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High throughput identification of human monoclonal antibodies and heavy-chain-only antibodies to treat snakebite

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ABSTRACT

Snakebite envenoming is a priority Neglected Tropical Disease that causes an estimated 81,000–135,000 fatalities each year. The development of a new generation of safer, affordable, and accessible antivenom therapies is urgently needed. With this goal in mind, rigorous characterisation of the specific toxins in snake venom is key to generating novel therapies for snakebite. Monoclonal antibodies directed against venom toxins are emerging as potentially strong candidates in the development of new snakebite diagnostics and treatment. Venoms comprise many different toxins of which several are responsible for their pathological effects. Due to the large variability of venoms within and between species, formulations of combinations of human antibodies are proposed as the next generation antivenoms. Here a high-throughput screening method employing antibody-based ligand fishing of venom toxins in 384 filter-well plate format has been developed to determine the antibody target/s The approach uses Protein G beads for antibody capture followed by exposure to a full venom or purified toxins to bind their respective ligand toxin(s). This is followed by a washing/centrifugation step to remove non-binding toxins and an in-well tryptic digest. Finally, peptides from each well are analysed by nanoLC-MS/MS and subsequent Mascot database searching to identify the bound toxin/s for each antibody under investigation. The approach was successfully validated to rapidly screen antibodies sourced from hybridomas, derived from venom-immunised mice expressing either regular human antibodies or heavy-chain-only human antibodies (HCAbs).

1. Introduction

Venomous snakebite is a WHO-listed priority neglected tropical disease, resulting in up to 138,000 deaths and triple that number of permanent disablements each year (Harrison et al., 2011; Warrell, 2010; Gutiérrez et al., 2017). Snake venoms are highly complex and comprise a multitude of toxins from different protein families, causing a variety of distinct pathologies (Harrison et al., 2011; Warrell, 2010; Calvete et al., 2007; Slagboom et al., 2017). This makes developing a broad-purpose therapy extremely difficult. Currently, the only effective therapies for snakebite are animal-derived antivenoms which contain immunoglobulin G (IgG) or IgG-derived F(ab')$_2$ or Fab antibodies, which are administered intravenously (Laustsen et al., 2018a; H Laustsen et al., 2016). These antivenoms are produced by immunising large animals (often horses or sheep) with snake venom, which induces the formation of polyclonal antibodies. After immunisation, the antibodies are extracted from the animal's plasma and formulated into intact IgG, F(ab')$_2$- or Fab-fragment therapies. However, such antivenom treatment using non-human antibodies poses a significant risk of adverse effects. Acute allergic reactions can result in systemic anaphylaxis which can be fatal (Slagboom et al., 2017). Additionally, delayed onset reactions, known as serum sickness, can occur days after antivenom administration (de Silva et al., 2016).

Due to the wide variation in venom toxin constituents between

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different snake species, the antibodies have limited paraspecificity, and thus are not very effective in treating envenoming from snake species that were not used for the immunisation process (Halassy et al., 2011; Ainsworth et al., 2018). This limits the geographical breadth of the efficacy of any particular antivenom and hampers the development of globally effective therapeutics for use for treating snakebite. Socioeconomic factors exacerbate this issue since the majority of venomous snakebites occur in rural impoverished areas (Harrison et al., 2011; Warrell, 2010). Research has shown a significant correlation between snakebite mortality rates and the gross domestic product per capita, per capita expenditure on health and human developmental index of a country (Harrison et al., 2009). Accordingly, the health infrastructure of these countries is less developed, and antivenom treatments are often not available or affordable to those in need (Harrison et al., 2011).

The development of a new generation of antivenoms that can address some or all of these shortcomings would have great significance in treating snakebite envenoming. Toxin characterisation of relevant snake venoms is fundamental for generating novel therapies by identifying key targets for neutralisation. To facilitate this characterisation process, several established approaches have been developed of which venomics, toxicovenomics and antivenomics are currently the most important (Calvete et al., 2007, 2009; Lomonte et al., 2008; Lauridsen et al., 2016; Calvete, 2017; Slagboom et al., 2022). These methodologies aim to define venom toxin composition, define the pathological effects of specific toxins, and identify toxin recognition and depletion by antivenom antibodies, respectively.

Various approaches are currently being used in the discovery and development of next-generation antivenoms, including drug discovery, aptamer selection, design of decoy receptors and monoclonal antibody discovery (Chen and Murawsky, 2018; Laustsen, 2018; Laustsen et al., 2018b; Clare et al., 2021; Albulescu et al., 2019, 2020; Alomran et al., 2022). One of the most promising developments in antivenom research has been the identification of toxin-specific human monoclonal antibodies. These can be made using different experimental approaches, such as phage-display (Riaño-Umbarila et al., 2005; Ledsgaard et al., 2018), HCAbs via HEK cell library (Drabek et al., 2016) or by hybridoma cell lines originating from venom-immunised mice with an engineered human immunoglobulin locus (Chen and Murawsky, 2018; Brüggemann et al., 2015). The latter approach has already shown to be very successful in the development of biopharmaceutical human antibodies (mAbs) for targeting cancer, autoimmune and infectious diseases, including Covid (Du et al., 2022; Zahavi and Weiner, 2020; Green, 2014; Lu et al., 2020; Wang et al., 2022). Sources that produce human antibodies are either hybridomas originating from B cells derived from human individuals or from transgenic rodents expressing human antibodies. Although naïve libraries of human antibody repertoires can yield anti-venom toxin antibodies, immunisation by venom or venom toxins and *in vivo* maturation are preferable (Dias da Silva et al., 2022; Ledsgaard et al., 2023). Antibodies can be obtained using different methodologies such as phage display libraries, eukaryotic cell libraries, combined transcriptomic/serum proteomic approaches and single cell technologies (Ledsgaard et al., 2018; Doerner et al., 2014; Drabek et al., 2022; Le et al., 2020).

