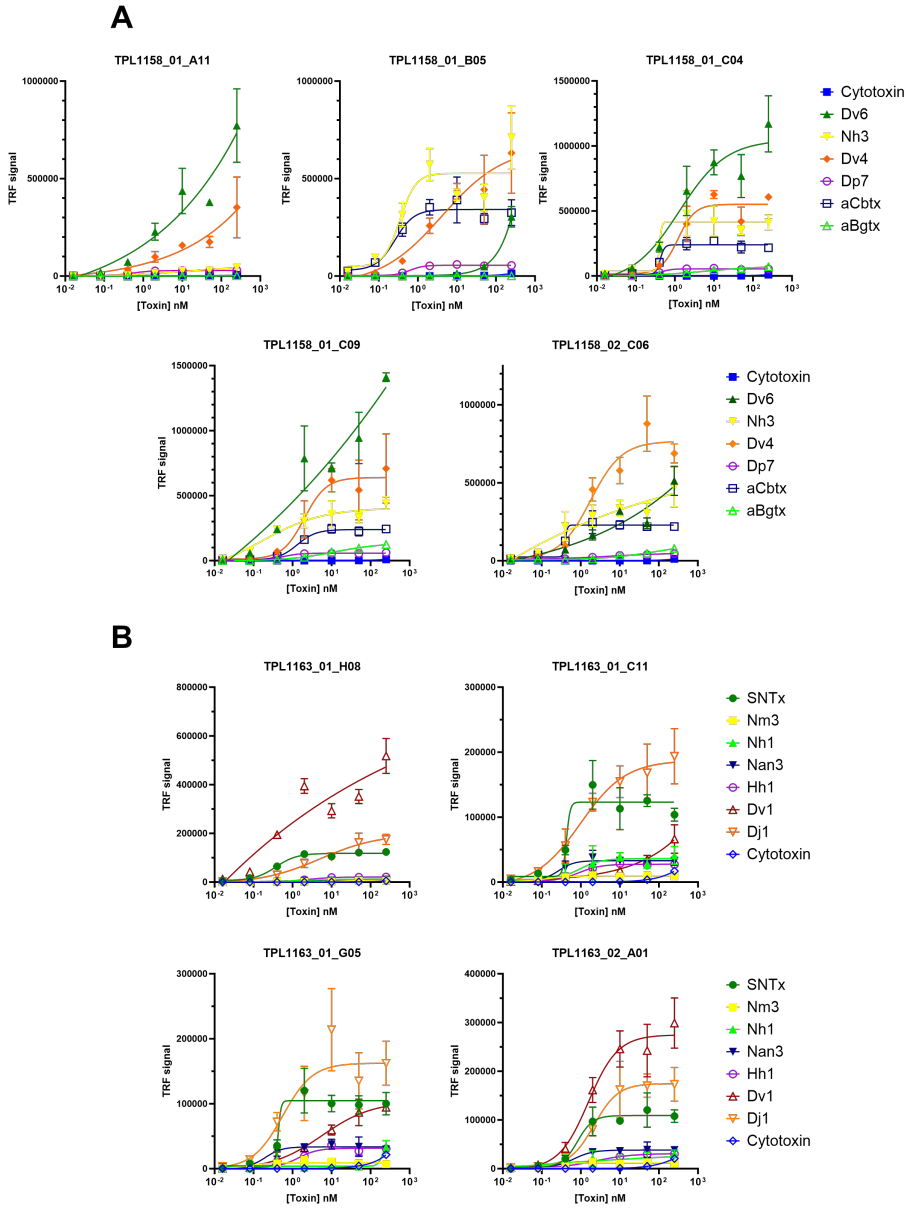


Figure 4 DELFIA assessment of cross-reactivity among nanobodies targeting LC- and SC-α-neurotoxins. *The binding capabilities of A) five nanobodies specific to LC-α-neurotoxins and B) four nanobodies specific to SC-α-neurotoxins were evaluated against a range of α-neurotoxins. The toxins were tested at concentrations ranging from 250 nM to 16 pM.*

The results indicated that the TPL1158 nanobodies exhibited binding to most of the tested LC-α-neurotoxins, with C04, C09, and C06 also showing slight affinity towards α-Bungarotoxin, the LC-α-neurotoxin with the least sequence similarity to LCC among the tested LC-α-neurotoxins (Figure S4).

Remarkably, the TPL1158_01_A11 nanobody exhibited binding signals exclusively with venom fractions from *D. viridis*, while no signals were observed to any of the tested toxins derived from cobra species. This observation is consistent with the kinetic data obtained through BLI.

Among the TPL1163 nanobodies, TPL1163_01_H08 demonstrated a relatively limited recognition of toxins compared to the other three SC-α-neurotoxin binders. However, the remaining three nanobodies (C11, G05, and A01) exhibited a broad recognition of various SC-α-neurotoxins. These three nanobodies not only demonstrated recognition and binding to a diverse range of SC-α-neurotoxins but also exhibited binding to the tested cytotoxin. This finding suggests that these nanobodies possess a broader binding capability beyond their specificity for α-neurotoxins.

The nanobodies show neutralization in vitro

Functional *in vitro* neutralization assays were performed to assess the neutralization capabilities of the nanobodies. Automated patch-clamp electrophysiology was used to determine the effectiveness of various α-neurotoxins in functional inhibiting the nAChR response (Figure S5 and S6). Subsequently, the α-neurotoxins were preincubated with the nanobodies at different molar ratios to evaluate the nanobodies' ability to neutralize the current-inhibiting activity of these α-neurotoxins (Figure 5 and S7). As a negative control, no toxin was added to the nanobodies.

All the clones exhibited broad neutralization capabilities, with TPL1158_01_C04 showing the ability to neutralize all tested LC-α-neurotoxins. TPL1158_01_A11 only neutralized toxins originating from mambas, consistent with the data obtained from BLI and DELFIA. Conversely, none of the nanobodies targeting SC-α-neurotoxins were able to neutralize Nn4, which closely resembles Nm3 used in the cross-binding DELFIA, where these nanobodies also displayed low binding affinity.

Unfortunately, due to limited material availability, it was not possible to test the neutralization of TPL1163_01_H08.

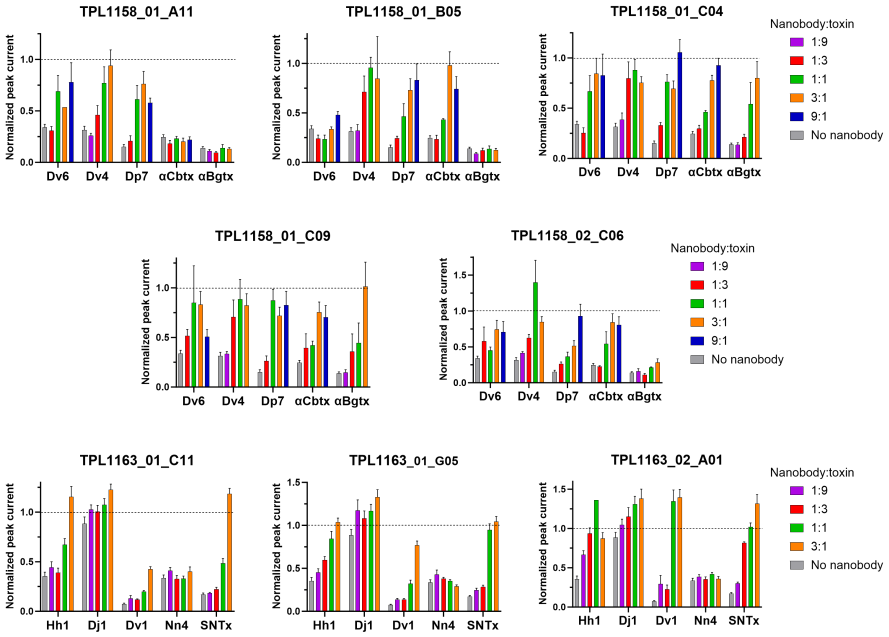


Figure 5 The in vitro neutralization capabilities of nanobodies against various α-neurotoxins. *The nanobodies were preincubated with different molar ratios of nanobody:toxin, and the resulting normalized peak current is shown. Toxins were used at concentrations ranging between* IC_{10} *and* IC_{90}*, as indicated by the peak current obtained when no nanobody was present.*

Discussion

In this study, we successfully expressed consensus α-neurotoxins using a yeast expression system based on the host *K. phaffii*. These consensus toxins were designed to mimic the structural features of native snake α-neurotoxins while presenting a well-defined antigenic target. Building on the knowledge that consensus toxins can be used as immunogens to generate polyclonal antibodies capable of neutralizing venom from different snake species,[23] we focused on leveraging this concept for the discovery of monoclonal nanobodies using phage display technology.

The use of consensus toxins as antigens in our phage display campaigns offered several advantages. The primary benefit was their utility as antigens for the discovery of cross-reactive nanobodies with broad neutralization capabilities compared to nanobodies and antibodies discovered using traditional cross-panning methods.[22] In the cross-panning method, the level of antigen variability that the library may encounter during successive panning rounds is restricted to only a few targets. On the other hand, consensus proteins are deliberately designed to present the features of multiple targets, thereby substantially broadening the possibilities for discovering cross-reactive binding proteins. Even though the cross-panning strategy has previously been shown to be useful for discovering broadly neutralizing antibodies against other toxins, the antigens exchanged in the successive panning rounds have often shared a high degree of sequence identity and function in these prior studies[22,24]. When this is not the case , the antigen exchange leads to a decrease in the amount of phages displaying antibodies capable of binding to multiple targets.[25] The capability of the consensus toxins to select antibodies that are able to bind multiple toxins is evident from the data presented here. In particular, the ability of the discovered nanobodies to cross-bind α-neurotoxins from snake species beyond those native to Africa indicates the potential for these nanobodies to target venom toxins from

diverse geographical regions and snake genera. By mimicking multiple similar antigens, it thus seems that the use of consensus proteins may enable a potentially more facile discovery approach for monoclonal antibodies and nanobodies with broadly neutralizing capacities. In relation to snakebite envenoming, this may help make recombinant antivenom products simpler to formulate, at they can be comprised of fewer antibodies, as well as it may enable the development of even more polyvalent recombinant antivenoms that cover snake species from vaster geographic regions. In this relation, we note that an exceptional broad recognition of toxins was seen in this study, with the discovery of nanobodies capable of binding cytotoxic 3FTx toxins. that share down to 43% sequence identity to SCC. Observations like these further emphasize the potential of using consensus toxins to select antibodies with very broad specificity.

To assess the neutralization capacities of the discovered nanobodies, functional neutralization assays were conducted using automated patch-clamping. Notably, all the tested clones showed broad neutralization capacities against LC-α-neurotoxins, with one particular clone, TPL1158_01_C04, demonstrating the ability to neutralize all tested LC-α-neurotoxins. Furthermore, the nanobodies targeting LC-α-neurotoxins exhibited similar IC_{50} values to an affinity-matured IgG previously discovered in our group, which had performed exceptionally well *in vivo*.[13] This observation highlights the potency of these nanobodies, especially in the light of them being monovalent and therefore not benefitting from potential avidity effect unlike the bivalent IgGs. Surprisingly, it also shows that the broad cross-reactivity observed for these nanobodies does not seem compromise affinity or potency. The discovered nanobodies may therefore pose as promising leads for further antivenom development.

In the context of consensus toxin design for uncovering nanobodies with broad neutralization capacities against snake toxins, it could be relevant

to examine the paratope-epitope binding interface between toxins and nanobodies to understand the basis for the broad specificity. In this regard, a previous study showed that broad neutralization can be achieved through receptor mimicry.[26] Such information on the mechanism of neutralization might not only be relevant for understanding how the discovered nanobodies potentially might neutralize α-neurotoxins, but it could potentially also be used to optimize the nanobodies further via *in silico* methods. Structural information could also provide cues to whether the nanobodies might neutralize a even wider spectrum of toxins beyond those evaluated in this study. Moreover, knowing the epitope could also be relevant for diagnostic applications, particularly in lateral flow assays where a pair of antibodies in a sandwich configuration is crucial for precise detection.[27] Here, epitope information could be used to guide the selection of such sandwich pairs to ensure that the two binding partners indeed bind different epitopes and do not compete for the same one.

Beyond the specific discovery of nanobodies that can broadly neutralize snake toxins, our strategy of utilizing consensus antigens and phage display technology may find broader application within general antibody discovery.[1] This versatile approach can be extended to identify antibodies effective against hypermutable targets in different cancers, providing potential multi-targeting therapeutics.[28,29] Additionally, the use of consensus antigens could lead to the development of antibodies capable of broadly neutralizing bacterial serotypes, offering a more comprehensive defense against infectious diseases.[30] Furthermore, our strategy could be employed to discover antibodies for targeting parasites with high antigenic variation, a crucial aspect in combating parasitic infections effectively.[31,32] Likewise, in the context of viral diseases, where escape mutants may often arise, the use of consensus antigens could enable the identification of antibodies with robust neutralization capacities that the viral particles cannot easily escape, thereby enhancing the

efficacy of antiviral therapies.[33] The continued exploration and refinement of the use of consensus antigens for antibody and nanobody discovery is therefore warranted, as it may ultimately benefit patients and equip healthcare systems to tackle a wide range of malignancies.

