



## Characterisation of two snake toxin-targeting human monoclonal immunoglobulin G antibodies expressed in tobacco plants

**Moore, Catherine M.; Ljungars, Anne; Paul, Matthew J.; Dahl, Camilla Holst; Ahmadi, Shirin; Adams, Anna Christina; Grav, Lise Marie; Schoffelen, Sanne; Voldborg, Bjørn Gunnar; Laustsen, Andreas Hougaard**

*Total number of authors:*  
11

*Published in:*  
Toxicon

*Link to article, DOI:*  
[10.1016/j.toxicon.2023.107225](https://doi.org/10.1016/j.toxicon.2023.107225)

*Publication date:*  
2023

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

### *Citation (APA):*

Moore, C. M., Ljungars, A., Paul, M. J., Dahl, C. H., Ahmadi, S., Adams, A. C., Grav, L. M., Schoffelen, S., Voldborg, B. G., Laustsen, A. H., & Ma, J. K-C. (2023). Characterisation of two snake toxin-targeting human monoclonal immunoglobulin G antibodies expressed in tobacco plants. *Toxicon*, 232, Article 107225. <https://doi.org/10.1016/j.toxicon.2023.107225>

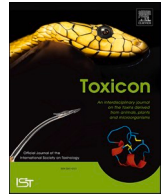
---

### General rights

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

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

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



## Characterisation of two snake toxin-targeting human monoclonal immunoglobulin G antibodies expressed in tobacco plants

Catherine M. Moore<sup>a,\*</sup>, Anne Ljungars<sup>b</sup>, Matthew J. Paul<sup>c</sup>, Camilla Holst Dahl<sup>b</sup>, Shirin Ahmadi<sup>b</sup>, Anna Christina Adams<sup>b</sup>, Lise Marie Grav<sup>b</sup>, Sanne Schoffelen<sup>b</sup>, Bjørn Gunnar Voldborg<sup>b</sup>, Andreas Hougaard Laustsen<sup>b,\*\*</sup>, Julian K-C Ma<sup>c</sup>

<sup>a</sup> School of Life Sciences, College of Liberal Arts and Sciences, University of Westminster, London, W1W 6UW, United Kingdom

<sup>b</sup> Department of Biotechnology and Biomedicine, Technical University of Denmark, Kongens Lyngby, Denmark

<sup>c</sup> Hotung Molecular Immunology Unit, Institute for Infection & Immunity, St George's University of London, Cranmer Terrace, London, SW17 0RE, United Kingdom

### ARTICLE INFO

Handling Editor: Denise Tambourgi

#### Keywords:

Snakebite envenoming  
Snake toxins  
Monoclonal antibodies  
Recombinant antibody expression  
Plant-based production

### ABSTRACT

Current snakebite antivenoms are based on polyclonal animal-derived antibodies, which can neutralize snake venom toxins in envenomed victims, but which are also associated with adverse reactions. Therefore, several efforts within antivenom research aim to explore the utility of recombinant monoclonal antibodies, such as human immunoglobulin G (IgG) antibodies, which are routinely used in the clinic for other indications. In this study, the feasibility of using tobacco plants as bioreactors for expressing full-length human monoclonal IgG antibodies against snake toxins was investigated. We show that the plant-produced antibodies perform similarly to their mammalian cell-expressed equivalents in terms of *in vitro* antigen binding. Complete neutralization was achieved by both the plant and mammalian cell-produced anti- $\alpha$ -cobratoxin antibody. The feasibility of using plant-based expression systems may potentially make it easier for laboratories in resource-poor settings to work with human monoclonal IgG antibodies.

### 1. Introduction

Snakebite envenoming continues to claim tens of thousands of lives globally every year and leaves many more maimed for life (Gutiérrez et al., 2017). While antivenoms have existed for more than 125 years and remain the mainstay of snakebite envenoming therapy (Pucca et al., 2019), newer approaches, involving monoclonal antibodies, nanobodies, and recombinant DNA technology, are being pursued in the attempt to develop improved antivenom products (Laustsen et al., 2016; Casewell et al., 2020; Laustsen, 2021). Some of the more promising attempts involve the use of phage display technology for the discovery of human monoclonal immunoglobulin G (IgG) antibodies (Ledsgaard et al., 2022, 2023; Miersch et al., 2022) and single-domain antibody constructs (Richard et al., 2013; Bailon Calderon et al., 2020) with superior neutralization capacities (Ljungars and Laustsen, 2023), which have been speculated to be more compatible with the human immune system compared to conventional animal plasma-derived antivenoms (Laustsen et al., 2018a; Knudsen et al., 2019; Hamza et al., 2021).

Thereby, some of the drawbacks with conventional antivenoms, including their associated risk of adverse reactions, batch-to-batch variation, dependence on venom for manufacture, low amount of therapeutically active antibodies, and high cost can be addressed.

While the discovery, characterization, and preclinical assessment of snake toxin-targeting monoclonal antibodies have received increasing attention in the last decade (Pucca et al., 2019), only few efforts have involved the use of full-length IgG antibodies. One of the reasons for this is that the cost of development and manufacture of IgG antibodies using industry standard mammalian cell cultivation systems has so far largely limited their accessibility to those in developed countries. In the case of snakebite envenoming therapy, a more costly combination of multiple monoclonal antibodies would be required to effectively neutralize the many toxins of medical importance that are typically present in a snake venom (Casewell et al., 2020). Furthermore, snakebite envenoming is primarily a problem in low- and middle-income countries (LMICs) for resource-poor communities (Gutiérrez et al., 2017). To this end, plant biotechnology has been proposed as an alternative solution to the use of mammalian cell cultivation to express snake toxin-neutralizing

\* Corresponding author.

\*\* Corresponding author.

E-mail addresses: [c.moore@westminster.ac.uk](mailto:c.moore@westminster.ac.uk) (C.M. Moore), [ahola@bio.dtu.dk](mailto:ahola@bio.dtu.dk) (A.H. Laustsen).