In this study we describe antivenom antibodies obtained by classical hybridoma fusion following venom protein immunisation of mice with a transgenic human/rat immunoglobulin loci producing heavy and light chain antibodies (H2L2) where all variable regions of the antibodies are of human origin or from mice expressing human heavy chain only antibodies (HCAbs) (Drabek et al., 2016, 2022). To avoid a long screening procedure a rapid analytical method is needed to characterise the venom toxin binding capacities of these antivenom monoclonal antibodies. Here we combined a bioaffinity-based screening technique known as ligand fishing (Moaddel et al., 2007; Zhuo et al., 2016; Guo et al., 2022) with peptide mass fingerprinting analysis using nanoLC-MS/MS to determine which monoclonal antibodies bind which toxins in the venom under study. In this technique, the target monoclonal antibodies are immobilised on a solid support, usually protein beads (in our case protein G coated beads that bind to the Fc-region of the antibodies (Kato et al., 1995), and then exposed to a ligand mixture (a venom in our case). Any compound in a mixture with an affinity to the immobilised target is retained for analysis while non-binding compounds remain in solution and are subsequently washed away from the filter plates. To determine cross-binding activity of large libraries of antibodies, these must be screened against numerous venoms and toxins, which again requires a rapid and sensitive high-throughput analytical method. Here we developed, validated, and then applied this high-throughput screening method employing ligand fishing with peptide mass fingerprinting nanoLC-MS/MS analysis to rapidly screen antibodies with affinity towards venom toxins.

2. Experimental

2.1. Chemical and biological materials

2.1.1. Chemicals

LC solvents and chemicals were of analytical grade. Acetonitrile (ACN) and formic acid (FA) were purchased from Biosolve (Valkenswaard, The Netherlands) and water was purified using a Milli-Q plus system (Millipore, Amsterdam, The Netherlands). Iodoacetamide, β-mercaptoethanol and ammonium bicarbonate were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Sequencing-grade modified trypsin was purchased from Promega Benelux B.V. (Leiden, The Netherlands). Protein G Agarose Fast Flow packed beads 50% slurry and 0.45 μm Durapore (PVDF) Membrane MultiScreen 384-well filter plates were purchased from Merck Milllipore Ltd (Amsterdam, The Netherlands). The venom used in this study were from captive bred specimens of *Calloselasma rhodostoma* and *Echis ocellatus* maintained in the herpetarium of the Centre for Snakebite Research & Interventions at the Liverpool School of Tropical Medicine, UK.

2.1.2. H2L2 immunisation and antibody isolation

Hybridoma cell lines were derived from Harbour BioMed H2L2 mice after immunisation with *Calloselasma rhodostoma* venom: Six H2L2 mice were immunised bi-weekly with an increasing dose of venom, starting at 5 μg/mouse and ending after six immunisations at a dose of 25 μg/mouse using Stimune Adjuvant (Prionics) freshly prepared according to the manufacturer's instruction for the first injection, whereas boosting was performed using Ribi (Sigma) adjuvant. Injections were delivered subcutaneously into the left and right groin (each (50 μl) and 100 μl intraperitoneally. Four days after the last injection, spleen and lymph nodes were harvested, and hybridomas made by the standard method using SP 2/0 myeloma cell line (ATCC#CRL-1581) as a fusion partner as described in detail in Drabek et al. (2022). Hybridomas were screened in a venom specific Elisa. 96 well Elisa plates were coated overnight at 4 °C with 50 μL of antigen (5 μg/mL crude venom dissolved PBS). After removing the antigen, wells were blocked for 30 min at RT with 1% fat free milk/1% BSA and subsequently washed 3 times with 300 μL of PBS/0.01%Tween 20. Each well was filled with 20 μL of blocking buffer to which 40 μL of the hybridoma supernatant was added. After at least 2 h of incubation at RT, the plates were 5 times washed with PBS/0.1% Tween 20 before adding 50 μL of 1:1500 diluted anti rat IgG-HRP (IgG1, IgG2b, IgG2c, Absea Biotechnology Ltd) in PBS with 1% BSA, 1% fat free milk powder/0.1% Tween-20 was added to each well and further incubated for 2 h at room temperature. Five washes were performed with 300 μL of PBS/0.1% Tween-20 followed by addition of 50 μL of POD substrate (Roche, BM Blue POD substrate soluble, # 11484281001). After incubation of 5–30 min the reaction was stopped with 50 μL of 1M H2SO4 and absorbance was read on an Elisa reader at 450 nm. Next, positive hybridomas were subcloned, expanded and cultured in serum- and protein-free medium (PFHM-II (1 ×), Gibco) with addition of non-essential amino acids 100 × NEAA, Biowhittaker Lonza, Catalog# BE13-114E). H2L2 antibodies were purified from

hybridoma culture supernatants using Protein-G affinity chromatography (Merck KGaA, Catalog# 16–266). For more detailed information the reader is referred to Drabek et al. (2022).

2.1.3. HCAb immunisation and antibody isolation

HCAb mice were immunised according to the protocol approved by the Dutch Experimental animal committee DEC Nr SP2100274. Briefly, different mice were injected with full venom from the *C. rhodostoma* and *E. ocellatus*. The first injection was 10 μg venom per snake per mouse dissolved in phosphate buffered saline (PBS) (Thermo, Catalog#14190144), cross-linked with 0,1% glutardehyde (EMS, Catlog#16220)1:1 in Freunds oil (Merck, Catlog#F5506). The 2nd and 3rd injections were 25 μg venom per mouse dissolved in PBS cross-linked with 0.1% glutardehyde in Sigma's Adjuvant (Merck, Catlog#s6322) according to the formulation provided by the supplier. After this full venom without crosslinking was used for immunisations. Starting with 5 μg snake venom per mouse up to 25 μg per mice for immunisation 6 and the booster 7. Every 2 weeks the mice were immunised and blood was collected after immunisation 4, 6 and on sacrificing day. Mice were sacrificed 1 week after the 7th booster immunisation. The details of immunisation, blood collection and sacrifices are described in Drabek et al. with a minor modification (Drabek et al., 2022). The protocol for antigen specific Elisa to the one given for H2L2 mice was modified. When testing the blood titres, we use as the secondary antibody anti mouse IgG-HRP 1:1500 because HCAbs have a mouse constant region. When screening the HEK cell library, we use anti human IgG-HRP since we clone the library in the expression plasmid containing human IgG1 Fc.