Author contributions:
ADJ, ERdT, and AHL conceptualized the project. ADJ and ERdT designed the experiments. ERdT, ADJ, AV, IB, TT, and KB performed the experiments. ADJ, ERdT, and AHL, wrote the manuscript. All authors revised and approved the manuscript.

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

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Accession	Species	Amino acid sequence
P01418	*Dendroaspis viridis*	RICYNHQSTTPATTKSC--GENSCYKKTWSDHRGTIIERGCGCPKVKRGVHLHCCQSDKCNN
P01417	*D. jamesoni kaimosae*	RICYNHQSTTPATTKSC--GENSCYKKTWSDHRGTIIERGCGCPKVKQGIHLHCCQSDKCNN
P01416	*D. polylepis*	RICYNHQSTTRATTKSC--EENSCYKKYWRDHRGTIIERGCGCPKVKPGVGIHCCQSDKCNY
P01419	*D. jamesoni kaimosae*	RICYNHQSNTPATTKSC--VENSCYKSIWADHRGTIIKRGCGCPRVKS--KIKCCKSDNCNL
P01425	*Hemachatus haemachatus*	LECHNQQSSQPPTTKSCP-GDTNCYNKRWRDHRGTIIERGCGCPTVKPGINLKCCTTDRCNN
P01433	*H. haemachatus*	LECHNQQSSQTPTTQTCP-GETNCYKKQWSDHRGSRTERGCGCPTVKPGIKLKCCTTDRCNK
P34075	*Naja annulata*	KICYNQPSSQHPTTKACP-GEKNCYRKQWSDHRGTIIERGCGCPTVKPGVKLHCCTTEKCNN
P01420	*N. annulifera*	MICYKQQSLQFPITTVCP-GEKNCYKKQWSGHRGTIIERGCGCPSVKKGIEINCCTTDKCNR
P01421	*N. annulifera*	MICYKQRSLQFPITTVCP-GEKNCYKKQWSGHRGTIIERGCGCPSVKKGIEINCCTTDKCNR
P01422	*N. annulifera*	MICHNQQSSQPPTIKTCP-GETNCYKKRWRDHRGTIIERGCGCPSVKKGVGIYCCKTNKCNR
P68417	*N. annulifera*	LECHNQQSSQPPTTKTCP-GETNCYKKRWRDHRGSITERGCGCPSVKKGIEINCCTTDKCNN
P34076	*N. christyi*	MECHNQQSSQPPTTTHCSGGETNCYEKRWHDHRGTIIERGCGCPTVKPGVKLNCCTTDKCNN
P25675	*N. haje*	MICHNQQSSQPPTIKTCP-GETNCYKKQWRDHRGTIIERGCGCPSVKKGVGIYCCKTDKCNR
P68418	*N. haje*	LECHNQQSSQPPTTKTCP-GETNCYKKRWRDHRGSITERGCGCPSVKKGIEINCCTTDKCNN
P01424	*N. melanoleuca*	MECHNQQSSQPPTTKTCP-GETNCYKKQWSDHRGTIIERGCGCPSVKKGVKINCCTTDRCNN
P01432	*N. mossambica*	LNCHNQMSAQPPTTTRCSRWETNCYKKRWRDHRGYKTERGCGCPTVKKGIQLHCCTSDNCNN
P01431	*N. mossambica*	LECHNQQSSEPPTTTRCSGGETNCYKKRWRDHRGYRTERGCGCPTVKKGIELNCCTTDRCNN
P01423	*N. nivea*	MICHNQQSSQRPTIKTCP-GETNCYKKRWRDHRGTIIERGCGCPSVKKGVGIYCCKTDKCNR
P68419	*N. nivea*	LECHNQQSSQPPTTKTCP-GETNCYKKRWRDHRGSITERGCGCPSVKKGIEINCCTTDKCNN
P01426	*N. pallida*	LECHNQQSSQPPTTKTCP-GETNCYKKVWRDHRGTIIERGCGCPTVKPGIKLNCCTTDKCNN
Consensus sequence		LICHNQQSSQPPTTKTCP-GETNCYKKQWRDHRGTIIERGCGCPTVKKGIKLNCCTTDKCNN

Table S1. Multiple sequence alignment for the design of SCC. 20 different short α-neurotoxins from African snakes were used to obtain the consensus sequence shown at the bottom of the table. Gaps are shown as a dash, and the cysteine residues are highlighted.

Accession	Species	Amino acid sequence
P01390	*N. nivea*	IR**C**FITPDVTSQA**C**PDGH-V**C**YTKMW**C**DNF**C**GMRGKRVDLG**C**AAT**C**PKVKPGVNIK**CC**SRDN**C**NPFPTRKRS-
P01388	*N. melanoleuca*	IR**C**FITPDVTSQI**C**ADGH-V**C**YTKTW**C**DNF**C**ASRGKRVDLG**C**AAT**C**PTVKPGVNIK**CC**STDN**C**NPFPTRNRP-
P01383	*N. melanoleuca*	KR**C**YRTPDLKSQT**C**PPGEDL**C**YTKKW**C**ADW**C**TSRGKVIELG**C**VAT**C**PKVKPYEQIT**CC**STDN**C**NPHPKMKP--
P25674	*N. haje*	IR**C**FITPDVTSQA**C**PDGH-V**C**YTKMW**C**DNF**C**GMRGKRVDLG**C**AAT**C**PTVKPGVDIK**CC**STDN**C**NPFPTRKRS-
P34074	*N. annulata*	IR**C**FITPRVSSQA**C**PDGH-V**C**YTKTW**C**DNF**C**GINGKRVDLG**C**AAT**C**PTVKPGVDIK**CC**STDN**C**NPFPTRKRP-
P01389	*N. anchietae*	IR**C**FITPDVTSQA**C**PDGQNI**C**YTKTW**C**DNF**C**GMRGKRVDLG**C**AAT**C**PTVKPGVDIK**CC**STDN**C**NPFPTRERS-
P01395	*D. viridis*	RT**C**YKTPSVKPET**C**PHGENI**C**YTETW**C**DAW**C**SQRGKRVELG**C**AAT**C**PKVKAGVGIK**CC**STDN**C**NPFPVWNPRG
P01394	*D. viridis*	RT**C**YKTPSVKPET**C**PHGENI**C**YTETW**C**DAW**C**SQRGKREELG**C**AAT**C**PKVKAGVGIK**CC**STDN**C**DPFPVKNPR-
C0HJD7	*D. polylepis*	RT**C**NKTFSDQSKI**C**PPGENI**C**YTKTW**C**DAF**C**SQRGKRVELG**C**AAT**C**PKVKAGVEIK**CC**STDN**C**NKFQFGKPR-
P01396	*D. polylepis*	RT**C**NKTFSDQSKI**C**PPGENI**C**YTKTW**C**DAW**C**SQRGKRVELG**C**AAT**C**PKVKAGVEIK**CC**STDD**C**DKFQFGKPR-
P01397	*D. polylepis*	RT**C**NKTPSDQSKI**C**PPGENI**C**YTKTW**C**DAW**C**SQRGKIVELG**C**AAT**C**PKVKAGVEIK**CC**STDN**C**NKFKFGKPR-
P25667	*D. polylepis*	RT**C**NKTFSDQSKI**C**PPGENI**C**YTKTW**C**DAW**C**SRRGKIVELG**C**AAT**C**PKVKAGVGIK**CC**STDN**C**NLFKFGKPR-
P01393	*D. jamesoni kaimosae*	RT**C**YKTYSDKSKT**C**PRGEDI**C**YTKTW**C**DGF**C**SQRGKRVELG**C**AAT**C**PKVKTGVEIK**CC**STDY**C**NPFPVWNPR-
P25670	*Aspidelaps scutatus*	RI**C**YIAPYDHKT-**C**AAGENI**C**YLKAW**C**DAW**C**SSRGKKLEFG**C**AAT**C**PTVKPGVDIS**CC**DTDN**C**NPHPKL----
Consensus sequence		RTCYKTPSDTSQICPPGENICYTKTWCDAWCSQRGKRVELGCAATCPKVKAGVDIKCCSTDNCNPFPTRKPR

Table S2. Multiple sequence alignment for the design of LCC. 14 different LCα-neurotoxins from African snakes were used to obtain the consensus sequence shown at the bottom of the table. Gaps are shown as a dash, and the cysteine residues are highlighted.

			TPL1158_01_A11	TPL1158_01_B05	TPL1158_01_C04	TPL1158_01_C09	TPL1158_02_C06
K_D	(M)	α-Cobratoxin (*N. kauothia*)	$2.03 \cdot 10^{-7}$	$<1.0 \cdot 10^{-12}$	$1.54 \cdot 10^{-9}$	$1.20 \cdot 10^{-9}$	$<1.0 \cdot 10^{-12}$
K_D	error		$1.16 \cdot 10^{-9}$	$<1.0 \cdot 10^{-12}$	$4.56 \cdot 10^{-12}$	$3.62 \cdot 10^{-12}$	$<1.0 \cdot 10^{-12}$
k_{on}	(1/M·s)		$2.59 \cdot 10^{4}$	$5.52 \cdot 10^{4}$	$8.80 \cdot 10^{4}$	$9.72 \cdot 10^{4}$	$5.37 \cdot 10^{4}$
k	error		$1.40 \cdot 10^{2}$	$1.28 \cdot 10^{2}$	$1.08 \cdot 10^{2}$	$1.08 \cdot 10^{2}$	$1.19 \cdot 10^{2}$
k_{off}	(1/s)		$5.28 \cdot 10^{-3}$	$<1.0 \cdot 10^{-7}$	$1.36 \cdot 10^{-4}$	$1.17 \cdot 10^{-4}$	$<1.0 \cdot 10^{-7}$
k_{off}	error		$1.00 \cdot 10^{-5}$	$<1.0 \cdot 10^{-7}$	$3.64 \cdot 10^{-7}$	$3.27 \cdot 10^{-7}$	$<1.0 \cdot 10^{-7}$
Full R^2			0.995	0.997	0.998	0.998	0.997
K_D	(M)	Dv4 (*D. viridis*)	$4.07 \cdot 10^{-10}$	$<1.0 \cdot 10^{-12}$	$9.33 \cdot 10^{-11}$	$1.20 \cdot 10^{-9}$	$<1.0 \cdot 10^{-12}$
K_D	error		$6.55 \cdot 10^{-12}$	$<1.0 \cdot 10^{-12}$	$2.49 \cdot 10^{-12}$	$4.70 \cdot 10^{-12}$	$<1.0 \cdot 10^{-12}$
k_{on}	(1/M·s)		$9.03 \cdot 10^{4}$	$6.53 \cdot 10^{4}$	$1.72 \cdot 10^{5}$	$2.32 \cdot 10^{5}$	$2.96 \cdot 10^{4}$
k_{on}	error		$2.43 \cdot 10^{2}$	$3.10 \cdot 10^{2}$	$2.61 \cdot 10^{2}$	$2.92 \cdot 10^{2}$	$4.99 \cdot 10^{2}$
k_{off}	(1/s)		$3.67 \cdot 10^{-7}$	$<1.0 \cdot 10^{-7}$	$1.60 \cdot 10^{-5}$	$2.81 \cdot 10^{-4}$	$<1.0 \cdot 10^{-7}$
k_{off}	error		$5.83 \cdot 10^{-5}$	$<1.0 \cdot 10^{-12}$	$4.28 \cdot 10^{-7}$	$1.03 \cdot 10^{-6}$	$<1.0 \cdot 10^{-12}$
Full R^2			0.996	0.995	0.997	0.998	0.986
K_D	(M)	Nn8 (*N. nivea*)	$4.56 \cdot 10^{-8}$	$<1.0 \cdot 10^{-12}$	$5.15 \cdot 10^{-10}$	$6.30 \cdot 10^{-10}$	$<1.0 \cdot 10^{-12}$
K_D	error		$1.15 \cdot 10^{-10}$	$<1.0 \cdot 10^{-12}$	$2.41 \cdot 10^{-12}$	$1.87 \cdot 10^{-12}$	$<1.0 \cdot 10^{-12}$
k_{on}	(1/M·s)		$6.70 \cdot 10^{4}$	$8.97 \cdot 10^{4}$	$1.83 \cdot 10^{5}$	$2.18 \cdot 10^{5}$	$2.18 \cdot 10^{4}$
k_{on}	error		$1.36 \cdot 10^{2}$	$2.41 \cdot 10^{2}$	$2.68 \cdot 10^{2}$	$2.70 \cdot 10^{2}$	$2.46 \cdot 10^{2}$
k_o	(1/s)		$3.04 \cdot 10^{-3}$	$<1.0 \cdot 10^{-7}$	$9.44 \cdot 10^{-5}$	$1.37 \cdot 10^{-4}$	$<1.0 \cdot 10^{-7}$
k_o	error		$4.58 \cdot 10^{-6}$	$<1.0 \cdot 10^{-12}$	$4.21 \cdot 10^{-7}$	$3.72 \cdot 10^{-7}$	$<1.0 \cdot 10^{-12}$
Full R^2			0.997	0.996	0.997	0.997	0.995