<https://doi.org/10.1016/j.toxicon.2023.107225>

Received 4 May 2023; Received in revised form 6 July 2023; Accepted 10 July 2023

Available online 11 July 2023

0041-0101/© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

## Abbreviations

$\alpha$ -cbtx	$\alpha$ -cobratoxin
DELFA	Dissociation-Enhanced Lanthanide Fluorescence Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
HRP	Horse Radish Peroxidase
IgG	Immunoglobulin G
OD	Optical Density
PBS	Phosphate-Buffered Saline
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
RLU	Relative Light Units
RU	Response Units
SDS PAGE	Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis
SPR	Surface Plasmon Resonance

antibodies (Murad et al., 2020; Julve Parreño et al., 2018). Here, plants like tobacco are used as living bioreactors in a low-tech approach, involving either stable transgenic plants or transient expression in plants (Moore et al., 2021; van Dolleweerd et al., 2014; Dent et al., 2016; Hull et al., 2005; Komarova et al., 2011; Gomes et al., 2019; Souza et al., 2020), either of which would be amenable to transfer to facilities in resource-poor settings.

In this study, the feasibility of expressing snake toxin-targeting antibodies in tobacco plants was investigated. We studied two antibodies targeting  $\alpha$ -cobratoxin ( $\alpha$ -cbtx) and a phospholipase A<sub>2</sub> (PLA<sub>2</sub>), which derive from the venoms of *Naja kaouthia* and *Naja nigricollis*, respectively.  $\alpha$ -cbtx causes paralysis by blocking the binding of acetylcholine to the nicotinic acetylcholine receptor (Laustsen et al., 2015; Alkondon and Albuquerque, 1990), while the PLA<sub>2</sub> disrupts cell membranes leading to severe tissue damage (Lauridsen et al., 2017). In this work, plant-produced full-length monoclonal IgG antibodies were expressed, characterised, and compared to the same antibodies produced by a mammalian cell expression system, by examining antigen binding, binding kinetics, and blocking of  $\alpha$ -cbtx binding to the nicotinic acetylcholine receptor. Thereby, we showcase that both expression systems can be used to produce functional human monoclonal IgG antibodies against snake toxins.

## 2. Material and methods

### 2.1. Toxins

The PLA<sub>2</sub>-containing fraction Nn19 was prepared from the whole venom of *N. nigricollis* using RP-HPLC as described previously (Ahmadi et al., 2020) and used as antigen in the experiments. Whole venom of *Naja nigricollis* and the purified  $\alpha$ -cobratoxin ( $\alpha$ -cbtx) (L8114) from *N. kaouthia* venom were purchased from Latoxan SAS, France. Toxins were biotinylated using biotin linked N-hydroxysuccinimide as described previously (Ahmadi et al., 2020).

### 2.2. Antibodies

The TPL004\_01\_A11 (anti-PLA<sub>2</sub>) antibody was discovered using phage display technology where a naïve human antibody library was used for selection on biotinylated PLA<sub>2</sub> (fraction Nn19) in a similar campaign as described by Ahmadi et al. (2020). The discovery of 2554\_01\_D11 (anti- $\alpha$ -cbtx) has been described previously (Ledsgaard et al., 2023). Briefly, a light chain shuffled library, constructed from a scFv binding a toxin ( $\alpha$ -elapitoxin) that share high homology to  $\alpha$ -cbtx, was used for selection on biotinylated  $\alpha$ -cbtx. The control IgG mAb used was a commercially-sourced human IgG1 kappa (Sigma). Protein

sequences are provided in Supplementary Material.

### 2.3. Production of antibodies in mammalian cells

The antibodies TPL004\_01\_A11 and 2554\_01\_D11 were produced in mammalian cells as described previously (Ledsgaard et al., 2023). Briefly, CHO-S cell lines stably expressing the respective IgGs were cultured for 144 h followed by harvest of the supernatant and purification of IgGs on a MabSelect column (Cytiva).

### 2.4. Cloning of antibodies for plant expression

The DNA sequences for the variable heavy and light chain coding regions of TPL004\_01\_A11 and 2554\_01\_D11 were codon-optimised for *Nicotiana benthamiana* and synthesised by GeneArt (Thermo Fisher Scientific). The heavy and light chain sequences were inserted into the pTRAK.6 vector using the MIDAS cloning system (Pinneh et al., 2022). Briefly, heavy and light chain variable regions were ligated into the donor plasmids containing the human IgG1 constant domains, using golden gate cloning (BsaI). The donor plasmids were then digested with NcoI/XbaI and ligated into pWhite and pBlue entry vectors respectively, and then both inserted, using golden gate cloning (BsaI/BsmBI), into the pTRAK.6 *Agrobacterium* binary vector. The pTRAK.6 vectors were used to transform *Agrobacterium tumefaciens* strain GV3101:pM90RK by electroporation (Pinneh et al., 2022).

### 2.5. *N. benthamiana* infiltration

*N. benthamiana* plants were germinated and maintained in the greenhouse with a 16/8-h day/night cycle at 24–28 °C and infiltrated with recombinant agrobacteria after 4–6 weeks. Infiltrations were carried out as described previously (Moore et al., 2021; Teh et al., 2014). Briefly, recombinant *Agrobacterium tumefaciens* were grown until an OD<sub>600</sub> nm of >1 was achieved. The bacteria were then resuspended in infiltration solution (10 mM MgCl<sub>2</sub>, 10 mM MES) at an OD<sub>600</sub> nm of 0.1 and incubated at room temperature for a minimum of 30 min with 200  $\mu$ M acetosyringone. Plants were infiltrated manually using a syringe or by vacuum infiltration for larger scale expression. Five days after the infiltration, the plant leaves were harvested.

### 2.6. Antibody purification

Antibodies were purified from plants as described previously (Webster et al., 2018). Briefly, plant tissue was homogenised in a Waring blender and filtered through miracloth (Sigma) to remove plant debris. The filtrate was centrifuged for 40 min at 16,000 g, before sterilising the supernatant by filtration through a 0.22  $\mu$ m filter. Antibodies in the filtrate were purified using affinity chromatography on a Protein A agarose column (Sigma). Eluates were dialysed against PBS (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) overnight at 4 °C and concentrated using 100k Centricon® centrifugal filters. Antibodies were filter-sterilised and the concentration determined by measuring A<sub>280</sub> using a Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific) before storage at –80 °C.