Mice were sedated with isoflurane and Evans blue dye (Thermo, A16774.18), 10 μL of 10 mg/mL PBS was injected in the back paws of the mice. After 10 min mice were euthanized by cervical dislocation. Lymphocytes were collected from Lymph nodes and bound to biotinylated snake venom (Thermo, Catlog#21312) coated on biotin binder Dynabeads according to protocol (Thermo, Catlog#11047) (Drabek et al., 2016). Total RNA extraction was done with TRI Reagent (Merck, T9424) followed by reverse transcription/cDNA synthesis and the amplification of human VH regions (Gan et al., 2022). The VHs were ligated into a mammalian expression vector and was cleaned up with Nucleospin Extract II (Macherey-NagelGmBH) according to protocol provided by the manufacturer. The VH fragments were first gel purified and a band was cut out at 380bp. This was ligated into a cut PvuII/BstEII phosphatase treated linearised pCAG hygro mG1 vector (Harbour Ab). This plasmid was transformed into *E.coli* and plated on LB plates. 960 mini's per snake venom were picked and grown for 24hrs. at 37 °C in Terrific broth in 96 well plates (Thermo, 249946). Plasmid DNA was isolated and transfected with Xtreme gene HP (Roche, 06366546001) into HEK293T cells in a 96 well format. After 48hrs. media were tested in ELISA with an anti-human IgG1 secondary antibody (Absea KT46, Catlog#031801A04H) and positive clones were sequenced and used for further analysis. Protocols for the ligation, mini prep and transfection are found in SI document: Protocols ligation miniprep and transfection. docx

The following resulting antibodies were used for ligand fishing experiments: H2L2: mAb 1 (3e12), mAb 2 (5h8), mAb 3 (8g7), mAb 4 (24a6), mAb 5 (38h3), mAb 6 (42h12), mAb 7 (47h4), mAb 8 (49d8), mAb 9 (50b8), mAb 10 (54c9), mAb 11 (57h6), mAb 12 (62e9), mAb 13 (62f10), mAb 14 (63h1), mAb 15 (68b3), mAb 16 (70b2), mAb 17 (71e2), mAb 18 (73f1) and mAb 19 (83e9). HCAbs: 4, 7, 12, 13, 15, 17, 18, 20, 21, 23, 24, 26, 27, 30, 31, 32, 35, 36, 37, 38, 39 and 40. Antibodies were stored at 4 °C until the ligand fishing was performed. The animal studies were performed under the animal permit AVD101002016512, approved by the CCD (central committee for animal experiments at Harbour BioMed).

2.1.4. Ligand fishing protocol

For the initial ligand fishing experiments, a standard low-throughput immunoprecipitation protocol was slightly adapted. First, antibody solutions (10 μg antibody in total) were mixed with the venom (10 μg) and then PBS (0.01 M pH 7) was added to reach a total volume of 200 μL in Eppendorf tubes. Trastuzumab (1 mg/mL stock) a human IgG1 antibody against human HER2, was used as a negative control, alongside the isolated antibodies listed above, since HER2 is not present in *Calloselasma rhodostoma* venom. Antibody-venom mixtures were incubated for 1h. In parallel, 20 μL portions of protein G beads slurry was added to other Eppendorf tubes. Note: all steps were performed at RT. Washing step one of the protein G beads slurries was carried out by adding 200 μL of 0.01 M PBS/0.1% Tween 20/0.5M NaCl pH 7, spinning the beads down for 5 s at 14,000×*g* and discarding the supernatant. This was twice repeated. Washing step two was performed three times following the same procedure, but with 0.01 M PBS pH 7. This was followed by a 1h incubation with gentle rocking of the beads in 200 μL 0.01M PBS buffer, 0.5M NaCl containing 2% bovine serum albumin to occupy the non-specific binding sites. Washing steps one and two mentioned above were then repeated three times to remove the unbound bovine serum albumin. Next the antibody-toxin complexes were added to the Eppendorf tubes containing the beads and 0.01 M PBS was added to an end volume of 200 μL. This was followed by gentle rocking for 2h to capture the complexes with the beads. The beads were then washed again according to washing steps one and two as mentioned previously, followed by an additional washing step with Milli-Q water. 50 μL 1% formic acid (pH ~2) was added to the beads-antibody-toxin complexes followed by incubation for 15 min to disrupt the toxin-antibody complexes and dissociate the toxins from the antibodies. Next the Eppendorf tubes were centrifuged for 5 s at 14,000×*g* and the supernatants containing the released toxins were transferred to new Eppendorf tubes which were vacuum centrifuge freeze-dried overnight to remove the water and formic acid. Finally, the toxins were subjected to tryptic digestion followed by proteomics analysis as described by Slagboom et al. (2023). After demonstrating that this procedure was successful, samples were transferred to 384-well filter plate format to develop the high-throughput methodology.

The first step in the high-throughput 384-well filter plate format protocol was to add 6 μL of Protein G Agarose Fast Flow packed beads 50% slurry (EMD Millipore) to 0.45 μm Durapore (PVDF) Membrane MultiScreen 384-well filter plates (Merck Millipore Ltd.). The beads were then rinsed with 100 μL of 0.01M PBS and the plates were subsequently centrifuged at 700×*g* for 1 min at RT with an Eppendorf Centrifuge 5810 R after which the flow through was discarded. This washing step was repeated twice. The beads were then incubated in 100 μL of 2% bovine serum albumin in 0.5M NaCl and 0.01M PBS, for 1 h at room temperature. Beads were washed twice with 100 μL PBS-T (PBS, 0.1% Tween-20) and twice with 100 μL PBS and the plates were subsequently centrifuged as before after which the flow throughs were discarded. Next, 2 μg of purified antibody (stock concentration of antibodies in Dulbecco's PBS varied) or 100 μL serum free media was added to the appropriate wells and incubated for 1 h on a shaker at 200 rpm at room temperature. Beads were then washed twice with 100 μL PBS-T (PBS, 0.1% Tween-20) and twice with 100 μL PBS, using the same centrifuge procedure for the washing steps, the flow through was discarded. 100 μg of crude venom (stock: 5 mg/mL in Milli-Q water) was added next to the appropriate wells and PBS was added to give a final volume of 100 μL. The plates were then incubated overnight at 4 °C. Beads were washed twice with 100 μL PBS-T (PBS, 0.1% Tween-20, 0.5M NaCl), twice with 100 μL PBS, and twice with water, using the same centrifuge procedure, the flow-throughs were discarded. The bound toxins were subsequently disrupted from their antibody binding partners by incubating the beads-antibody-toxin complexes for 10 min in 50 μL of 5% v/v formic acid after which the plates were centrifuged for 1 min at 700×*g*. The flow throughs containing the released venom toxins were collected on collection well plates (i.e., flat bottom transparent 384-well plates) and evaporated to dryness overnight using a Christ Rotational Vacuum Concentrator RVC 2–33 CD plus (Salm en

Kipp, Breukelen, The Netherlands) with a rotor for well-plates.