			TPL1163_01_H08	TPL1163_01_C11	TPL1163_01_G05	TPL1163_02_A01
K_D	(M)	Short Neurotoxin 1 (*N. pallida*)	$9.69 \cdot 10^{-10}$	$<1.0 \cdot 10^{-12}$	$1.88 \cdot 10^{-12}$	$1.0 \cdot 10^{-9}$
K_D	error		$1.18 \cdot 10^{-11}$	$<1.0 \cdot 10^{-12}$	$2.28 \cdot 10^{-12}$	$1.35 \cdot 10^{-11}$
k_{on}	(1/M·s)		$3.14 \cdot 10^{4}$	$6.48 \cdot 10^{4}$	$2.55 \cdot 10^{4}$	$2.78 \cdot 10^{4}$
k_{on}	error		$4.29 \cdot 10^{1}$	$1.16 \cdot 10^{2}$	$5.52 \cdot 10^{1}$	$4.31 \cdot 10^{1}$
k_{off}	(1/s)		$3.15 \cdot 10^{-5}$	$<1.0 \cdot 10^{-7}$	$<1.0 \cdot 10^{-7}$	$2.78 \cdot 10^{-5}$
k_{off}	error		$3.71 \cdot 10^{-7}$	$<1.0 \cdot 10^{-12}$	$<1.0 \cdot 10^{-7}$	$3.78 \cdot 10^{-7}$
Full R^2			0.998	0.997	0.997	0.998
K_D	(M)	Dv1 (*D. viridis*)	$<1.0 \cdot 10^{-12}$	$1.40 \cdot 10^{-8}$	$1.80 \cdot 10^{-8}$	$2.63 \cdot 10^{-9}$
K_D	error		$<1.0 \cdot 10^{-12}$	$6.78 \cdot 10^{-11}$	$1.45 \cdot 10^{-10}$	$1.34 \cdot 10^{-11}$
k_{on}	(1/M·s)		$4.71 \cdot 10^{4}$	$5.40 \cdot 10^{4}$	$1.71 \cdot 10^{4}$	$2.52 \cdot 10^{4}$
k_{on}	error		$4.52 \cdot 10^{1}$	$1.63 \cdot 10^{2}$	$1.30 \cdot 10^{2}$	$3.62 \cdot 10^{1}$
k_{off}	(1/s)		$<1.0 \cdot 10^{-7}$	$7.58 \cdot 10^{-4}$	$3.08 \cdot 10^{-4}$	$6.65 \cdot 10^{-5}$
k_{off}	error		$<1.0 \cdot 10^{-12}$	$2.85 \cdot 10^{-6}$	$8.44 \cdot 10^{-7}$	$3.27 \cdot 10^{-7}$
Full R^2			0.999	0.996	0.994	0.999
K_D	(M)	Dp4 (*D. polylepis*)	$8.19 \cdot 10^{-8}$	NB	NB	NB
K_D	error		$3.92 \cdot 10^{-10}$	-	-	-
k_{on}	(1/M·s)		$5.88 \cdot 10^{4}$	NB	NB	NB
k_{on}	error		$2.46 \cdot 10^{2}$	-	-	-
k_{off}	(1/s)		$4.82 \cdot 10^{-3}$	NB	NB	NB
k_{off}	error		$1.27 \cdot 10^{-5}$	-	-	-
Full R^2			0.992	-	-	-

Table S3. Detailed kinetics table of the nanobodies binding to three different α-neurotoxin. Kinetics data obtained of the selected nanobodies from BLI.

Toxin abbreviation	Snake species	Geographical location	UniProt no.
Hh1	*Hemachatus haemachatus*	South Africa	P01433
Dp4	*D. polylepis*	Sub-Sharan Africa	P01416 + P00982
Dp7	*D. polylepis*	Sub-Sharan Africa	P01397 + P00982
Dj1	*D. jamesoni*	Sub-Sharan Africa	P01417
Dv1	*D. viridis*	West Africa	P01418
Dv4	*D. viridis*	West Africa	P00983 + P18328 + P01397
Dv6	*Dendroaspis viridis*	West Africa	P01397 + F8J2H1
Nh1	*Naja haje*	North and West Africa	P68418
Nh3	*N. haje*	North and West Africa	P01389
Nn4	*N. nivea*	South Africa	P01421 + P01390
Nn8	*N. nivea*	South Africa	P01390 + P01456
Nm3	*N. melanoleuca*	Central Africa	Q9YGJ6 + P01424
Nan3	*N. annulifera*	South Africa	P01421
Cytoxin (Nan13)	*N. annulifera*	South Africa	P01462
Short NTx	*N. pallida*	East Africa	P01426
αCBTx	*Naja kaouthia*	South and Southeast Asia	P01391
αBgtx	*Bungarus multicinctus*	Southeast Asia	P60615

Toxin abbreviation	Common toxin names	Toxin family
Hh1	sNTx-2	3FTx short-chain αNTx
Dp4	sNTx1 + δ-DTX	3FTx short-chain αNTx + Venom kunitz type
Dp7	α-elapitoxin-Dpp2C + δ-DTX	3FTx Long-chain αNTx + Venom kunitz type
Dj1	sNTx 1	3FTx short-chain αNTx
Dv1	sNTx 1	3FTx short-chain αNTx
Dv4	Mambaquaretin-7 + muscarinic toxin 2 + α-elapitoxin Dpp2c	venom Kunitz-type + 3FTx short-chain Aminergic toxin + 3FTx short-chain αNTx
Dv6	α-elapitoxin Dpp2c + Putative short chain NTX 61R	3FTx Long-chain αNTx + unknown 3FTx
Nh1	sNTx1	3FTx short-chain αNTx
Nh3	lNTx1	3FTx long-chain αNTx
Nn4	sNTx4 + lNTx1	3FTx short-chain αNTx + 3FTx long-chain αNTx
Nn8	lNTx1 + CTx1	3FTx long-chain αNTx + 3FTx cytotoxin
Nm3	α-neurotoxin NTX-1 + SNTx 1	3FTx short-chain αNTx
Nan3	sNTx4	3FTx short-chain αNTx
Cytoxin (Nan13)	CTx2	3FTx cytotoxin
Short NTx	SNTx1	3FTx short-chain αNTx
αCBTx	α-cobratoxin	3FTx long-chain αNTx
αBgtx	α-Bungarotoxin	3FTx long-chain αNTx

Table S4. Toxin and venom fraction components. The table provides information on the toxins and venom fractions utilized in this study, including the snake species from which the venom was obtained and their geographical distribution. The UniProt accession number, common toxin name, and toxin family are listed for the major components present in each venom fraction. The number associated with the toxin abbreviation corresponds to the fraction peak obtained during venom purification using RP-HPLC.

Toxin abbreviation	Amino acid sequence
LCC	SRTCYKTPS--DTSQICPPGENICYTKTWCDAWCSQRGKRVELGCAATCPKVKAGVDIKCCSTDNCNPFPTRKPRGS
Dp7	-...N....--.Q.K.....I.....E.....K.KFG...--
Dv4	-...N....--.Q.K.....I.....E.....K.KFG...--
Dv6	-...N....--.Q.K.....I.....E.....K.KFG...--
Nh3	-IR.FI..D--V...A..D.Q.....NF.GM.....D.....T..P.....ERS--
aCBTx	-IR.FI..D--I..KD..N.-HV.....F..I.....D.....T..T....Q.....RP--
aBgtx	-IV.HT.ATSPISAVT.....L..R.M....F..S...V.....SK.PYEEVT.....K...H.KQR.G--
Cytoxin (Nan13)	-LK.H.LVP--PFWKT..E.K.L..K----MYMVATPMLP.KR..IDV...DS.L.KYM..N..K..----------
SCC	LICHNQQSSQPPTTKTCPGETN-CYKKQ---WRDHRGTIIERGC--GCPTVKKGIKLNCCTTDKCNN
Hh1	.E.....T...Q.....-.....---.S....SRT....--.....P....K.....R..K
Dj1	R..Y.H..TT.A...S.-..NS-....T---.S.....--...K..Q..H.H..QS.....
Dv1	R..Y.H..TT.A...S.-..NS-....T---.S.....--...K..R.VH.H..QS.....
Nh1	.E.....-....R---.....S.T....--...S.....EI.....
Nm3	ME.....-.....---.S.....--...S....V.I.....R...
Nan3	M..YK.R.L.F.I.TV....K.-.....---.SG.....--...S.....EI.....R
Short NTx1	.E.....-....V---.....--.....P.....
Cytoxin (Nan13)	.K..K---LV..FW....EGK.L...MY---MVATPMLPVK...IDV..KDSALV.YM..N.....-

Table S5. Alignment of the major toxin components present in the venom fractions used for cross-reactive DELFIA. The alignment compares the toxin components against either LCC and SCC, which were utilized in the DELFIA assay to evaluate the binding capabilities of the nanobodies towards various toxins and assess their cross-reactivity.

	LCC	Dp7	Dv6	Dv4	Nh3	aCbtx	aBgtx	Cytotoxin
LCC	100	88	88	88	78	76	63	40
Dp7	88	100	100	100	70	70	66	37
Dv6	88	100	100	100	70	70	66	37
Dv4	88	100	100	100	70	70	66	37
Nh3	78	70	70	70	100	82	56	38
aCbtx	76	70	70	70	82	100	58	40
aBgtx	63	66	66	66	56	58	100	42
Cytotoxin	40	37	37	37	38	40	42	100

	SCC	Hh1	Dj1	Dv1	Nh1	Nm3	Nan3	SNTx	Cytotoxin	Nn4
SCC	100	83	72	70	89	89	75	95	43	75
Hh1	83	100	62	60	82	82	64	85	37	63
Dj1	72	62	100	97	66	68	64	71	36	64
Dv1	70	60	97	100	64	69	63	69	38	63
Nh1	89	82	66	64	100	87	73	89	40	73
Nm3	89	82	68	69	87	100	77	87	40	77
Nan3	75	64	64	63	73	77	100	70	37	100
SNTx	95	85	71	69	89	87	70	100	41	70
Cytotoxin	43	37	36	38	40	40	37	41	100	37
Nn4	75	63	64	63	73	77	100	70	37	100

Table S6. Amino acid sequence similarity between the major toxin components present in the venom fractions used for cross-reactive DELFIA. The percentage of amino acid identity between each tested toxin. Nn4 used for the neutralization assays are also included.

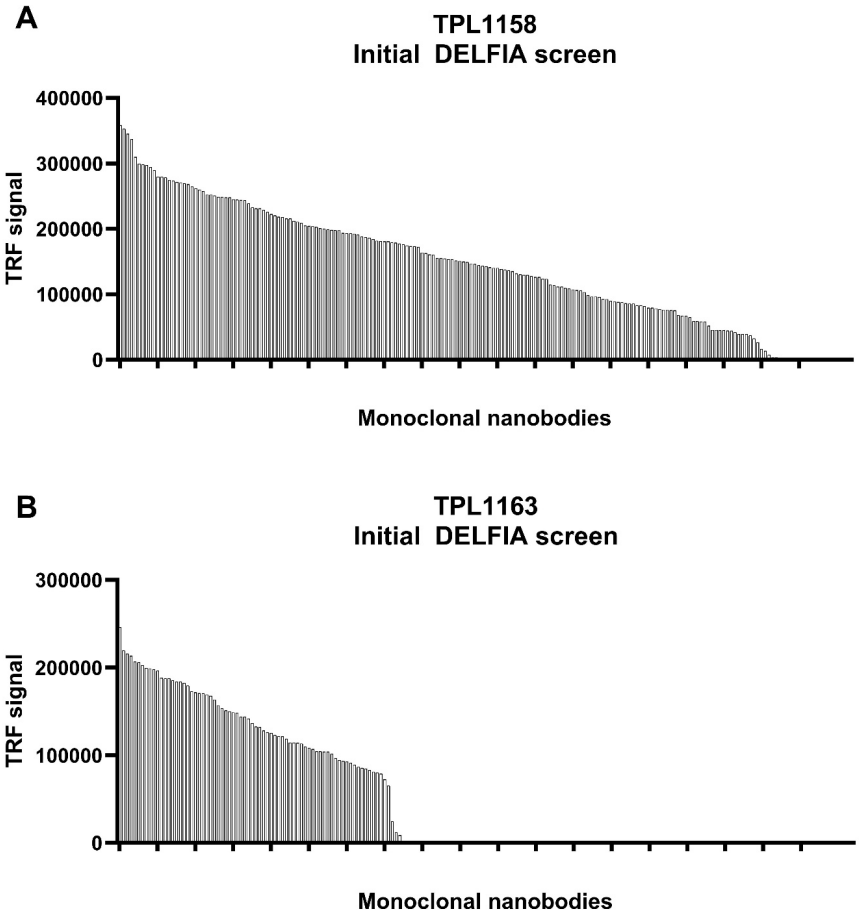

Figure S1. Initial DELFIA screen. The initial DELFIA screen involved the screening of cherry-picked clones from TPL1158 and TPL1163. A total of 186 clones from each selection round were tested against either a) LCC or b) SCC. The X-axis represents the clones, with 10 clones marked between each tick.