### 2.7. SDS-PAGE and western blots

All SDS-PAGE gels and western blots were performed following the Invitrogen NuPAGE manufacturer's instructions (NuPAGE). 4–12% Bis-Tris SDS-PAGE gels were run in MOPS buffer and stained with InstantBlue® Coomassie Protein Stain (Abcam). For western blots, the gels were blotted onto nitrocellulose and blocked with LI-COR® Odyssey® PBS blocking buffer. Antibodies were detected with IRDye® 800CW tagged goat anti-human IgG secondary antibody, diluted 1:10,000 (LI-COR®). Blots were visualised using the LI-COR® Odyssey® CLx scanner and analysed using Image Studio.

## 2.8. Antibody concentration ELISA

To estimate the concentration of the purified antibodies, sandwich ELISAs were performed as previously described (Webster et al., 2018). Briefly, ELISA plates were coated with anti-human IgG1 Fc antiserum (The Binding Site) and blocked with PBS + 5% skimmed milk powder. Plant antibody samples were diluted ten-fold and added in a two-fold dilution series and as positive control, a human IgG1 mAb (Sigma) (500 ng/mL) was used. A plant extract from a non-infiltrated *N. benthamiana* plant was included as the negative control. Incubation was performed for a minimum of 2 h at 37 °C. Bound antibodies were detected using anti-human IgG1( $\kappa$ ) antiserum conjugated with HRP (The Binding Site), diluted in PBS + 5% skimmed milk powder. Developing solution (3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate) (Sigma) was added and incubated until colour development was complete, and the reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub>. Plates were read in a Tecan Infinite F200 Pro. Data were analysed and concentrations calculated with GraphPad Prism 9 using the Michaelis-Menten equation for curve fitting.

## 2.9. Antigen binding ELISA

To assess antigen recognition, antigen binding ELISAs were performed. ELISA plates were coated overnight at 4 °C with 10  $\mu$ g/mL of Streptavidin (Thermo Fisher Scientific) in PBS (50  $\mu$ L/well). Biotinylated toxins were added at 5  $\mu$ g/mL (60  $\mu$ L/well) in PBS + 3% skimmed milk powder, and left to bind for 1 h at room temperature. Purified antibody preparations were diluted to 10  $\mu$ g/mL in PBS + 3% skimmed milk powder and titrated 1:2 before they were added to the washed plates. After washing, bound antibodies were detected using  $\alpha$ -human-IgG-HRP (Jackson ImmunoResearch) diluted to 0.05  $\mu$ g/mL in PBS + 3% skimmed milk powder. Substrate (Pico Supersignal, Pierce, Thermo Fisher Scientific) was diluted 1:10 in TRIS buffer, 50  $\mu$ L/well was added to the washed plate, and luminescence was read in a Victor Nivo plate reader after a 10 min incubation at room temperature.

## 2.10. Binding kinetics

Surface plasmon resonance (SPR) was employed to calculate binding kinetics, according to Stelter et al. (2020), using a BIAcore™ X-100 instrument (GE healthcare). All proteins were diluted in HBS-EP + buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P-20). Protein A (Sigma) was immobilised onto a CM5 chip in two flow channels with standard amine coupling to 1000 response units (RU). Thereafter, plant or mammalian cell-produced antibodies were bound to the active flow channel (Fc2) to 1000 RU. The antigens,  $\alpha$ -cbtx or the PLA<sub>2</sub>, were passed over both flow channels at different concentrations in each cycle (PLA<sub>2</sub> containing Nn19: 1000, 333.3, 111.1, 37.0, and 12.3 nM.  $\alpha$ -cbtx: 100, 33.3, 11.1, 3.7, and 1.23 nM) at 30  $\mu$ L/min for 50 s ( $\alpha$ -cbtx) and 100 s (PLA<sub>2</sub>). Dissociation was thereafter followed for 400 s. Buffer only was included as a blank control. The chip was regenerated between each cycle with 10 mM glycine-HCl (pH 1.5) for 30 s. All reference and blank subtracted sensorgrams were fitted to Langmuir model of binding (1:1) using BIAcore™ Evaluation software.

## 2.11. Receptor blocking DELFIA

The blocking of  $\alpha$ -cbtx binding to the nicotinic acetylcholine receptor was performed as described previously (Ledsgaard et al., 2022). Briefly, 5  $\mu$ g/mL (100  $\mu$ L/well) of the nicotinic acetylcholine receptor was coated onto plates overnight. A fixed concentration of biotinylated  $\alpha$ -cbtx (0.1  $\mu$ g/mL) was pre-incubated with different concentrations of IgG 2554\_01\_D11. After addition to the washed plate, bound biotinylated  $\alpha$ -cbtx was detected using europium-conjugated streptavidin followed by addition of enhancement solution and reading of fluorescence in a Victor Nivo plate reader.

## 3. Results

### 3.1. Expression and yield of plant produced antibodies

TPL004\_01\_A11 and 2554\_01\_D11 were plant codon-optimised, expressed in *N. benthamiana*, and after extraction, the yields of the antibodies were estimated to be 24.3 mg/kg and 4.0 mg/kg, respectively, calculated from the sandwich ELISA and A<sub>280</sub> measurements. To assess purity and quality, the antibodies were run on an SDS-PAGE gel (Fig. 1A) under both non-reducing and reducing conditions. For both plant expressed antibodies, under non-reducing conditions, the predominant band at approximately 150 kDa (top band) is the expected size for fully assembled IgG (black arrow). Under reducing conditions, two major bands were present at approximately 50 kDa and 25 kDa, the expected sizes for heavy and light chains, respectively (red and blue arrows). The fully assembled 150 kDa antibody is recognized by anti-human IgG antiserum in western blot (Fig. 1B). A number of smaller, much weaker bands are also detected, representing either assembly intermediates or degradation products of the antibody. A commercial purified human IgG1 $\kappa$  monoclonal antibody was included as positive control and showed similar results. Assembly of light and heavy chains was confirmed using a sandwich ELISA, where anti-heavy chain antiserum was used to capture the antibodies and anti-light chain antiserum was used for detection (Fig. 1C and D).