2.1.5. Tryptic digestion

25 μL of 0.05% β-mercaptoethanol in 25 mM ammonium bicarbonate pH 8.2 was added to each well using robotic pipetting. Plates were incubated at 95 °C for 15 min, then cooled to RT. 10 μL of 12.5 mM iodoacetamide in Milli-Q water was added to each well and incubated in the dark at room temperature for 30 min. 10 μL of 0.01 mg/mL trypsin solution (in 25 mM ammonium bicarbonate) was added to each well and the plate was incubated at 37 °C overnight. The digestion was quenched with 10 μL of 1.25% formic acid.

2.1.6. Proteomics

Tryptic digests from the H2L2 ligand fishing experiments were analysed with nanoLC separations using an UltiMate 3000 RPLCnano system (ThermoFischer Scientific, Ermelo, The Netherlands). 1 μL injection samples from the wells from the 384-well plates were separated on an Acclaim™ PepMap™ 100 C18 HPLC Column (150 mm × 75 μm) with a particle size of 2 μm and a pore size of 100 Å in combination with an Acclaim™ PepMap™ 100 C18 trapping column (5 mm × 0.3 mm), with a particle size of 5 μm and a pore size of 100 Å (Thermo Fischer Scientific) at a flow rate of 0.5 μL/min. The mobile phases comprised of eluent A (98% water, 2% ACN, 0.1% FA) and eluent B (98% ACN, 2% water, 0.1% FA). The gradient used for the separation of the digests was: 3 min isocratic separation at 1% B, linear increase to 40% in 7.5 min followed by a linear increase to 85% in 0.1 min, isocratic separation at 85% B for 0.7 min, linear decrease to 1% B in 0.2 min and finally the column was equilibrated for 3.7 min at 1% B. The column temperature was 45 °C. Mass detection was performed by using a maXis Q-TOF mass spectrometer (Bruker, Bremen, Germany) equipped with a Bruker Captivespray source operating in positive-ion mode. The source parameters were: Source temperature, 150 °C; capillary voltage, 1.6 kV; Dry gas flow, 3.0 L/min, nanoBooster pressure, 0.20 Bar. Spectral data were recorded at a rate of 2 Hz in the range of 50–3000 *m/z*. MS/MS spectra were obtained using collision induced dissociation (CID) in data-dependent mode using 10-eV collision energy. Bruker Compass software was used for instrument control and data analysis.

Tryptic digests from the HCAb ligand fishing experiments were analysed with microLC separations using an UltiMate 3000 RPLCnano system (ThermoFischer Scientific, Ermelo, The Netherlands). 0.6 μL injection samples from the wells from the 384-well plates were separated on an Kinetex 2.6u XB-C18 LC Column (150 mm × 0.3 mm) with a particle size of 2.6 μm and a pore size of 100 Å at a flow rate of 6 μL/min. The mobile phases comprised of eluent A (98% water, 2% ACN, 0.1% FA) and eluent B (98% ACN, 2% water, 0.1% FA). The gradient used for the separation of the digests was: 3 min isocratic separation at 1% B, linear increase to 40% in 7.5 min followed by a linear increase to 85% in 0.1 min, isocratic separation at 85% B for 0.7 min, linear decrease to 1% B in 0.2 min and finally the column was equilibrated for 3.7 min at 1% B. Mass detection was performed with a Sciex 7600 ZenoTOF (Framingham, MA, USA) and operated with the OptiFlow 1–50 μL Micro/MicroCal ion source in positive mode. The ZenoTOF method parameters were as follows: the workflow selected was peptides with a method duration of 13 min. The ionisation start time was set at 2 min while the ionisation stop time was set at 13 min. Source and gas parameters were as follows: curtain gas was set to 35, CAD gas was set to 7, ion source gas 1 was set to 15 psi and ion source gas 2 to 20 psi, temperature was set to 150 °C and the column temperature to 30 °C. IDA experiment settings were as follows: spray voltage 4500 V, TOF mass range: 400–1500 Da, accumulation time: 0.1 s, declustering potential: 80 V, DP spread: 0 V, collision energy:10 V, CE spread: 0 V, time. TOF MS Advanced experiment settings were: bins to sum: 8, channels 1–4: selected. The IDA criteria were: peptide workflow, maximum candidates ions: 45, intensity threshold exceeds of 300 counts/s, no dynamic background subtract, exclusion of former candidate ions for 6 s and after one occurrence. The dynamic CE for MS/MS was used. The maximum CE was set to 80 V and the minimum to 5 V. Charge states 2 to 5 were selected and the selected isotope was monoisotopic. In advanced IDA criteria a mass tolerance of ±50 mDa was set. TOF MS/MS settings were: fragmentation mode: CID, TOF mass range: 100–2000 Da, accumulation time: 0.01 s, declustering potential: 80 V, spread: 0 V, collision energy spread: 0 V, Q1 resolution set to unit, zeno pulsing on. TOF MS/MS Advanced experiment settings: zeno threshold:100000 cps, time bins to sum: 8, channels 1–4: on.