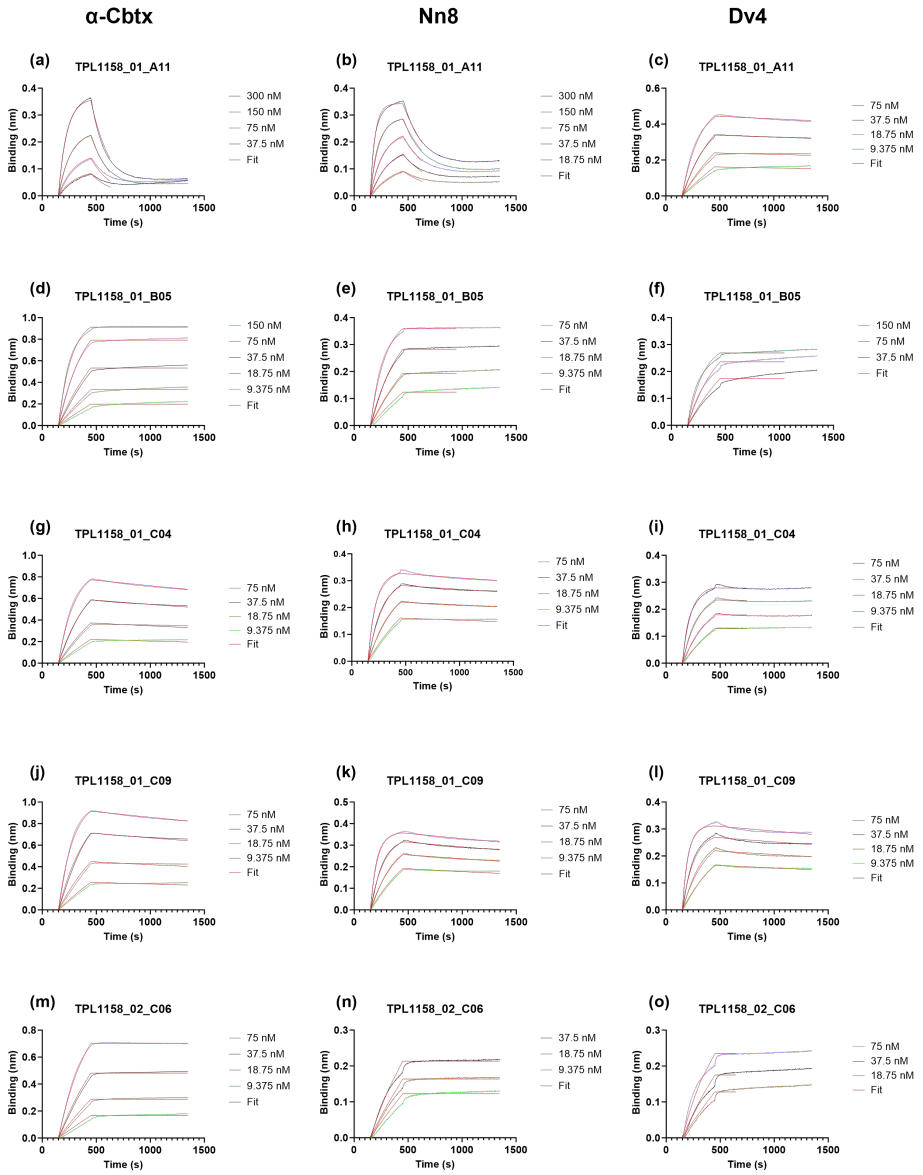

Figure S2. BLI binding curves from TPL1158. The measured binding curves of the nanobodies from the TPL1158 selection against three different LC-α-neurotoxins including their fitted curves.

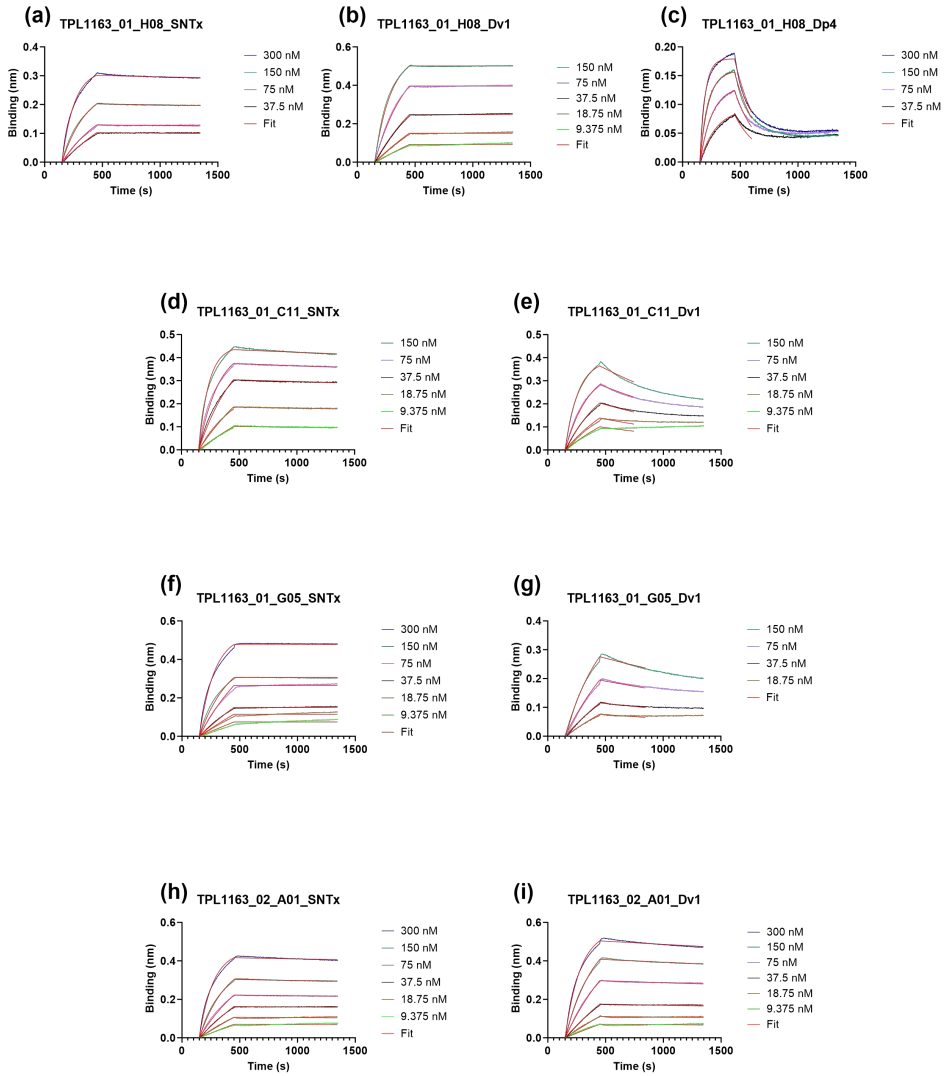

Figure S3. BLI binding curves from TPL1163. The measured binding curves of the nanobodies from the TPL1163 selection against three different short α-neurotoxins including their fitted curves. No binding was observed for TPL1163_01_C11, 01_G05, and 02_A01 to Dp4.

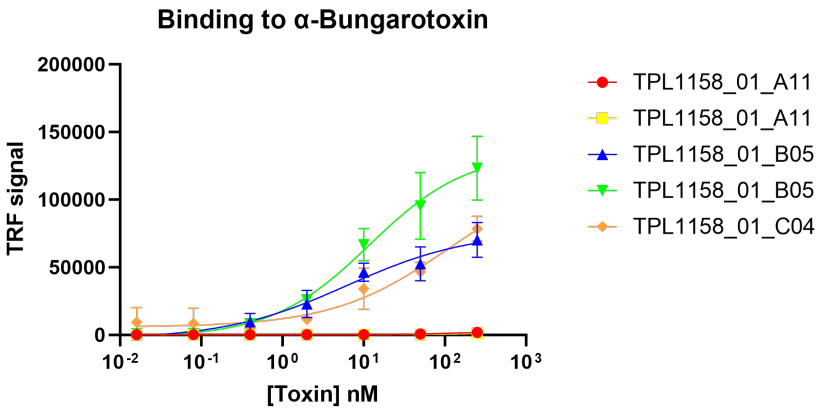

Figure S4. Nanobody binding to α-Bungarotoxin. Despite α-Bungarotoxin having only 62% (on average) sequence similarity compared to the other tested α-neurotoxins, three of the nanobodies exhibited binding towards α-Bungarotoxin.

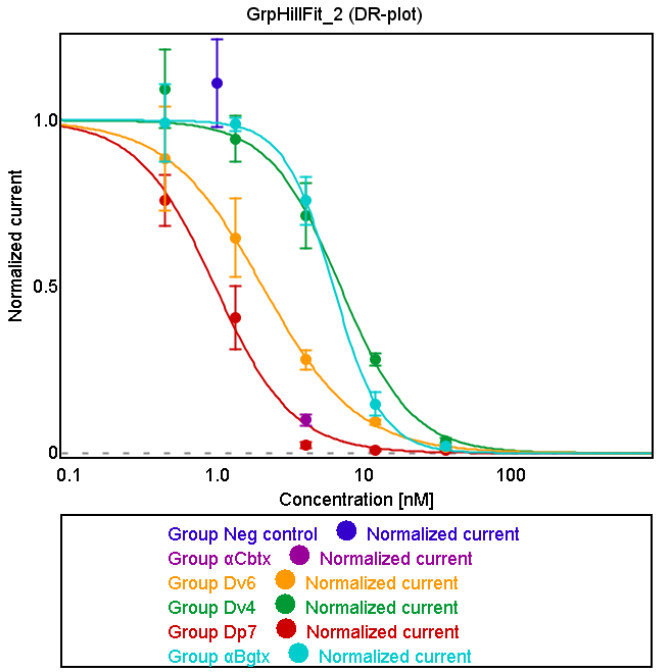

Figure S5. Inhibition curves of LC-α-neurotoxins. Using automated patch-clamp technology, we evaluated the functional inhibition of the nAChR on whole cells by

testing five different LC-α-neurotoxins. Concentration-dependent inhibition curves were plotted by measuring the acetylcholine-induced peak current at various toxin concentrations and normalizing it to the full response without toxin. For α-cobratoxin (αCbtx), previous inhibition curves had been measured, and its IC_{80} was determined to be 4 nM. Thus, in this study, we specifically tested this toxin at the IC_{80} concentration to confirm its functional inhibitory activity.

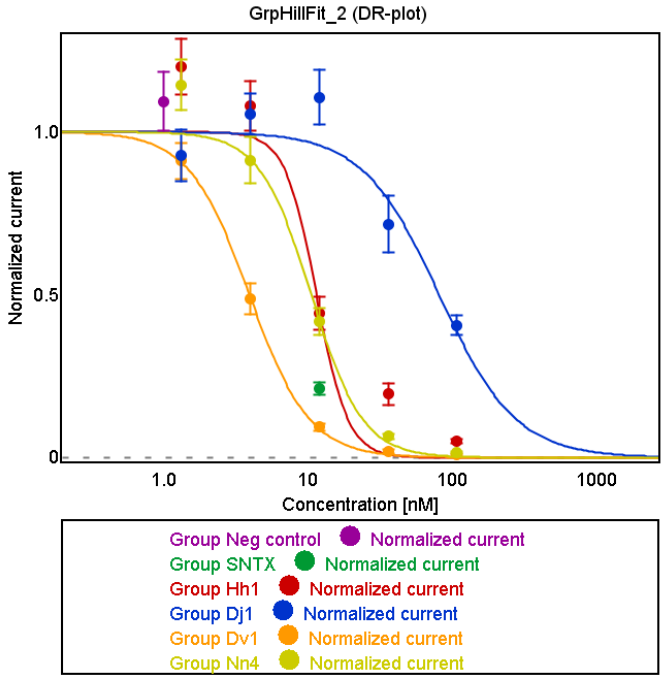

Figure S6. Inhibition curves of SC-α-neurotoxins. Using automated patch-clamp technology, we evaluated the functional inhibition of the nAChR on whole cells by testing five different SC-α-neurotoxins. Concentration-dependent inhibition curves were plotted by measuring the acetylcholine-induced peak current at various toxin concentrations and normalizing it to the full response without toxin. For Short neurotoxin 1 (SNTX), previous inhibition curves had been measured, and its IC_{80} was determined to be 12 nM. Thus, in this study, we specifically tested this toxin at the IC_{80} concentration to confirm its functional inhibitory activity.