### 3.2. Plant and mammalian cell-produced antibodies show similar binding to their antigens

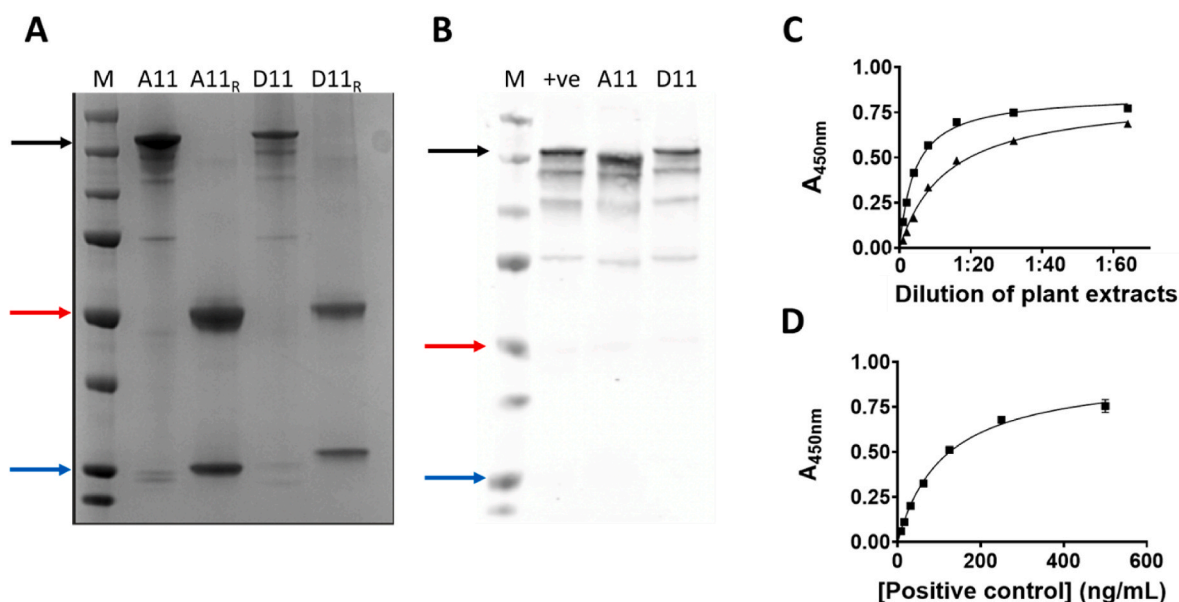
To confirm antigen binding, the plant and mammalian cell-produced antibodies were assessed side-by-side in ELISA experiments. As expected, TPL004\_01\_A11 and 2554\_01\_D11 bound to the PLA<sub>2</sub> and  $\alpha$ -cbtx, respectively, and similar binding was seen for the antibodies produced in plants and mammalian cells (Fig. 2A and B). Furthermore, the antigen binding kinetics of plant and mammalian cell-produced TPL004\_01\_A11 and 2554\_01\_D11 was compared using surface plasmon resonance. The affinity constants were calculated using the Langmuir 1:1 model of binding and were 1.3-times higher for plant-produced versus mammalian cell-produced antibody TPL004\_01\_A11, and identical for the plant-produced and mammalian cell-produced versions of antibody 2554\_01\_D11 (Fig. 3 A-D and Table 1).

### 3.3. Plant and mammalian-cell expressed 2554\_01\_D11 antibodies inhibit $\alpha$ -cbtx from binding to the nicotinic acetylcholine receptor

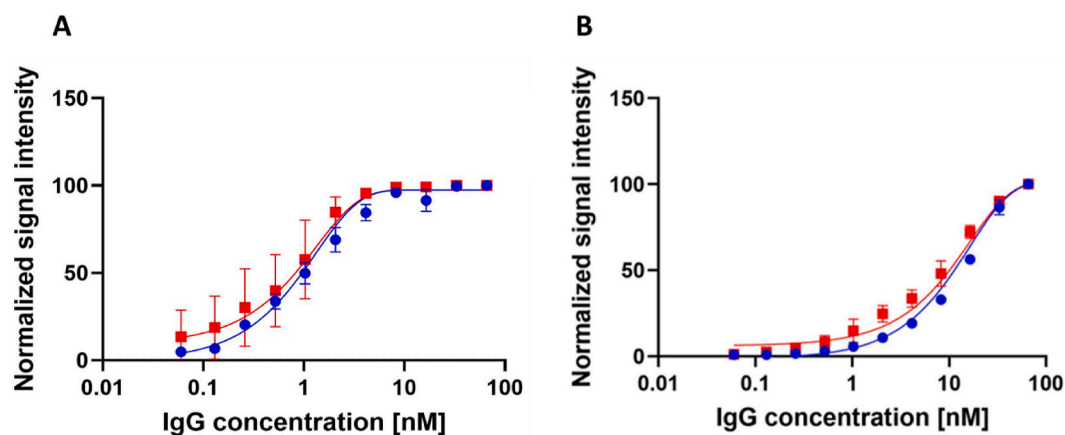
The mammalian cell-produced version of the antibody 2554\_01\_D11 is known to neutralize the lethal effects of  $\alpha$ -cbtx by inhibiting toxin binding to the nicotinic acetylcholine receptor *in vivo* (Ledsgaard et al., 2023). Therefore, in addition to antigen binding, the plant and mammalian-cell produced versions of this antibody were also tested for their ability to block  $\alpha$ -cbtx binding to the nicotinic acetylcholine receptor in a DELFIA assay. Both the plant and mammalian cell-produced antibodies fully inhibited the binding of  $\alpha$ -cbtx to the receptor. However, the mammalian cell-produced antibody was more potent and could be used at around four times lower concentrations (Fig. 4).