2.1.7. MS data processing and mascot database searches

The mass spectrometry data for the H2L2 measurements were processed by using Bruker DataAnalysis software (Version 5.1). The ProcessWithMethod function was used to generate MGF files for each sample, which were then processed using Mascot Daemon software. The mass spectrometry data obtained for the HCAb measurements were processed into mgf files by using ProteoWizard's msConvert. Search parameters used were: Instrument type: ESI-QUAD-TOF, Digestion enzyme: semiTrypsin, Allowing one missed cleavage, Carbamidomethyl on cysteine as a fixed modification, amidation (protein C-terminus) and oxidation on methionine as variable modifications, Fragment mass tolerance: ±0.05 Da, and ±0.2 Da peptide for mass tolerance. Database searches were done by searching two different databases. 1: Uniprot database containing only Serpentes accessions and 2: Species-specific venom gland transcriptomic database. The Mascot search information obtained from all the MGF files for one experiment was extracted and merged into a single Excel file using in-house written R scripts (Slagboom et al., 2023). The first R script extracted all the information obtained for each of the Mascot searches as individual Comma Separated Value (CSV) files for all samples. The second script merged and filtered information from all the CSV files into a single Excel file. The information that remained for each of the samples after merging and filtering was: protein accessions, protein scores, protein masses, protein sequence coverages, protein descriptions, full protein sequences, and peptide sequences. The scripts used are provided in the Supplementary Information named R scripts.

3. Results and discussion

In this study a high-throughput screening method, which employs an antibody-based ligand fishing technique for venom toxins was developed. This method is implemented in a 384-filter-well plate format and serves the dual purpose of identifying antibody targets and expediting the process of finding potential therapeutic antibody candidates for neutralisation. The procedure involves the utilisation of Protein G beads for antibody capture. Subsequently, the captured antibodies are exposed to a complete venom sample to bind with their respective toxin ligands. Following this, a washing and centrifugation step is employed to eliminate toxins that haven't bound to the antibodies. An in-well tryptic digest is then performed. In the final step, peptides from each well are subject to analysis through nanoLC-MS/MS, and the data is processed through a Mascot database search. This allows us to pinpoint the specific toxin(s) bound by each antibody under scrutiny.

The low-throughput ligand fishing method utilised an adapted standard immunoprecipitation protocol, as depicted in Fig. 1 and described in the Experimental section at the Ligand fishing protocol part. Nineteen monoclonal antibodies derived from Harbour H2L2 mice immunised against the venom of *Calloselasma rhodostoma* were included for method development and evaluation. These antibodies were selected based on initial crude venom specific Elisa from which an example is shown in the Supplementary information under the name ELISA hybridomas.TIFF. In addition, Trastuzumab was used as a control antibody. First the method was validated by testing antibodies 3e12, 8g7, 38h3 and Trastuzumab in triplicate. Antibodies mAb 1 (3e12) and mAb 5 (38h3) showed binding of venom toxins while antibodies mAb 3 (8g7) and Trastuzumab did not show any toxin binding. The methodology showed binding to the same toxins with high protein scores and the same

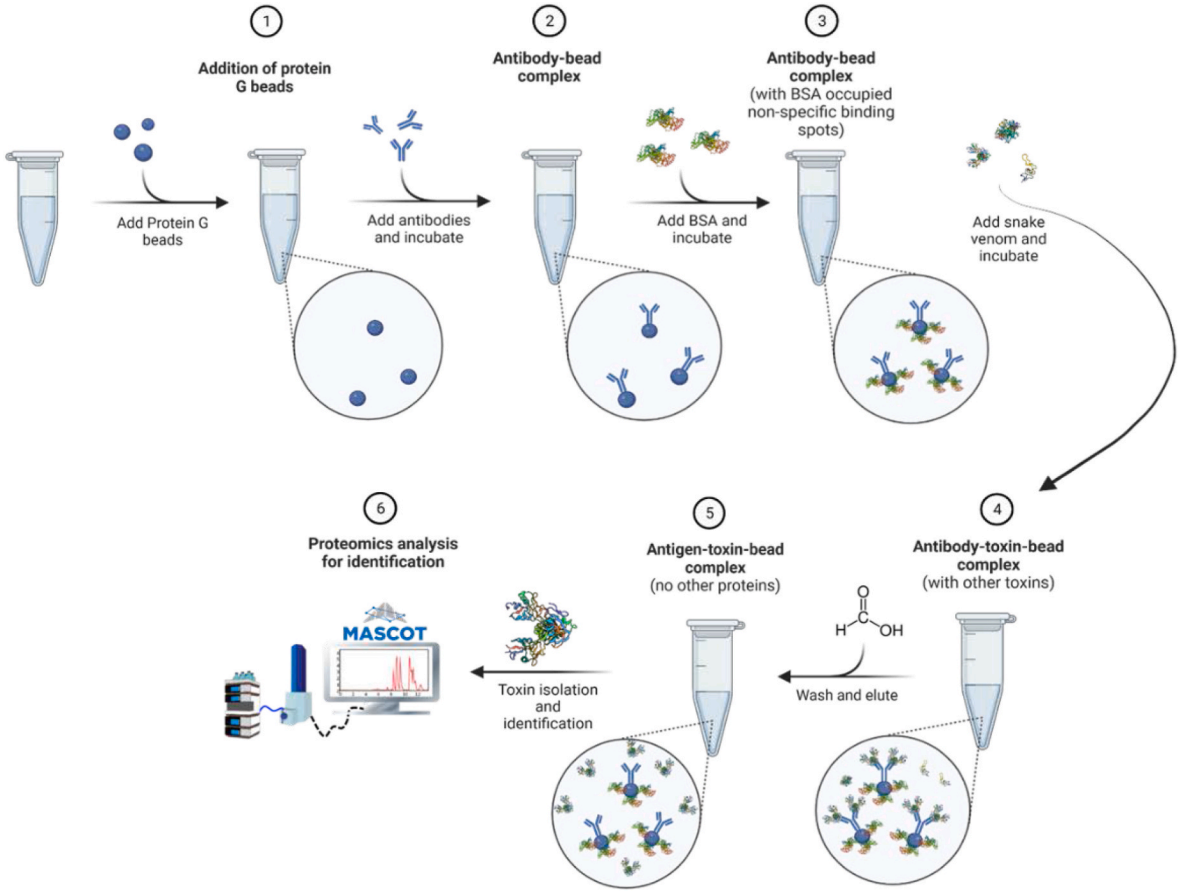

Fig. 1. The low-throughput ligand fishing workflow. Initially, protein G beads were introduced into Eppendorf tubes. Then, antibodies were added and mixed with the beads, leading to the formation of bead-antibody complexes. To prevent any non-specific binding, BSA was incorporated into the complexes. Next, crude venom was added and allowed to incubate, facilitating the creation of bead-antibody-toxin complexes. Following this step, unbound venom toxins were thoroughly washed away, after which the bound toxins were eluted from the bead-antibody-toxin complexes by means of acidification. The final stage involved isolating the toxins for further analysis through proteomics, enabling accurate identification of the toxins.