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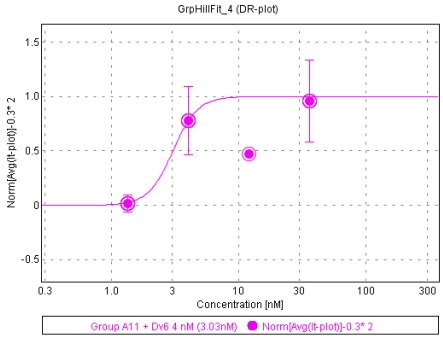
Dv6



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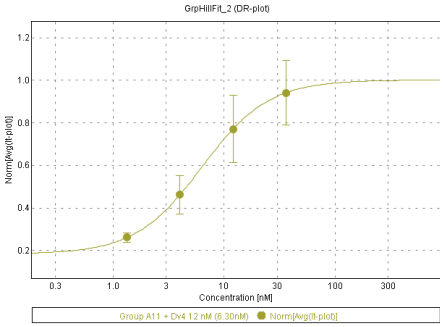

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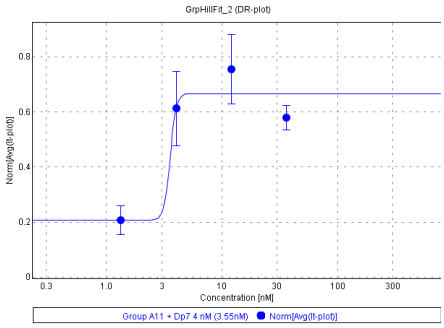
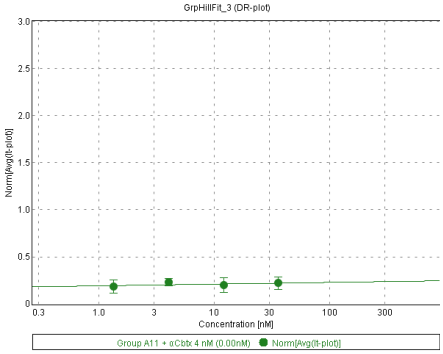


αCbtx



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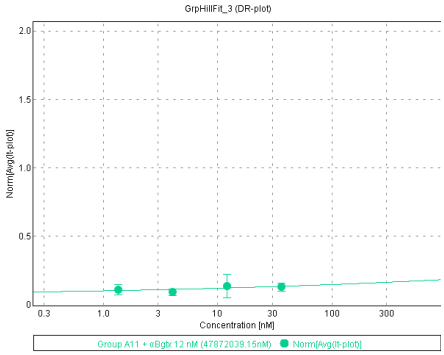

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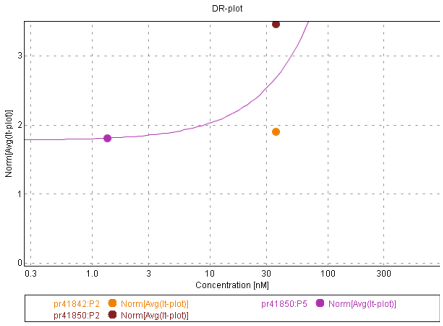

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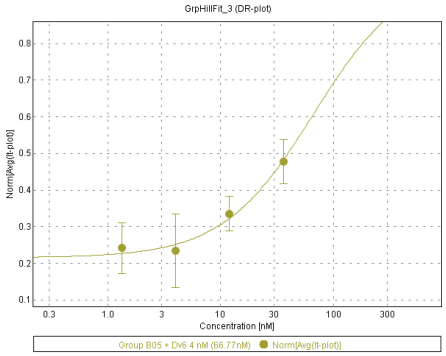

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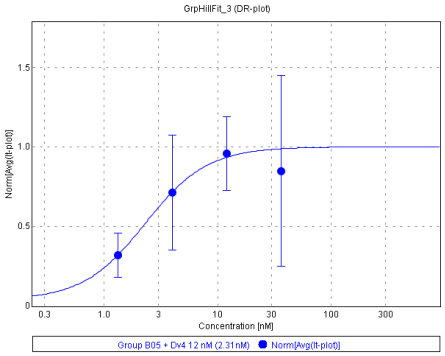

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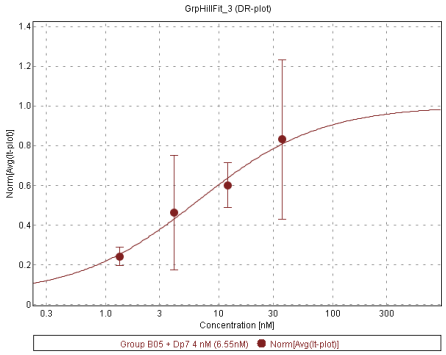

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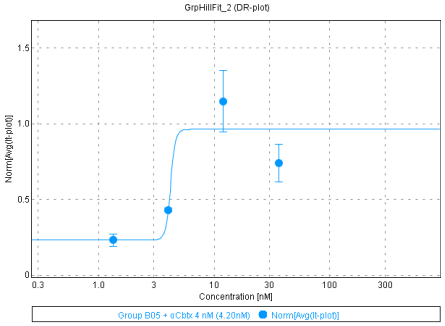

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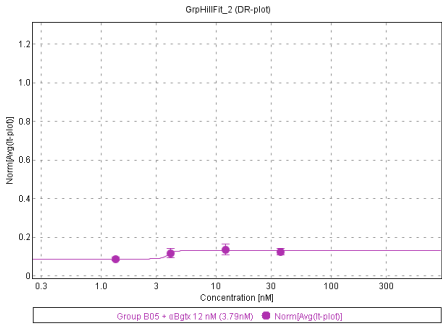

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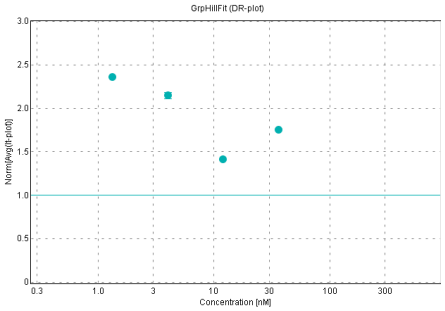

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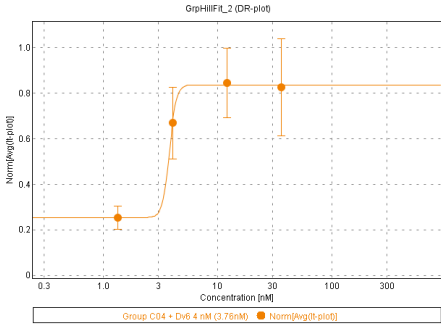

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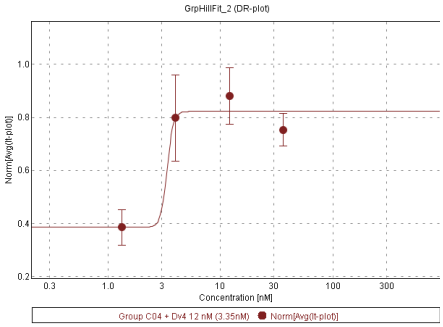

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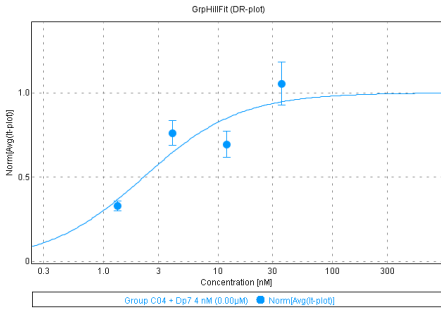

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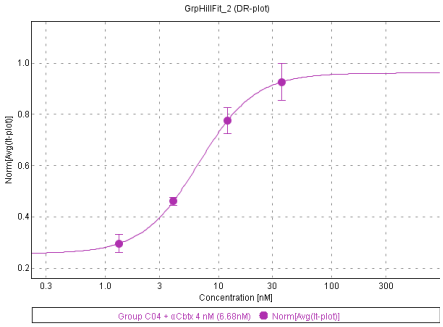

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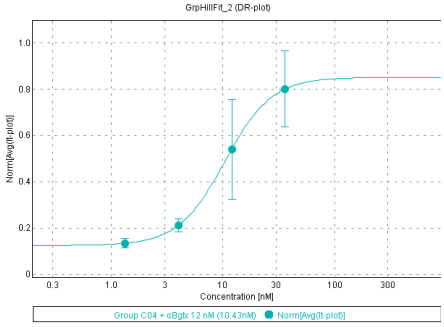

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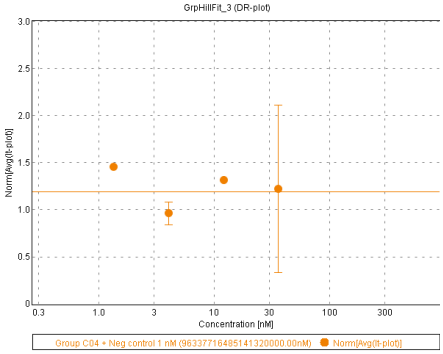

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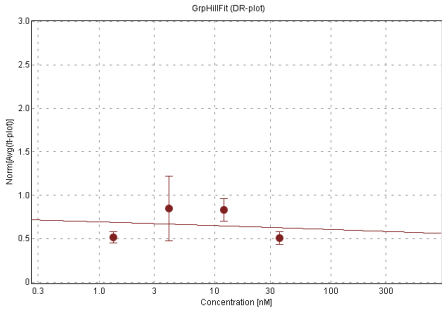

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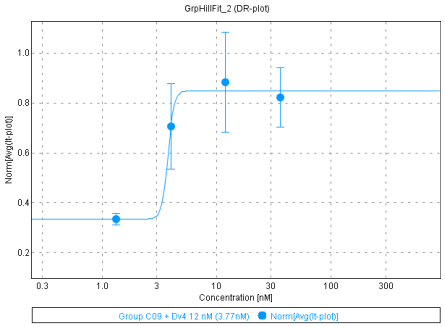

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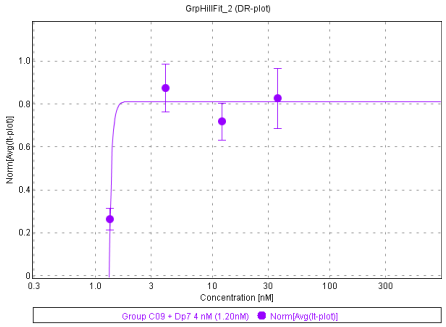

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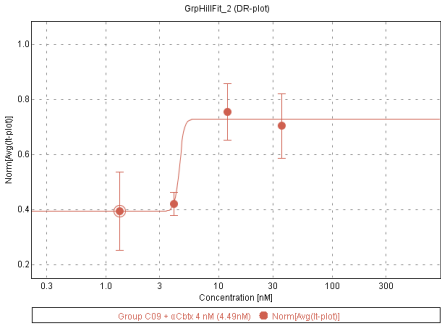

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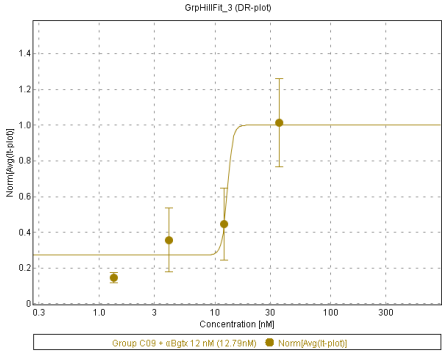

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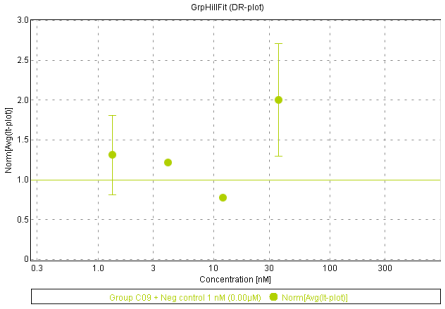

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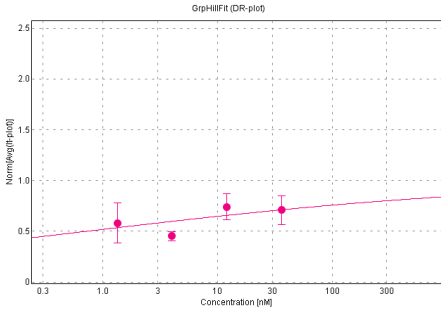

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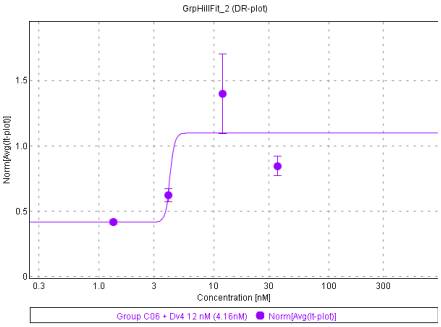

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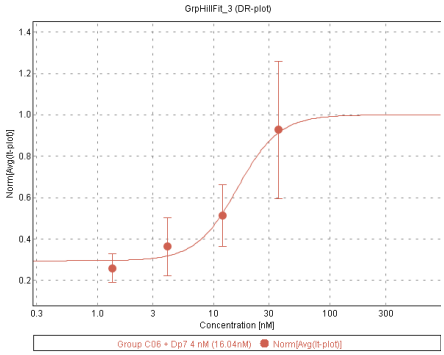

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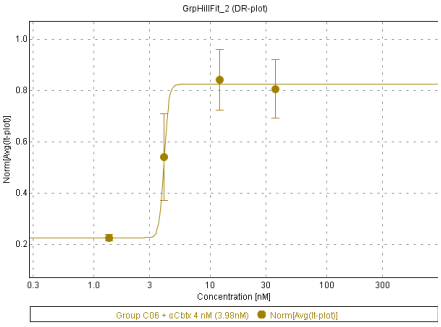