## 4. Discussion

Animal plasma-derived antivenoms remain the mainstay of envenoming therapy even though they are associated with adverse reactions. Due to this and other drawbacks, new therapeutic approaches relying on recombinantly expressed monoclonal antibodies are currently being investigated (Ledsgaard et al., 2023; Richard et al., 2013; Bailon Calderon et al., 2020; Laustsen et al., 2018b). Traditionally, recombinant proteins, such as IgG antibodies, are produced using mammalian cell cultivation (Walsh and Walsh, 2022). However, such expression systems are technically challenging and come with a relatively high cost. An



**Fig. 1.** Characterisation of monoclonal antibodies, TPL004\_01\_A11 (A11) and 2554\_01\_D11 (D11), produced in *N. benthamiana*. (A) SDS-PAGE of antibodies under non-reducing or reducing (R) conditions. (B) Western blot of antibodies with positive control (+ve) human IgG1 (100 ng). Marker (M) for SDS-PAGE and western blot was Precision Plus Protein™ All Blue Pre-stained Protein Standards. Black, red, and blue arrows indicate 150 kDa, 50 kDa, and 25 kDa (the expected sizes for fully assembled antibody, and heavy and light chains), respectively. Detection was with IRDye® 800CW goat anti-human IgG secondary antibody diluted 1:10,000 (LI-COR®). (C) Sandwich ELISA detecting fully assembled antibodies, TPL004\_01\_A11 (triangles) and 2554\_01\_D11 (squares), from plant extracts. Extracts were initially diluted 1:10, and then titrated 2-fold on the plate. (D) Positive control is human IgG1 (squares). ELISA plate was coated with goat anti-human IgG Fc domain antibody. Bound antibodies were detected using HRP-conjugated goat anti-human IgG kappa light chain antibody. Each ELISA was performed with 2 technical replicates. Means derived from two leaf disks per sample. Curves were calculated using GraphPad Prism software using the Michaelis-Menten equation for curve fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

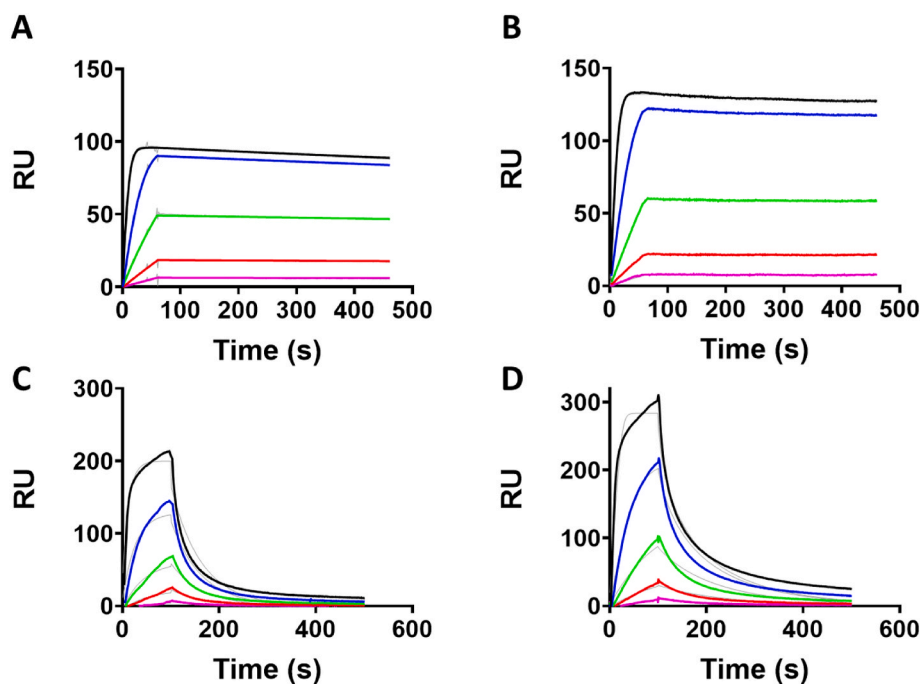


**Fig. 2.** Binding of plant (blue circles) and mammalian cell-produced (red squares) antibodies to their cognate antigens measured by ELISA. (A) 2554\_01\_D11 binding to  $\alpha$ -cbtX. (B) TPL004\_01\_A11 binding to the PLA<sub>2</sub>. Antibodies were diluted to 100 nM and titrated 1:2 followed by addition to biotinylated antigens bound to a streptavidin coated plate. Antibody binding was detected using an HRP-conjugated anti-human antibody followed by addition of a luminescent substrate. Samples were run as duplicates and error bars show the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

alternative solution, which is being developed for an increasing number of antibodies, is plant-based expression – a relatively simple technology that only requires modest investment in infrastructure and has a low setup cost. Other potential benefits that have been cited with manufacturing facilities in less developed regions in mind are the local availability of the main raw materials (light energy, growth media for plants, and water), and that upstream manufacturing is not completely dependent on a steady power supply, clean water, and clean air (Murad et al., 2020). These factors may make plant-based expression attractive to investors in LMICs (Murad et al., 2020), as evidenced by the recent emergence of Cape Bio Pharms in South Africa and Baiya Phytopharm in

Thailand. In these cases, the establishment of local manufacturing has also allowed a targeting of local endemic needs, which may not normally be prioritised by global biologics manufacturers.

Here, we demonstrate the successful use of *N. benthamiana* as an expression host for two human monoclonal IgG antibodies against snake toxins. The antibodies produced in tobacco showed specific recognition for their target antigens with binding affinities that were comparable to their mammalian cell-expressed equivalents. One of the antibodies, 2554\_01\_D11, has previously been shown to neutralize  $\alpha$ -cbtX *in vitro* as well as the lethal effects of this toxin *in vivo* (Ledsgaard et al., 2023). In this work, neutralization of  $\alpha$ -cbtX was assessed *in vitro* by analysing the

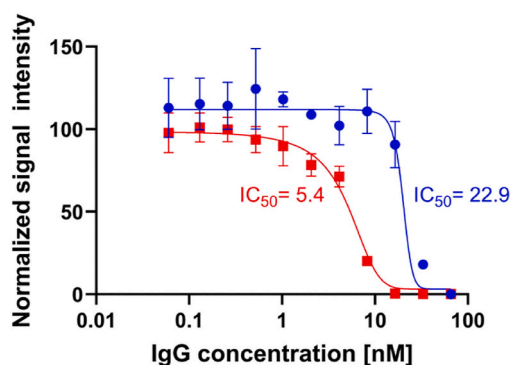


**Fig. 3.** Sensorgrams showing the binding kinetics of plant-produced (A, C) versus mammalian cell-produced antibodies (B, D) to  $\alpha$ -cbtx and the PLA<sub>2</sub>, respectively. Protein A was immobilised onto a CM5 chip and antibodies were captured to 1000 response units (RU). Antigens were passed over antibody-coated chips. Curves were fitted to the Langmuir model of binding (1:1). Antigen concentrations were as follows:  $\alpha$ -cbtx 100 nM (black), 33.3 nM (blue), 11.1 nM (green), 3.7 nM (red), and 1.23 nM (magenta); PLA<sub>2</sub> 1000 nM (black), 333.3 nM (blue), 111.1 nM (green), 37.0 nM (red), and 12.3 nM (magenta). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**

Association constant, dissociation constant, and affinity for plant and mammalian cell-produced antibodies binding to  $\alpha$ -cbtx and the PLA<sub>2</sub>, respectively. Affinity constants were calculated using BIAcore Evaluation software.