sequences found for antibodies mAb 1 (3e12) and mAb 5 (38h3) in the triplicate measurements, while for antibodies mAb 3 (8g7) and Trastuzumab no binding was observed for the triplicate measurements (details on this experiment are given in the SI document: Initial triplicate experiments). Table 1 shows typical results obtained from the standard immunoprecipitation protocol for all 19 antibodies and the negative control. Of the 19 purified antibodies, 12 showed binding of venom toxins from *Calloselasma rhodostoma*, namely mAbs 1 (3e12), 4 (24a6), 5 (38h3), 8 (49d8), 9 (50b8), 10 (54c9), 11 (57h6), 12 (62e9), 13 (62f10), 14 (63h1), 15 (68b3) and 17 (71e2). The control mAb Trastuzumab did not bind any toxin. Based on the toxin identities the proteomics analyses showed that mAbs 1 (3e12), 5 (38h3) and 10 (54c9) all bound to SLEC_CALRH and SLED_CALRH. These are gamma and delta subunits from a heterotetrameric C-type lectin (CTL) named rhodocetin. This toxin is a potent collagen-induced platelet aggregation inhibitor, with a mode of action mediated by binding to the integrin alpha2A domain and blocking collagen binding to integrin alpha-2/beta-1 (Eble et al., 2017). The gamma/delta (SLEC_CALRH/SLED_CALRH) heterodimeric subunits are the main contributors to this activity (Eble et al., 2017). mAbs 9 (50b8) and 14 (63h1) were found to bind SLEA_CALRH and SLEB_CALRH, which correspond to the alpha and beta subunits from the same heterotetrameric CTL (Wang et al., 1999). mAb 8 (49d8) binds SLYA_CALRH and SLYB_CALRH, a different CTL called rhodocytin, which is a dimer consisting of subunits alpha and beta and causes platelet aggregation by means of binding to the C-type lectin domain family 1 member B (Navdaev et al., 2001). mAbs 11 (57h6), 13 (62f10), 15 (68b3) and 17 (71e2) were found to bind to PA2HD_CALRH, a phospholipase A_2 (PLA_2) that is known to exhibit myotoxic and edema-inducing activities (Tsai et al., 2000). mAbs 12 (62e9) and 4 (24a6) both bind snake venom serine proteases (SVSPs) of which mAb 12 (62e9) binds SVSPs VSPF1_CALRH and VSPF2_CALRH, while mAb 4 (24a6) only binds SVSP VSPF2_CALRH. VSPF1_CALRH and VSPF2_CALRH are thrombin-like snake venom serine proteases that are known to have an effect on the blood coagulation cascade by means of cleaving fibrinogen to split off the A-fibrinopeptides A, AY and AP in the case of VSPF1_CALRH, while VSPF2_CALRH generates the fibrinopeptides AM, AO, and AY (Albulescu et al., 2019, 2020).

After the low-throughput method successfully identified antibody bound venom toxins, mAbs 4 (24a6), 8 (49d8), 9 (50b8), 11 (57h6), 12 (62e9), 13 (62f10) and 17 (71e2) were selected for development of the high-throughput workflow (see Fig. 2). First a matrix experiment was performed to investigate the optimal venom and mAb concentration for the high-throughput methodology. As expected, the matrix experiment showed that the optimal antibody amount of mAbs 4 (24a6), 8 (49d8), 9 (50b8), 11 (57h6), 12 (62e9), 13 (62f10) and 17 (71e2) was the maximal amount tested of 2 μg (see Table 2). When using 0.4 μg of mAb the same toxins were found for mAbs: 4 (24a6), 8 (49d8), 9 (50b8), 11 (57h6), 12 (62e9) and 17 (71e2) but with lower protein scores and sequence coverage. For concentrations lower than 0.4 μg mAb no venom toxins were detected. Detailed information on the results for all samples tested in the 384-filter plate matrix experiments are found in SI document: “All results matrix table”. Higher amounts of antibodies were not tested since testing this posted issues in terms of feasibility to rapidly acquire sufficient amounts of antibody from the hybridoma

Table 1
Initial ligand fishing results using the low throughput method.

Antibody (10 µg)	Uniprot accession	Protein score	Sequence coverage %	Toxin family[a]
mAb 1 (3e12)	SLEC_CALRH	327	38	CTL
	SLED_CALRH	93	21	
mAb 2 (5h8)	–	–	–	–
mAb 3 (8g7)	–	–	–	–
mAb 4 (24a6)	VSPF2_CALRH	148	10	SVSP
mAb 5 (38h3)	SLEC_CALRH	325	38	CTL
	SLED_CALRH	223	23	
mAb 6 (42h12)	–	–	–	–
mAb 7 (47h4)	–	–	–	–
mAb 8 (49d8)	SLYA_CALRH	753	33	CTL
	SLYB_CALRH	109	8	
mAb 9 (50b8)	SLEA_CALRH	100	51	CTL
	SLEB_CALRH	3122	53	
mAb 10 (54c9)	SLEC_CALRH	245	36	CTL
	SLED_CALRH	105	44	
mAb 11 (57h6)	PA2HD_CALRH	71	25	PLA_2
mAb 12 (62e9)	VSPF1_CALRH	370	32	SVSP
	VSPF2_CALRH	201	15	
mAb 13 (62f10)	PA2HD_CALRH	148	35	PLA_2
mAb 14 (63h1)	SLEA_CALRH	378	30	CTL
	SLEB_CALRH	83	13	
mAb 15 (68b3)	PA2HD_CALRH	157	35	PLA_2
mAb 16 (70b2)	–	–	–	–
mAb 17 (71e2)	PA2HD_CALRH	174	35	PLA_2
mAb 18 (73f1)	–	–	–	–
mAb 19 (83e9)	–	–	–	–
Negative control Antibody (Trastuzumab)	–	–	–	–

[a] CTL (C-Type Lectin), SVSP (Snake venom serine protease), PLA_2 (Phospholipase A_2).

supernatants. To ensure the detection of all toxin IDs, the maximum amount of venom (20 µg) was utilised. Lower concentrations would result into missing some toxin IDs during the analysis. A summary of the optimal results obtained from this experiment are given in Table 3.