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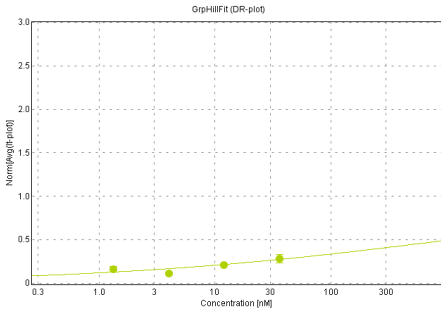

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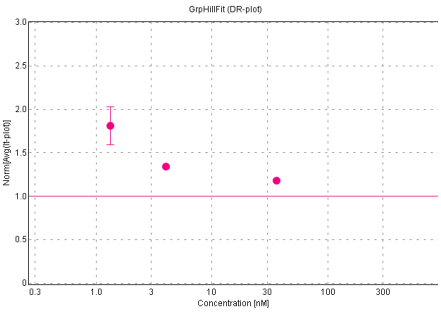

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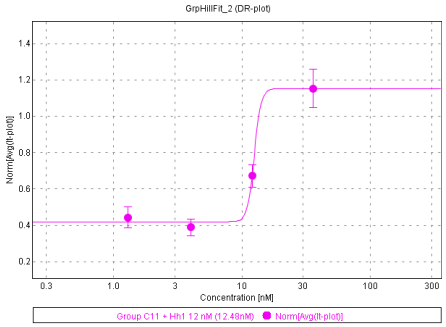

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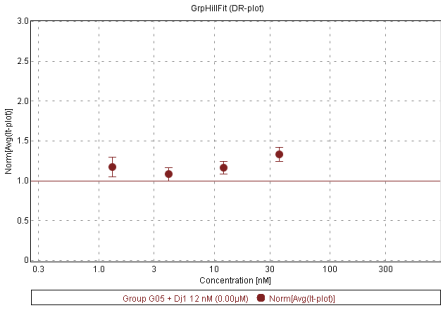

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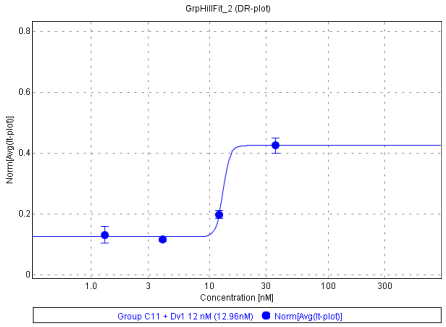

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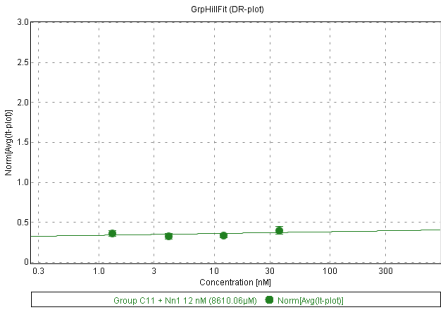

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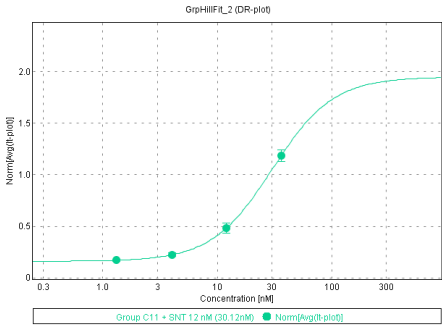

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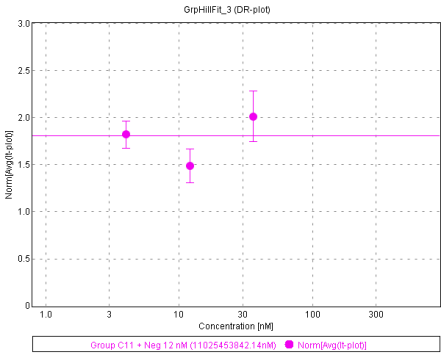

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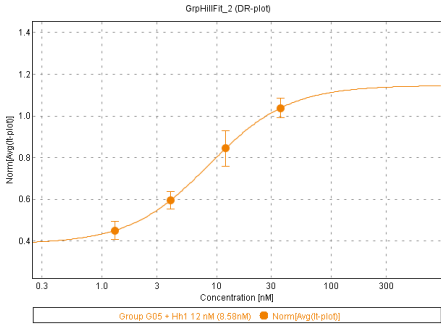

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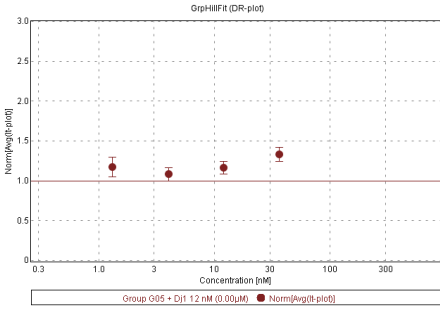

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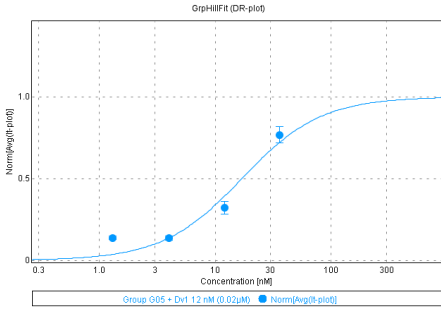

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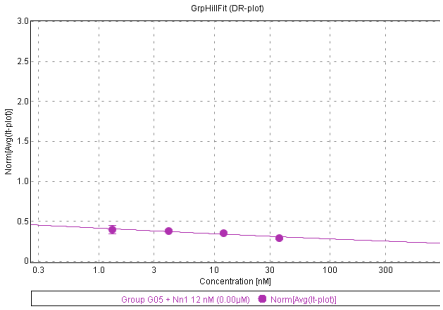

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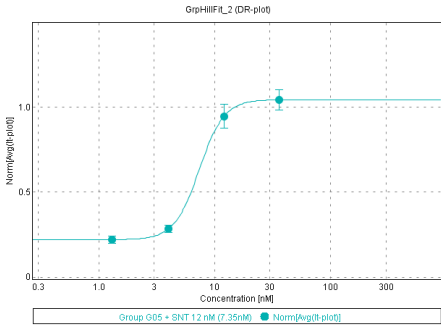

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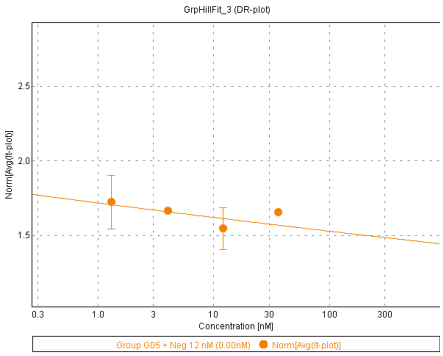

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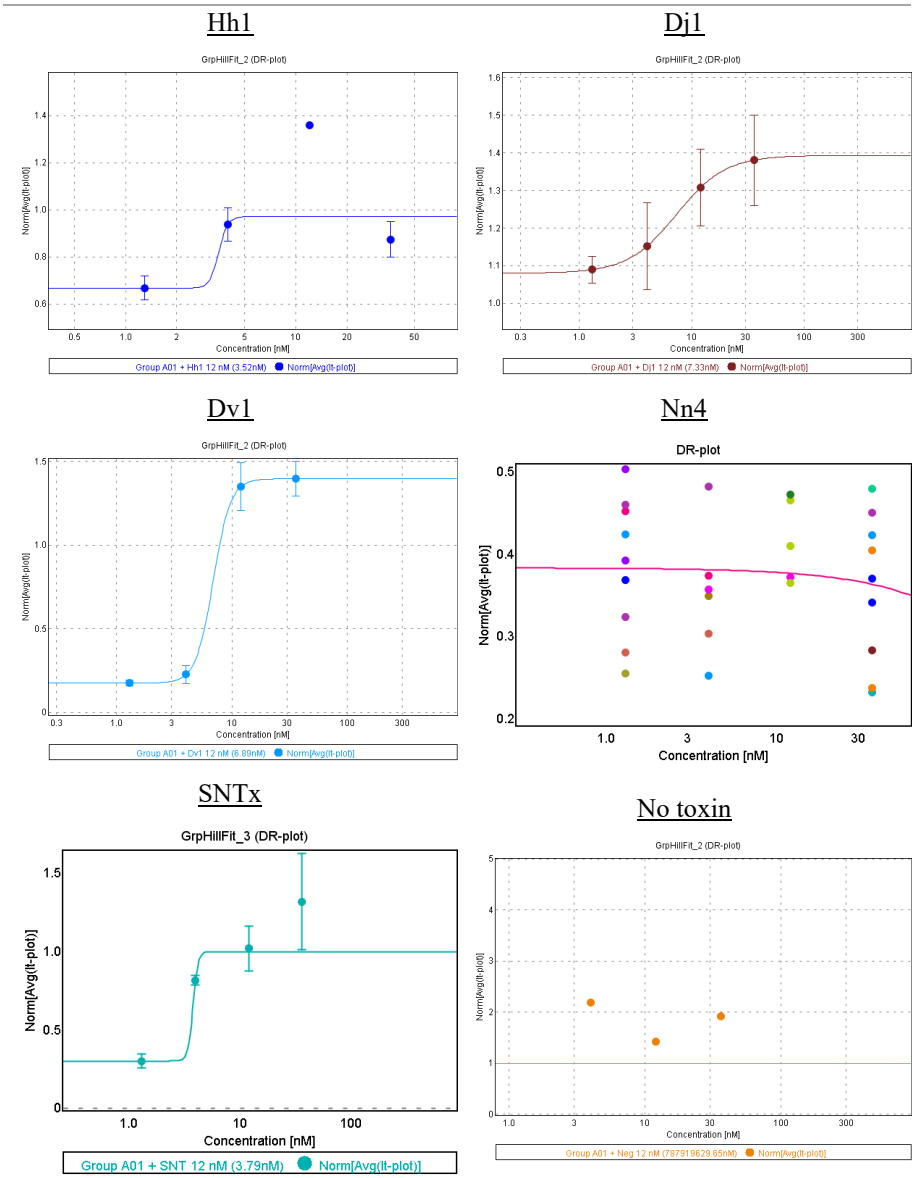

Figure S7. Neutralization of the nanobodies. The concentration-response curves demonstrate that increasing concentrations of the nanobodies, preincubated with various α-neurotoxins, led to enhanced protection of the nAChR. This protection was evident as a dose-dependent prevention of the loss of current mediated by the toxins.

Chapter 6 – Discussion

1. Advancing antivenom development

Snakebite envenoming poses a significant global health problem, particularly affecting vulnerable populations in rural and developing regions. Currently, the only available treatment for snakebite envenoming is traditional antivenom, which is derived from animal plasma and holds several drawbacks.[1] These include batch-to-batch variation, limited availability, potential adverse effects, and the need for careful storage and administration. Over the past few decades, researchers have endeavored to develop alternative therapies.[2] One promising strategy that is being explored is the discovery and development of recombinant antibodies against snake venom toxins.[3]

This thesis zeroes in on this strategy with a focus on pursuing the expression of recombinant α-neurotoxins from snake venom, with the aim of using these as antigens for discovering antibodies capable of neutralizing these toxins. Previous efforts in the field of recombinant antivenoms primarily relied on using toxins purified directly from venom through chromatographic techniques.[4–9] However, this approach has its limitations, as the venom fractions obtained in this process only rarely consist of a single pure toxin, making it challenging to isolate antibodies against specific targets.[10] For instance, our research group faced difficulties in identifying antibodies specific to short α-neurotoxins, possibly because these venom fractions frequently contained other toxins with properties that biased the selection of antibodies towards these (unpublished results).

To overcome these challenges, I explored the expression of snake toxins using heterologous systems, which offer several advantages for antivenom development. Heterologous expression enables the production of pure, homogenous toxins, effectively sidestepping impurity and cross-contamination issues. Additionally, it offers scalability and a reduction of batch-to-batch variation. I demonstrated the utility of this approach using recombinant α-cobratoxin, a well-characterized 3FTx, which could serve as an antigen for discovering scFvs with comparable affinities to those generated against the native toxin (Article II). This accomplishment was the first entirely *in vitro* antibody selection strategy reported for generating neutralizing monoclonal antibodies against a snake toxin. This achievement highlights the potential of using recombinant antibodies to revolutionize the field of antivenom research, thereby improving treatment options for snakebite envenoming by offering a more reliable and scalable method for producing potent antivenom therapies.