Antibody	Antigen	$k_a$ (1/(M×s))	$k_d$ (1/s)	$K_D$ (nM)
Plant 2554_01_D11	$\alpha$ -cbtx	$2.0 \times 10^6$	$2.0 \times 10^{-4}$	0.1
Mammalian 2554_01_D11	$\alpha$ -cbtx	$2.0 \times 10^6$	$1.1 \times 10^{-4}$	0.1
Plant TPL004_01_A11	PLA <sub>2</sub>	$6.6 \times 10^4$	0.02	244.0
Mammalian TPL004_01_A11	PLA <sub>2</sub>	$1.5 \times 10^6$	0.28	187.0



**Fig. 4.** Inhibition of  $\alpha$ -cbtx binding to nicotinic acetylcholine receptor by plant (blue circles) or mammalian cell-produced (red squares) 2554\_01\_D11. Plates were coated overnight with 5  $\mu$ g/mL of nicotinic acetylcholine receptor. After washing, IgG pre-incubated with biotinylated  $\alpha$ -cbtx was added to the plate and bound  $\alpha$ -cbtx was detected using europium conjugated streptavidin followed by addition of an enhancement solution. Samples were run as duplicates and error bars show the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ability of the produced antibodies to inhibit the binding of the toxin to the nicotinic acetylcholine receptor in a DELFIA assay. Both plant and mammalian cell-produced antibodies were able to completely inhibit this binding. However, higher concentrations of the plant-produced antibody appeared to be required, compared to the mammalian

cell-produced equivalent. This finding needs to be confirmed and explained. Whilst a four-fold difference in potency to inhibit binding of  $\alpha$ -cbtx to its receptor was determined, this was not reflected in reduced IgG binding to  $\alpha$ -cbtx or the binding kinetics, measured by SPR. In SPR, the observed binding to  $\alpha$ -cbtx could be fitted to a 1:1 binding model, and kinetic binding parameters representing the interaction could be calculated with a high degree of confidence. However, the observed binding data to the PLA<sub>2</sub> fits less precisely to the expected 1:1 binding model, resulting in some uncertainty in the calculated parameters ( $k_a$  and  $k_d$ ). Therefore, the relatively small difference in the calculated  $K_D$ s may not necessarily reflect inherent differences between the binding kinetics of the plant and mammalian cell-produced anti-PLA<sub>2</sub> antibodies.

In 2017, the World Health Organisation reinstated snakebite envenoming to its list of category A neglected tropical diseases (NTDs) ([The Lancet, 2017](#)). Despite the huge unmet need for new and better products to replace existing treatments that can be expensive to manufacture, are associated with serious adverse reactions, and are not always effective, there are many aspects surrounding antivenom products, which make them unattractive for financial investment ([Rappuoli et al., 2002](#)). One of these aspects is that snakebite envenoming is primarily a disease of poverty ([Harrison et al., 2009](#)), meaning that in the majority of cases, those victims that require treatment are the least likely to be able to pay, and they often live in regions with limited national healthcare. Furthermore, a snakebite therapeutic product would need to have geographic specificity, as different geographic regions are hosts to different snake species and venoms ([Casewell et al., 2020](#)). Therefore, it is infeasible to develop one universal antivenom product with global coverage of all snake species.

To improve snakebite envenoming therapy, new approaches and technologies are undoubtedly necessary ([Laustsen, 2021](#); [Williams et al., 2019](#)). To this end, antibody technologies may provide advantages by enabling the discovery and optimisation of snake toxin-targeting monoclonal antibodies and antibody fragments with improved neutralization capacity ([Ljungars and Laustsen, 2023](#)), broader specificities ([Pucca et al., 2019](#); [Ledsgaard et al., 2023](#)), improved product safety, and low batch-to-batch variation ([Knudsen et al., 2019](#)). But to unlock the potential of such molecules, manufacturing technologies that are affordable and accessible will also be critical ([Laustsen and](#)

Dorrestijn, 2018; Jenkins and Laustsen, 2020; Laustsen et al., 2017). Various approaches are being explored, including the simplification of the recombinant antivenom products by engineering antibody fragments or, as described here, by developing alternative expression technologies.

#### Credit author statement

Catherine M. Moore: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Roles/Writing – original draft; Writing – review & editing, Anne Ljungars: Conceptualization; Visualization; Roles/Writing – original draft; Writing – review & editing, Matthew J. Paul: Formal analysis; Data curation; Investigation; Methodology; Writing – review & editing, Camilla Holst Dahl: Data curation; Investigation; Methodology; Writing – review & editing, Shirin Ahmadi: Data curation; Investigation; Methodology; Formal analysis; Writing – review & editing, Anna Christina Adams: Data curation; Investigation; Methodology; Writing – review & editing, Lise Marie Grav: Data curation; Investigation; Methodology; Writing – review & editing, Sanne Schoffelen: Data curation; Investigation; Methodology; Writing – review & editing, Bjørn Gunnar Voldborg: Data curation; Investigation; Methodology; Writing – review & editing, Andreas Hougaard Laustsen: Funding acquisition; Project administration; Resources; Software; Supervision; Validation: Conceptualization; Roles/Writing – original draft; Writing – review & editing, Julian K-C. Ma: Funding acquisition; Project administration; Resources; Software; Supervision; Validation: Conceptualization; Roles/Writing – original draft; Writing – review & editing.

#### Ethical statement

No animal or human participants were involved in this study, thus no ethical considerations are relevant.

#### 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.

#### Acknowledgments

The authors would like to thank Karen Kathrine Brøndum and Daniel Duun from National Biologics Facility for the mammalian production of IgGs. A.H.L gratefully acknowledges funding from the Villum Foundation (00025302), the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (850974), and Wellcome (221702/Z/20/Z). B.V., A.C.A, S.S. and L.M.G. gratefully acknowledge funding from the Novo Nordisk Foundation (NNF20SA0066621) J.M. gratefully acknowledges support from the Hotung Charitable Foundation for this work. The research was also supported by EU Horizon 2020 (Grant Agreement No. 774078, PharmaFactory and Grant Agreement No. 760331, Newcotiana).