The comparison of the results obtained for the high-throughput ligand-fishing workflow with those of the low-throughput ligand-fishing workflow showed that very similar results were obtained. The same binding toxins were identified for each of the antibodies, together with high protein scores and sequence coverages. The observation that the protein scores found in the low-throughput method were overall higher is explained by the fact that the amount of mAb used in the high-throughput method was five times lower. These results demonstrate that the high-throughput ligand-fishing/immunoprecipitation method for the identification of venom toxins with affinity towards specific antibodies was successful.

Once the high-throughput methodology was successful, a total of 22 purified HCAbs underwent duplicate screening to determine their binding affinity against the venom of *Calloselasma rhodostoma*, following the procedures outlined in the materials and methods section. Following the promising outcome of this initial screening, the serum-free media containing the 22 HCAbs were also subjected to the high-throughput methodology. The rationale for utilising serum-free media was to emulate the methodology without the intricate process of antibody

Table 2
Optimal results from the high-throughput ligand fishing workflow procedure (i.e., using 2 µg mAbs and 20 µg venom).

Antibody	Uniprot accession	Protein score	Sequence coverage %	Toxin family
4 (24a6)	VSPF2_CALRH	75	4	SVSP
8 (49d8)	SLYA_CALRH	259	35	CTL
9 (50b8)	SLEA_CALRH	436	59	CTL
	SLEB_CALRH	273	47	CTL
11 (57h6)	PA2HD_CALRH	233	25	PLA_2
12 (62e9)	VSPF1_CALRH	658	44	SVSP
	VSPF2_CALRH	196	12	SVSP
13 (62f10)	PA2HD_CALRH	344	42	PLA_2
17 (71e2)	PA2HD_CALRH	462	52	PLA_2
Control (Trastuzumab)	–	–	–	–

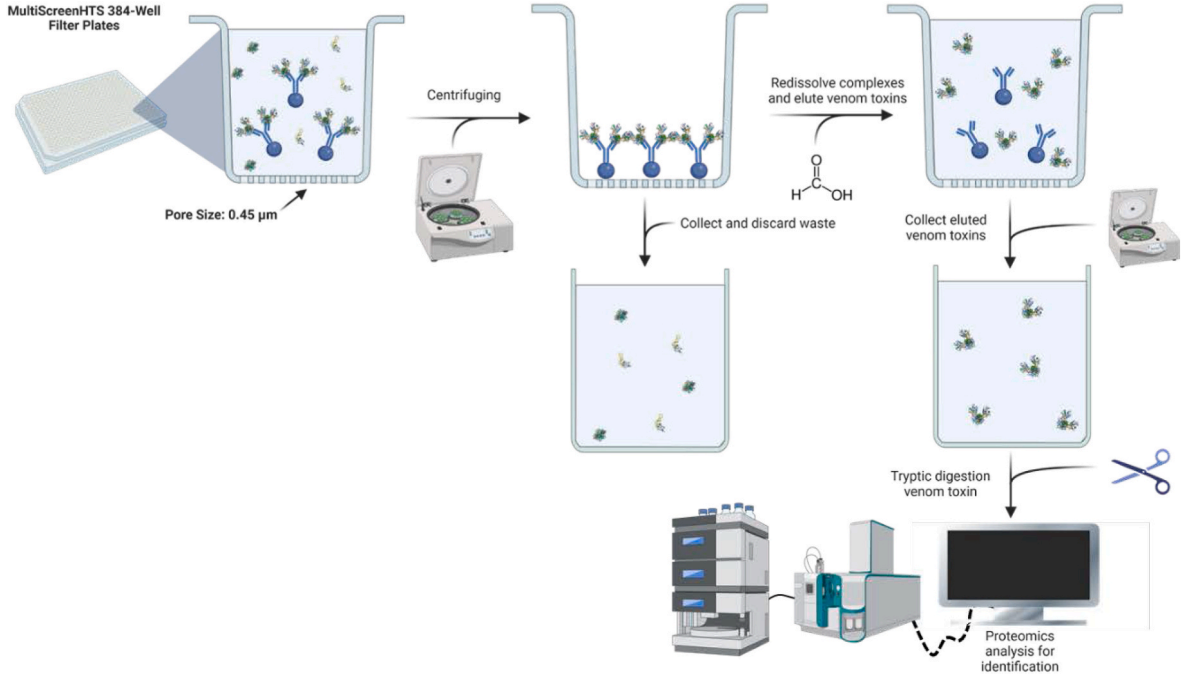

Fig. 2. High-throughput ligand fishing workflow. Following a similar process to the low-throughput method, protein G beads were employed to form complexes with antibodies and venom toxins. However, instead of using Eppendorf tubes, 384-well filter plates were utilised to enable efficient high-throughput screening. By applying centrifugation on the well plates, the liquids could be forced through the filter, facilitating bead washing and the removal of unbound toxins. Once the toxin of interest was eluted from the antibodies, the final flow-through was collected and subjected to proteomics analysis for precise toxin identification.

Table 3
Comparison of the results of ten purified and serum free media HCAbs with the high-throughput ligand fishing workflow procedure.