Since at least the 1980s, it has been speculated that only a few antibodies with broad neutralization capacity should be sufficient to neutralize snake venom toxins across different species.[11] However, finding such cross-reactive antibodies while keeping therapeutic binding affinity has remained largely unsuccessful to date. One strategy used to try to achieve this goal has been phage display combined with cross-panning, thus aiming to generate broadly neutralizing antibodies that can recognize multiple antigens. However, this approach poses challenges as it requires the use of several different antigens, making it difficult to determine which combination will lead to the desired cross-reactivity, and it is restricted to a handful of antigens that can be used on successive panning rounds. As a result, the applicability of this method may potentially be limited in certain cases.[12] As an alternative approach, I utilized consensus toxins, which represent artificial averages of several toxin variants, for my selection campaigns. The successful utilization of consensus toxins in the discovery of scFvs capable of neutralizing sphingomyelinases from both spiders and scorpions has been achieved in our research group.[13] The novel use of consensus toxins to discover broadly neutralizing scFvs is a unique contribution to the field, having only been reported this one time in the scientific literature. Moreover, consensus toxins have been utilized in immunization strategies, giving rise to polyclonal responses capable of neutralizing elapid venoms from diverse genera.[14] The antigen's structural integrity requirement may be lower during immunization, as conserved epitopes can be recognized even in partially unfolded proteins. It also remains unclear whether the observed broad response is primarily due to the polyclonality of the immune response, generating a diverse repertoire of antibodies with varying specificities, or whether it is attributed to the presence of truly broadly neutralizing monoclonal antibodies within the polyclonal response.

In this thesis, I utilized two different consensus α-neurotoxins, one short and one long, to discover nanobodies capable of binding and neutralizing toxins from several elapid species. This investigation highlights the potential of using recombinantly produced consensus toxins in the development of broadly neutralizing antibodies and could extend beyond toxin and antivenom research. It presents opportunities for antibody discovery in various therapeutic and diagnostic applications requiring cross-reactivity, such as infectious diseases, where consensus envelope proteins from e.g. dengue fever can be used to generate broad protection against different serotypes,[15] cancers, where consensus tumor-associated antigens can be used to elicit T cell responses against tumor cells,[16] and cross-species applications, where consensus proteins can aid in the discovery of antibodies that function both in preclinical models and in the clinical setting.

2. Advancements in recombinant toxin expression and antibody discovery for antivenom development

2.1 Previous research on the expression of recombinant α-neurotoxins

Throughout this thesis, a key objective has been to identify the optimal expression system for α-cobratoxin and other α-neurotoxins. However, this proved challenging, as I encountered various difficulties and inconsistencies in the expression process. For example, the amount of expressed protein could vary each time I performed the expression, and the efficiency of the purification process showed significant variability. In some instances, the first purification step resulted in almost pure toxins, while in other attempts, a substantial amount of impurities were present in the sample despite following the exact same protocol. This work underscores the importance of establishing a standardized and reproducible methodology, which involves not only following protocols but also considering factors like temporal precision, reactant purity, and equipment calibration. Despite facing challenges, my pursuit of recombinant toxins was motivated by the numerous advantages they offer over traditional purification from natural sources.

As discussed in detail in Article I, the heterologous expression of snake toxins can significantly reduce the need for animal venoms, alleviating ethical concerns and associated risks in research.[3,17] Additionally, this approach enables the investigation of toxins that are not readily available or challenging to purify from natural sources due to their low abundance in venom, low venom abundance, or purification complexities.[10] Moreover, the incorporation of fusion tags in the recombinant expression process may offer valuable control to the researcher during subsequent experiments. Lastly, recombinant expression also allows for the exploration of dormant genes, unlocking a wealth of possibilities for toxin research, taking advantage of the massive data generated by the recent advances on genomics and transcriptomic technologies.[18,19]

α-Neurotoxins have garnered significant interest in the field of toxin expression, to the extent that a specialized review has been published solely focusing on the expression of this subgroup of 3FTxs.[20] In addition to the insights gained from such review articles, recent studies have further contributed to our understanding of recombinant toxin expression. Glanville *et al.* published a preprint one year ago on BioRxiv, where they successfully expressed three long α-neurotoxins using HEK cells. Insights from their research involved a crystal structure analysis of the recombinant α-neurotoxin, revealing that the recombinantly produced α-

neurotoxins exhibited a native-like structure, along with a deeper understanding of how antibodies interact with these toxins through receptor mimicry.[21]

Another relevant study by Liu *et al.* focused on the expression of α-neurotoxins in *E. coli*.[22] Here, CD spectra were used to analyze the secondary structure of the expressed toxin, offering insights into the formation of native-like structures. Furthermore, tandem mass spectrometry (MS/MS) was employed to characterize the disulfide linkages in the recombinant 3FTxs, confirming the successful formation of native-like structures in the expressed toxins. This study provides valuable insights into the evaluation of the structure of recombinant toxins and demonstrates the suitability of using *E. coli* as a host for producing α-neurotoxins with native-like structures, which may be crucial for their potential application in research and therapeutic contexts.

Similarly, Makarova *et al.* also utilized *E. coli* to express α-neurotoxins.[23] They evaluated the recombinant toxins through different types of electrophysiology techniques. This investigation highlighted that their recombinant α-neurotoxins were functionally active when expressed in *E. coli*. However, this study did not provide any structural analysis of the produced α-neurotoxins.

The studies mentioned above, along with existing literature, provide valuable knowledge about successful expression systems for 3FTxs and α-neurotoxins, as well as insights into how the outcome of the expressions could be evaluated and analyzed. As there is no gold-standard methodology shared among these studies, ensuring the quality control of toxin products is paramount, but challenging. The varying approaches in toxin expression and purification highlight the need for meticulous quality assessment to maintain consistency and reliability in research findings. These findings have not only guided the progress of this thesis, but have also contributed to the broader field of recombinant toxin expression, particularly in the context of discovering antibodies targeting 3FTxs and thus antivenom.

2.2 Challenges and further optimization for the expression of α-neurotoxins

Throughout this thesis, I encountered a critical issue related to overall lack of reproducibility in toxin expression, leading to variable expression levels each time I conducted an expression experiment. This unpredictability highlights the need for further optimization of the recombinant expression process to achieve consistent and high yields of the desired toxins. In addition to optimizing expression, it became evident that the purification methods employed could also benefit from refinement. In addition to immobilized-metal affinity chromatography (IMAC), which was presented in Manuscripts I and II, both size-exclusion chromatography (SEC) and ion-exchange chromatography (IEX) were

utilized for purification. While these methods offered some insights into finding the optimal purification method, they had their limitations and were not effective purification strategies for α-neurotoxins, as they did not consistently yield pure and reproducible results. Despite the challenges faced during the investigation, the exploration of different purification methods yielded information on the efficiency, reproducibility, and potential pitfalls of each approach. This provided valuable knowledge on the challenges and considerations for the advancement of toxin purification strategies, which hopefully can pave the way for improved methods to isolate pure and functional toxins in future research.

The protease cleavage step with TEV and Ulp1, which is essential for obtaining the native-like toxin if expressed with a tag, required the use of reducing agents for activation. Upon investigation, I discovered that the concentration of 1 mM DTT used was too high and likely affected the disulfides of α-cobratoxin. To address this issue, future research could explore alternative proteases, such as thrombin, which is a serine protease, or reducing the DTT concentration, to improve the efficiency of maintaining the disulfides and enhancing toxin yield during purification. By further optimizing both the expression and purification aspects of the process, it might be possible to achieve consistent and reliable production of recombinant α-neurotoxins for further studies and applications. The expression system in *K. phaffii* may also require optimization, to achieve optimal expression. For instance, utilizing codon-optimized toxins could be another potential approach to improve expression efficiency. In my hands, *K. phaffii* generally outperformed bacterial expression systems in expressing complex proteins, such as α-neurotoxins with intricate disulfide patterns, while being cheaper and less high-maintenance compared to mammalian cells. The success of the *K. phaffii* system for the expression of consensus toxins, as demonstrated in Manuscript II, further supports the potential for this expression system.

The csCyDisCo system, which was the first expression system I explored in the work behind this thesis, was effective in producing the α-cobratoxin used in Article II. While csCyDisCo is optimized for conotoxins from cone snails,[24] I hypothesize that incorporating snake PDIs may enhance its performance for α-neurotoxin expression. Although this system may not be the optimal choice, it still provided enough α-cobratoxin of sufficiently high quality for successful antibody phage display campaigns. During my thesis, attempts were made in our lab to discover antibodies using consensus toxins expressed in *E. coli* with csCyDisCo. However, the scFvs that were obtained recognized only the recombinantly expressed α-neurotoxins and not their native counterparts, indicating potential differences in folding when using this expression system.

2.3 Standardized quality criteria and functional assessment of recombinant toxins

To enhance the transparency in the scientific literature and the efficiency of recombinant toxin expression, I propose a standardized set of quality criteria. Among these criteria, structural integrity assessment using CD analysis emerges as a cheap and rapid method that preserves protein samples for further investigations. Additionally, methods like MS/MS could be utilized to determine disulfide patterns. During this project, we attempted MS analysis, similar to the work by Liu et al., with the assistance from ETH Zürich and the Proteomics Core DTU. However, the complex disulfide patterns of α-neurotoxins complicated the data analysis substantially. When using trypsin for cleavage while the disulfides were intact, all the peptides remained connected, which complicated the analysis process. To improve the MS analysis of α-neurotoxins with intact disulfide bonds, future research could explore alternative proteases and methods that allow for more efficient cleavage of the peptides or MS acquisition methods that allow for the detection of larger peptides.

Functional assays for α-neurotoxins are difficult to perform, as their mode of action involves binding to and inhibiting the nAChR, rather than performing enzymatic activities (in contrast to other medically important snake toxin families, such as SVMPs, SVSPs, and PLA_2s). In this context, *in vitro* assays utilizing a chimera subunit of the receptor, α7, proved valuable, specifically for long-chain α-neurotoxins that, unlike short-chain α-neurotoxins, demonstrate high binding affinity to this particular subunit.[25] These *in vitro* assays offer valuable information about the toxin's ability to bind to the receptor. However, to assess the true functionality of the α-neurotoxins, a different method is needed. At the moment, patch-clamp assays can provide this crucial insight into toxin behavior, but it requires specialized equipment. As part of this project, a collaboration was established with Sophion Bioscience to conduct high-throughput automated patch-clamp experiments. This collaboration enabled a more comprehensive assessment of the functionality of recombinant α-neurotoxins, providing valuable insights into their interaction with nAChRs and potential effects on the activation of the receptor and the cellular activity. Additionally, the neutralizing capacity of discovered nanobodies was tested by evaluating their ability to block the toxic effects of α-neurotoxins on nAChRs. The use of automated patch-clamp experiments through this collaboration significantly enhanced our ability to assess whether we could express functional recombinant toxins, what their potency was, and what the neutralization capacity our discovered nanobodies and antibodies possessed.

A recently published preprint introduced an *in vitro* assay that utilizes immortalized TE671 cells, expressing the fetal muscle type nAChR, and a membrane potential dye to indicate receptor activation.[26] This approach measures nAChR activation through dye uptake, making use of a standard plate reader without requiring specialized electrophysiology equipment or facilities. This method might offer an intriguing alternative for studying the functionality of α-neurotoxins without the need for investment in expensive equipment.

Establishing a low-cost, standardized method to validate the functionality of recombinant toxins requires the adoption of more accessible *in vitro* methods to evaluate the function of α-neurotoxins. This should ideally be one that does not rely on specialized equipment like patch-clamp experiments – at least if the purpose is mostly quality control purposes and not deeper biological or biochemical insight. In the future, the beforementioned *in vitro* method could serve as a standard experiment to validate the function of α-neurotoxins, while CD analysis should be employed to investigate the structural integrity. By combining CD analysis and *in vitro* assessments, we propose a robust and accessible standard for comparing toxin expression systems, enabling an evaluation of both the structure and functional activity of the produced α-neurotoxins.

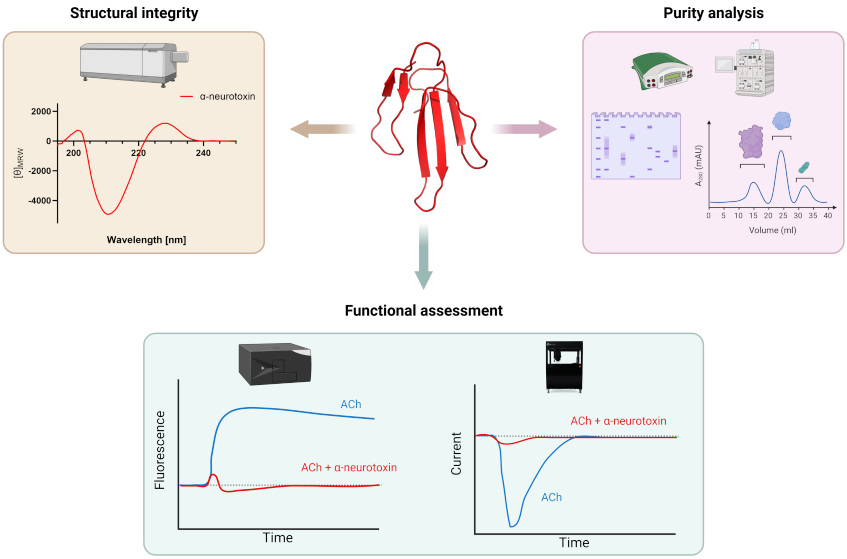

Figure 1. Graphical illustration of suggested assessment strategy. *The structural integrity can be evaluated using CD spectroscopy by comparing the spectra obtained from the recombinant α-neurotoxin with a native toxin. The function of the α-neurotoxins could be assessed using the newly developed in vitro method, where the nAChR activation can be measured through fluorescence or by path-clamping. The level of purity achieved for the purified α-neurotoxin could be assessed using SDS-PAGE and chromatographic techniques.*

While the suggested analysis would offer valuable information, a more comprehensive insight can be achieved through additional methods. X-ray crystallography allows obtaining the actual crystal structure of the α-neurotoxins, while patch-clamp measurements can be used to assess the blocking of the nAChR. However, it is important to note that both these methods may have limitations in terms of being low-throughput and/or relatively resource-consuming.