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxicol.2023.107225>.

#### References

- Ahmadi, S., Pucca, M.B., Jürgensen, J.A., Janke, R., Ledsgaard, L., Schoof, E.M., Sørensen, C.V., Çalişkan, F., Laustsen, A.H., 2020. An *in vitro* methodology for discovering broadly-neutralizing monoclonal antibodies. *Sci. Rep.* 10, 10765.
- Alkondon, M., Albuquerque, E.X., 1990.  $\alpha$ -Cobratoxin blocks the nicotinic acetylcholine receptor in rat hippocampal neurons. *Eur. J. Pharmacol.* 191, 505–506.
- Bailon Calderon, H., Yaniro Coronel, V.O., Cáceres Rey, O.A., Colque Alave, E.G., Leiva Duran, W.J., Padilla Rojas, C., Montejo Arevalo, H., García Neyra, D., Galarza Pérez, M., Bonilla, C., et al., 2020. Development of nanobodies against hemorrhagic and myotoxic components of *Bothrops atrox* snake venom. *Front. Immunol.* 11, 655.
- Casewell, N.R., Jackson, T.N.W., Laustsen, A.H., Sunagar, K., 2020. Causes and consequences of snake venom variation. *Trends Pharmacol. Sci.* 41, 570–581.
- Dent, M., Hurtado, J., Paul, A.M., Sun, H., Lai, H., Yang, M., Esqueda, A., Bai, F., Steinkellner, H., Chen, Q., 2016. Plant-produced anti-dengue virus monoclonal antibodies exhibit reduced antibody-dependent enhancement of infection activity. *J. Gen. Virol.* 97, 3280–3290.
- Gomes, M., Alvarez, M.A., Quellis, L.R., Becher, M.L., Castro, J.M. de A., Gameiro, J., Caporino, M.C., Moura-da-Silva, A.M., de Oliveira Santos, M., 2019. Expression of an scFv antibody fragment in *Nicotiana benthamiana* and *in vitro* assessment of its neutralizing potential against the snake venom metalloproteinase BaP1 from *Bothrops asper*. *Toxicol.* 160, 38–46.
- Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017. Snakebite envenoming. *Nat. Rev. Dis. Prim.* 3, 17063.
- Hamza, M., Knudsen, C., Gnanathasan, C.A., Monteiro, W., Lewin, M.R., Laustsen, A.H., Habib, A.G., 2021. Clinical management of snakebite envenoming: future perspectives. *Toxicol X* 11, 100079.
- Harrison, R.A., Hargreaves, A., Wagstaff, S.C., Faragher, B., Laloo, D.G., 2009. Snake envenoming: a disease of poverty. *PLoS Neglected Trop. Dis.* 3, e569.
- Hull, A.K., Criscuolo, C.J., Mett, V., Groen, H., Steeman, W., Westra, H., Chapman, G., Legutkí, B., Baillie, L., Yusibov, V., 2005. Human-derived, plant-produced monoclonal antibody for the treatment of anthrax. *Vaccine* 23, 2082–2086.
- Jenkins, T.P., Laustsen, A.H., 2020. Cost of manufacturing for recombinant snakebite antivenoms. *Front. Bioeng. Biotechnol.* 8, 703.
- Julve Parreño, J.M., Huet, E., Fernández-Del-Carmen, A., Segura, A., Venturi, M., Gandía, A., Pan, W.-S., Albaladejo, I., Forment, J., Pla, D., et al., 2018. A synthetic biology approach for consistent production of plant-made recombinant polyclonal antibodies against snake venom toxins. *Plant Biotechnol. J.* 16, 727–736.
- Knudsen, C., Ledsgaard, L., Dehli, R.L., Ahmadi, S., Sørensen, C.V., Laustsen, A.H., 2019. Engineering and design considerations for next-generation snakebite antivenoms. *Toxicol.* 167, 67–75.
- Komarova, T.V., Kosorukov, V.S., Frolova, O.Y., Petrunia, I.V., Skrypnik, K.A., Gleba, Y. Y., Dorokhov, Y.L., 2011. Plant-made trastuzumab (herceptin) inhibits HER2/Neu+ cell proliferation and retards tumor growth. *PLoS One* 6, e17541.
- Lauridsen, L.P., Laustsen, A.H., Lomonte, B., Gutiérrez, J.M., 2017. Exploring the venom of the forest cobra snake: toxicovenomics and antivenom profiling of *Naja melanoleuca*. *J. Proteomics* 150, 98–108.
- Laustsen, A.H., 2021. Antivenom in the age of recombinant DNA technology. In: *Handbook of Venoms and Toxins of Reptiles*, second ed. CRC Press, Boca Raton, pp. 499–510. CRC Press; 2021.
- Laustsen, A., Dorrestijn, N., 2018. Integrating engineering, manufacturing, and regulatory considerations in the development of novel antivenoms. *Toxins* 10, 309.
- Laustsen, A.H., Gutiérrez, J.M., Lohse, B., Rasmussen, A.R., Fernández, J., Milbo, C., Lomonte, B., 2015. Snake venomomics of monocled cobra (*Naja kaouthia*) and investigation of human IgG response against venom toxins. *Toxicol.* 99, 23–35.
- Laustsen, A.H., Engmark, M., Milbo, C., Johannesen, J., Lomonte, B., Gutiérrez, J.M., Lohse, B., 2016. From fangs to pharmacology: the future of snakebite envenoming therapy. *Curr. Pharmaceut. Des.* 22, 5270–5293.
- Laustsen, A.H., Johansen, K.H., Engmark, M., Andersen, M.R., 2017. Recombinant snakebite antivenoms: a cost-competitive solution to a neglected tropical disease? *PLoS Neglected Trop. Dis.* 11, e0005361.
- Laustsen, A.H., María Gutiérrez, J., Knudsen, C., Johansen, K.H., Bermúdez-Méndez, E., Cerni, F.A., Jürgensen, J.A., Ledsgaard, L., Martos-Esteban, A., Öhlenschläger, M., et al., 2018a. Pros and cons of different therapeutic antibody formats for recombinant antivenom development. *Toxicol.* 146, 151–175.
- Laustsen, A.H., Karatt-Vellatt, A., Masters, E.W., Arias, A.S., Pus, U., Knudsen, C., Oscoz, S., Slavny, P., Griffiths, D.T., Luther, A.M., et al., 2018b. *In vivo* neutralization of dendrotoxin-mediated neurotoxicity of black mamba venom by oligoclonal human IgG antibodies. *Nat. Commun.* 9, 3928.
- Ledsgaard, L., Laustsen, A.H., Pus, U., Wade, J., Villar, P., Boddum, K., Slavny, P., Masters, E.W., Arias, A.S., Oscoz, S., et al., 2022. *In vitro* discovery of a human monoclonal antibody that neutralizes lethality of cobra snake venom. *mAbs* 14, 2085536.
- Ledsgaard, L., Wade, J., Jenkins, T.P., Boddum, K., Oganessian, I., Harrison, J.A., Villar, P., Leah, R.A., Zenobi, R., Schoffelen, S., et al., 2023. Discovery and optimization of a broadly-neutralizing human monoclonal antibody against long-chain  $\alpha$ -neurotoxins from snakes. *Nat. Commun.* 14, 682.
- Ljungars, A., Laustsen, A.H., 2023. Neutralization capacity of recombinant antivenoms based on monoclonal antibodies and nanobodies. *Toxicol.* 222, 106991.
- Miersch, S., de la Rosa, G., Friis, R., Ledsgaard, L., Boddum, K., Laustsen, A.H., Sidhu, S. S., 2022. Synthetic antibodies block receptor binding and current-inhibiting effects of  $\alpha$ -cobratoxin from *Naja kaouthia*. *Protein Sci.* 31, e4296.
- Moore, C.M., Grandits, M., Grünwald-Gruber, C., Altmann, F., Kotouckova, M., Teh, A.Y.-H., Ma, J.K.-C., 2021. Characterisation of a highly potent and near pan-neutralising anti-HIV monoclonal antibody expressed in tobacco plants. *Retrovirology* 18, 17.