3. The role of broadly neutralizing antibodies

Broadly neutralizing antibodies are expected to play a pivotal role in modern antivenom development. Unlike monospecific antibodies that target a single toxin, broadly neutralizing antibodies can recognize and neutralize multiple toxins, sometimes even across different snake species. The significance of these antibodies lies in their ability to provide broad therapeutic effectivity against a range of venoms, potentially offering a more comprehensive and cost-effective antivenom therapy.[27]

The feasibility of introducing recombinant antivenoms as a therapeutic option in impoverished regions highly affected by snakebite is heavily dependent on affordability. The costs associated with antivenom development encompass discovery, development, manufacturing, regulatory processes, and profit margins of the final product. While the latter two factors are largely influenced by business aspects, technology, and scientific advancements can significantly impact the cost of product development and manufacture.

Several variables influence the overall cost of antivenom development. Firstly, the choice of antibody format used in the production process plays a critical role. Immunoglobulins (IgGs), typically expressed in mammalian expression systems, and nanobodies, produced using bacterial expression systems, differ significantly in size and complexity. Consequently, the cost per produced molecule varies between these formats. Throughout this thesis, we successfully investigated the discovery of scFvs (which can be reformatted to IgGs) and nanobodies utilizing recombinant α-neurotoxins, leading to promising and successful discoveries in both cases.

The dose needed and the number of unique antibodies required in the oligoclonal antivenom significantly impact development costs. Higher affinity antibodies are desirable, as they can neutralize venom toxins effectively at lower concentrations, potentially reducing the dose required for antivenom treatment. Jenkins and Laustsen estimated that the use of highly efficacious antibodies (2:1 toxin to high-affinity antibody molar ratio) or less efficacious antibodies (1:3 toxin to lower affinity antibody molar ratio) in a polyvalent recombinant antivenom against ten

venoms could lead to a five-fold difference in the cost of goods manufactured of the final drug product.[28]

My findings could also contribute to the advancement of antivenom production methods, particularly when compared to traditional methods reliant on animal plasma-derived antivenoms. Recombinant expression offers the potential to produce consistent and reliable toxin components, provided a robust process can be established. This could lead to a more standardized and reliable antivenom product, with a better balance of antibodies targeting medically relevant toxins.

One of the main intentions of this thesis was to discover broadly neutralizing nanobodies capable of neutralizing α-neurotoxins found in African elapids. In pursuit of this goal, I employed consensus toxins as antigens in a phage display campaign to discover nanobodies (Manuscript II). Although my primary aim was not to pursue the discovery of high-affinity nanobodies, I was able to obtain nanobodies that were both broadly neutralizing and had high affinities.

A study by Richard *et al.* highlighted the significance of nanobodies in antivenom therapy.[29] They used a nanobody with a 400 pM affinity to α-cobratoxin and tested its efficacy in preventing α-cobratoxin-induced lethality in mice. The nanobody, when preincubated with LD_{100} of α-cobratoxin in a 1:0.75 molar ratio, successfully prevented lethality even at this low dose. Additionally, in a rescue experiment, where mice were first injected with the venom and later treated with the nanobody, this same low dose effectively prevented lethality. These results underscore the potential of nanobodies as potent therapeutic agents for antivenom development.

In Manuscript II, several nanobodies were assessed for their ability to neutralize α-neurotoxins *in vitro*. Some of these nanobodies displayed complete inhibition of α-cobratoxin at a 1:3 molar ratio (toxin:nanobody). This finding suggests that these nanobodies have potent neutralizing capabilities, potentially making them effective at lower dosages. Furthermore, the evaluated nanobodies exhibited a broad neutralization spectrum capable of neutralizing several α-neurotoxin from different snakes. These features make these nanobodies promising leads for antivenom development, as they might pose as a more effective and versatile treatment option for snakebite envenoming.

To further assess the neutralization abilities of the discovered nanobodies, *in vivo* experiments are needed in mice. These experiments will shed light on the nanobodies' capacity for neutralizing venom toxins. In addition, larger animal models, such as sheep, can be employed to assess both the neutralization capacity and pharmacokinetics of the antibodies. Understanding both

neutralization capacity and pharmacokinetic properties is vital for advancing the development of effective and safe antivenom treatments.[30]

4. Consensus proteins: potential applications beyond antivenom development

Throughout the research behind this thesis, I have come to recognize the extensive potential of consensus proteins in diverse scientific domains. Beyond their significant impact on antivenom development and production, consensus proteins present versatile applications across different fields, demonstrating their adaptability and importance in advancing science and medicine.

One key area where consensus proteins hold promise is vaccine development. Their potential ability to represent an average of multiple pathogenic variants of an antigen enables them to cover conserved epitopes shared across e.g. bacterial strains, parasites, or cancers.[16,31] As antigens, consensus proteins have the potential to induce a broader immune response, enhancing protection against infectious diseases that exhibit antigenic variability and possibly even be more robust against escape mutations occurring both in infectious diseases and oncology. Their application in vaccine development can therefore possibly play a crucial role in global health efforts by providing more effective immunization strategies against pathogens with high mutation rates and cancers.

In the realm of diagnostics, consensus proteins might also serve as valuable tools for detecting pathogens or toxins. By designing diagnostic assays based on consensus antigens, rapid and sensitive identification of infections or envenoming with low false negative rates can possibly be achieved. These assays can aid healthcare professionals in making timely and accurate diagnoses, leading to improved patient outcomes. For instance, in the case of influenza, a consensus antigen could be designed to represent an average of multiple variants of the viral hemagglutinin protein, which is known to exhibit antigenic variability. By using this consensus antigen in diagnostic assays, it might be possible to detect various strains of influenza virus using a single test.[32] This approach could lead to early detection, enabling timely and accurate treatment decisions and potentially reducing the spread of the virus in the population.

Another aspect of the versatility of consensus proteins lies in their capacity to represent an amalgamation of protein variants from multiple species. This characteristic makes them attractive targets for antibody discovery with cross-species functionality, especially in the context of translational medicine, *i.e.*, the transition from preclinical to clinical drug development, as it can reduce the risk of trial failures during the transition between different model organisms.

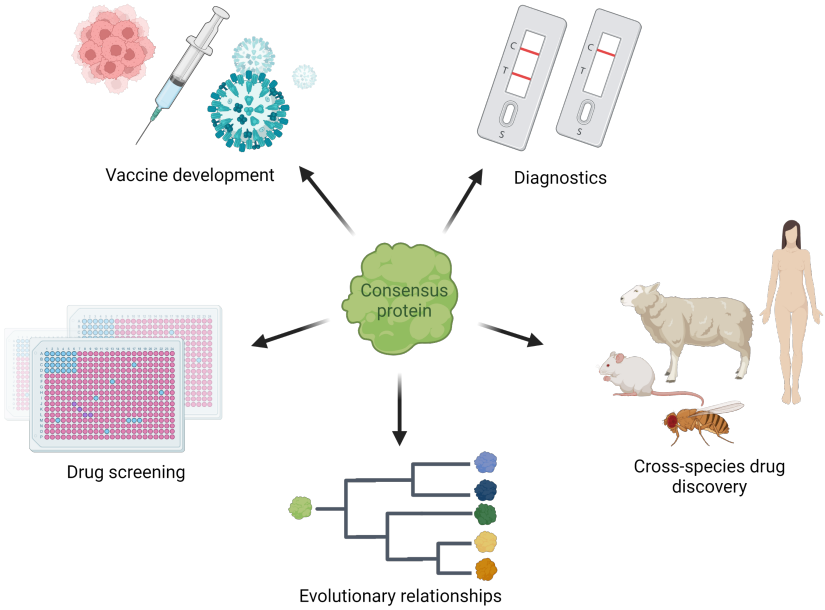

Figure 2. Broad application possibilities of consensus proteins. *Consensus proteins could offer a diverse range of applications, including the development of vaccines targeting cancers, bacteria, parasites, and viruses, where antigen variation is a challenge, as well as diagnostic tools capable of detecting various antigenic variations, thereby avoiding false negatives. Consensus proteins might potentially also serve as an effective tool to screen potential drug candidates when cross-reactivity is desired and to create antibodies with functionality across different species. Additionally, consensus proteins may enable the investigation of evolutionary relationships and ancestral proteins.*

Consensus proteins also present an opportunity in drug screening and discovery, particularly when applied in high-throughput screening assays. Acting as essential tools, they could aid in the identification of small molecules or other compounds that effectively interact with target proteins across related species or protein variants. Streamlining the initial screening steps, these proteins represent an average of multiple variants of the target protein, enabling researchers to focus on a single representative consensus protein. This strategy may potentially optimize the time and resources of potential drug candidates. The versatility of consensus proteins extends to the identification of compounds with cross-reactivity against diverse disease variants. This applicability broadens the potential scope of therapeutic agents, offering novel opportunities for addressing various diseases and medical conditions.

Finally, in the field of bioinformatics and comparative genomics, consensus proteins may also offer essential insights into natural selection. They can potentially provide hypothesis-driving data for studying protein evolution, identifying conserved domains, and establishing evolutionary relationships

among diverse species. By comparing the amino acid sequences of consensus proteins from various organisms, researchers can identify key mutations and sequence variations that have occurred over time. This information provides valuable clues about the evolutionary processes that have shaped these proteins and the organisms they belong to. This contributes to a deeper understanding of the genetic diversity and evolutionary history of organisms.

Chapter 7 – Conclusion and perspectives

This thesis marks a significant advancement in the field of recombinant toxin expression for antibody discovery and antivenom development. Notably, it marks the first-ever discovery of an antibody completely *in vitro* against a toxin from the animal kingdom. The use of consensus toxins for monoclonal antibody discovery was also a pioneering endeavor, marking the first time such an approach has been successfully employed. By focusing on heterologous expression of α-neurotoxins and consensus toxins and their use as antigens in antibody and nanobody discovery campaigns, the generation of broadly neutralizing and potent antibodies and nanobodies was achieved. Moreover, the potential of consensus proteins goes beyond snakebite envenoming, presenting opportunities in various fields where antibodies with cross-reactivity are advantageous.

However, there are still important milestones to be achieved. The optimal expression system for α-neurotoxins and other 3FTxs is yet to be developed. Further optimization is crucial to maximizing the production of pure, functional toxins for efficient antibody discovery and other applications.

In addition to their promising *in vitro* binding and neutralization capabilities, the true potential of the discovered nanobodies awaits full realization and validation *in vivo*. To thoroughly assess their effectiveness, *in vivo* experiments using animal models are essential, as they provide crucial insights into the dynamics and kinetics of neutralization and offer a deeper understanding of their therapeutic potential within living organisms.

By proposing a set of quality criteria for recombinant toxin expression, this thesis aims to streamline future research efforts and facilitate the discovery of effective antivenoms. It is imperative to continue investigating different expression strategies and purification methods to optimize the yield and functionality of recombinant toxins.

Within the realm of consensus proteins, the impact of my research extends beyond toxinology, encompassing various fields. Consensus proteins offer potential in vaccine development, inducing broader immune responses against infectious diseases with genetic variability. They hold promise in diagnostics, enabling rapid and accurate detection of infections or envenoming, leading to timely treatments. As therapeutic agents, they serve as targets for drug discovery, leading to novel drugs effective against diverse diseases. High-throughput screening assays with consensus proteins could potentially be used to expedite drug discovery and provide more robust therapeutic leads. Moreover, in

bioinformatics and comparative genomics, consensus proteins offer valuable data for studying protein evolution and establishing evolutionary relationships among species. Furthermore, advancements in automation and *in silico* design techniques could streamline and enhance the process of designing and utilizing consensus proteins, making the development of diagnostics and therapeutics even more efficient, potentially even eliminating the need for expression and purification. The concepts developed in the work behind this thesis thus hold transformative potential for addressing critical global health challenges and driving innovative solutions across several scientific disciplines.

In conclusion, this thesis has advanced the field of modern snakebite antivenom research with the discovery of antibodies against toxins entirely *in vitro* and has hinted towards the vast potential of consensus proteins across diverse scientific domains. These new insights offer hope that safer and more effective antivenom therapies can be developed, thereby improving human and animal health and well-being. There is still much work to be conducted, but with dedication and collaboration, we hope to pave the way for a future with safer and more effective therapies against snakebite envenoming, and potentially other devastating diseases, bringing hope to vulnerable patients and populations worldwide.

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