- Murad, S., Fuller, S., Menary, J., Moore, C., Pinneh, E., Szeto, T., Hitzeroth, I., Freire, M., Taychakhoonavudh, S., Phoolcharoen, W., et al., 2020. Molecular Pharming for low and middle income countries. *Curr. Opin. Biotechnol.* 61, 53–59.
- Pinneh, E.C., van Dolleweerd, C.J., Görtzer, K., Drake, P.M.W., Ma, J.K.-C., Teh, A.Y.-H., 2022. Multiple gene expression in plants using MIDAS-P, a versatile type II restriction-based modular expression vector. *Biotechnol. Bioeng.* 119, 1660–1672.
- Pucca, M.B., Cerni, F.A., Janke, R., Bermúdez-Méndez, E., Ledsgaard, L., Barbosa, J.E., Laustsen, A.H., 2019. History of envenoming therapy and current perspectives. *Front. Immunol.* 10, 1598.
- Rappuoli, R., Miller, H.I., Falkow, S., 2002. Medicine. The intangible value of vaccination. *Science* 297, 937–939.
- Richard, G., Meyers, A.J., McLean, M.D., Arbabi-Ghahroudi, M., MacKenzie, R., Hall, J. C., 2013. *In vivo* neutralization of  $\alpha$ -cobratoxin with high-affinity llama single-domain antibodies (V<sub>H</sub>Hs) and a V<sub>H</sub>H-Fc antibody. *PLoS One* 8, e69495.
- Souza, J.B., Cardoso, R., Almeida-Souza, H.O., Carvalho, C.P., Correia, L.I.V., Faria, P.C. B., Araujo, G.R., Mendes, M.M., Rodrigues, R.S., Rodrigues, V.M., et al., 2020. Generation and *In-planta* expression of a recombinant single chain antibody with broad neutralization activity on *Bothrops pauloensis* snake venom. *Int. J. Biol. Macromol.* 149, 1241–1251.
- Stelter, S., Paul, M.J., Teh, A.Y.-H., Grandits, M., Altmann, F., Vanier, J., Bardor, M., Castilho, A., Allen, R.L., Ma, J.K.-C., 2020. Engineering the interactions between a plant-produced HIV antibody and human Fc receptors. *Plant Biotechnol. J.* 18, 402–414.
- Teh, A.Y.-H., Maresch, D., Klein, K., Ma, J.K.-C., 2014. Characterization of VRC01, a potent and broadly neutralizing anti-HIV mAb, produced in transiently and stably transformed tobacco. *Plant Biotechnol. J.* 12, 300–311.
- The Lancet, 2017. Snake-bite envenoming: a priority neglected tropical disease. *Lancet* 390, 2. [https://doi.org/10.1016/S0140-6736\(17\)31751-8](https://doi.org/10.1016/S0140-6736(17)31751-8).
- van Dolleweerd, C.J., Teh, A.Y.-H., Banyard, A.C., Both, L., Lotter-Stark, H.C.T., Tsekoa, T., Phahladira, B., Shumba, W., Chakauya, E., Sabeta, C.T., et al., 2014. Engineering, expression in transgenic plants and characterisation of E559, a rabies virus-neutralising monoclonal antibody. *J. Infect. Dis.* 210, 200–208.
- Walsh, G., Walsh, E., 2022. Biopharmaceutical benchmarks 2022. *Nat. Biotechnol.* 40, 1722–1760.
- Webster, G.R., van Dolleweerd, C., Guerra, T., Stelter, S., Hofmann, S., Kim, M.-Y., Teh, A.Y.-H., Diogo, G.R., Copland, A., Paul, M.J., et al., 2018. A polymeric immunoglobulin-antigen fusion protein strategy for enhancing vaccine immunogenicity. *Plant Biotechnol. J.* 16, 1983–1996.
- Williams, D.J., Faiz, M.A., Abela-Ridder, B., Ainsworth, S., Bulfone, T.C., Nickerson, A. D., Habib, A.G., Junghanss, T., Fan, H.W., Turner, M., et al., 2019. Strategy for a globally coordinated response to a priority neglected tropical disease: snakebite envenoming. *PLoS Neglected Trop. Dis.* 13, e0007059.