HCAb	Uniprot accession	Purified		Serum free media		Toxin family
		Protein score	Sequence coverage %	Protein score	Sequence coverage %	
4	OXLA_CALRH	879	49	346	29	LAAO
	VGT4456_L_300_LAAO	249	33	108	7	LAAO
7	VGT1180_L_800_SVSP	900	60	64	4	SVSP
	VSPF2_CALRH	162	4	275	50	SVSP
13	SLYA_CALRH	657	87	63	4	CTL
	SLYB_CALRH	393	52	431	71	CTL
20	SLEA_CALRH	370	63	225	45	CTL
	SLEB_CALRH	566	54	139	36	CTL
30	SLYA_CALRH	658	81	197	54	CTL
	SLYB_CALRH	419	53	142	22	CTL
31	V5NTD_NAJAT	131	10	94	5	Nucleotidase
32	PA2AA_CALRH	282	61	123	14	PLA2
	PA2A_BOTPC	227	19	65	32	PLA2
36	VGT1180_L_800_SVSP	658	60	161	36	SVSP
	VSPF1_CALRH	559	45	110	14	SVSP
	VSPF2_CALRH	548	35	59	11	SVSP
38	PA2HD_CALRH	759	66	176	52	PLA2
	VGT0797_R_1.0959_L_966_PLA2	219	12	66	11	PLA2
40	OXLA_CALRH	978	52	594	40	LAAO
	VGT4456_L_300_LAAO	305	39	209	9	LAAO

purification and concentration measurement. Instead, our objective is to employ unpurified materials, specifically the supernatants from serum-free medium. The serum from FCS introduces a concern due to its potential to interfere with protein G binding and concentration measurement within the sample. It is worth noting, however, that antibody production levels are not uniform, resulting in varying antibody quantities within the same volume of serum-free medium. Our examination of 22 HCAbs revealed adequate antibody production within serum-free medium. Additionally, the outcomes achieved using unprocessed antibodies align with those obtained from purified antibodies, underscoring that the purification step at this stage was unnecessary. The results obtained from this step were then compared to those from the purified HCAbs, as detailed in Table 4 (for comprehensive information on all 22 analysed antibodies, refer to the Supplementary Information document: HCAb purified and medium results.xlsx).

In order to assess the sensitivity of the methodology and instrumentation for detecting toxins bound on HCAbs obtained from serum-free media, we compared the averaged protein scores and sequence coverages of the duplicates of the top ten binders. The selection process for these top ten binders involved two criteria: first, based on the uniqueness of proteins, and second, on their protein scores.

The HCAbs were able to capture a diverse array of toxin families, including CTLs, nucleotidases, PLA_2s (phospholipases A_2), and SVSPs (serine proteases). Note that the H2L2 antibodies used in this study did not exhibit binding to LAAOs and nucleotidases from these toxin families. Snake venom 5′-nucleotidases have a broad distribution among venomous snake species, yet knowledge regarding their biological activities remains limited. Existing evidence indicates that they possess the ability to hinder platelet aggregation, potentially achieved by releasing inhibitory AMP or adenosine through their interaction with ADP, which is released during the initiation of aggregation (Dhananjaya and D'Souza, 2010; Ouyang and Huang, 1983; Dhananjaya et al., 2010). Furthermore, these venom 5′-nucleotidases are recognised for their *in vivo* synergistic action with other toxins such as ADPases, phospholipases, and disintegrins, collectively resulting in a more notable anti-coagulant effect (Dhananjaya and D'Souza, 2010; Ouyang and Huang, 1983; Dhananjaya et al., 2010). LAAOs facilitate the oxidative deamination of predominantly hydrophobic and aromatic L-amino acids. As a result of this process, hydrogen peroxide is produced, which may contribute to the enzyme's various toxic effects. Additionally, LAAOs exhibit a wide range of biological activities, including causing hemorrhage, hemolysis, edema, apoptosis of vascular endothelial cells or tumor cell lines, and anti-parasitic actions (Du and Clemetson, 2002; Guo et al., 2012; Izidoro et al., 2014). Furthermore, LAAOs are involved in the regulation of platelet aggregation, but their effect on platelets is a subject of controversy. Depending on the specific experimental conditions, LAAOs have been observed to either induce platelet aggregation or inhibit agonist-induced aggregation (Du and Clemetson, 2002; Guo et al., 2012; Izidoro et al., 2014). These conflicting effects are likely attributed to the differences in experimental setups and conditions (Macheroux et al., 2001). The remaining toxin families bound by the HCAbs were previously described in the low-throughput results section. In the Supplementary Information three documents were added that comprise of additional details of the obtained proteomics results such as MS/MS derived peptide sequences, matched MH+, mass delta, matched number of peptides.

In summary, the antibodies generated in this study demonstrated binding affinity to toxins belonging to the CTL, PLA_2s, SVSP, LAAO, and nucleotidase toxin families. This alignment largely corresponds to the toxin families known to be accountable for the hemotoxic pathologies induced by the venom of *C. rhodostoma*, including SVSPs, CTLs, PLA_2s, LAAOs, and SVMPs (Tang et al., 2019). Nonetheless, a noteworthy finding in this study was the lack of binding exhibited by both H2L2s and HCAbs towards SVMPs, a significant toxin group in the venom known for its pivotal role in envenomation. Subsequent immunisations of H2L2 mice with *C. rhodostoma* venom and HCAb mice with *E. ocellatus* venom, however, led to the identification of several antibodies capable of binding to SVMPs. Two H2L2 antibodies were found against VM2RH_CALRH, a zinc metalloproteinase from *C. rhodostoma*, from which the details are described in the Supplementary Information document named "Calloselasma rhodostoma H2L2 SVMP binders.xlsx" and for the HCAbs twelve binders were found against SVMPs from *E. ocellatus* from which the details are described in the Supplementary Information document, with the file name "Echis ocellatus HCAb SVMP binders.xlsx. While SVMP binders were identified in the supplementary screening, it is notable that this crucial toxin class is underrepresented in this study. Although the antibodies produced in this study have the potential to address the majority of relevant toxins, considering treatment applications, an alternative antibody or a small molecule inhibitor, such as marimastat, should be considered to potentially compensate for the underrepresentation of SVMP binding antibodies. (Layfield et al., 2020).

The comparison of toxin results obtained from the purified and serum-free media analysis of the top ten HCAb binders revealed that all toxins were detected in both analyses. This finding indicates that the methodology used in this study is sensitive, allowing for the

identification of toxins bound by antibodies even in serum-free media. As a result, this approach offers a significant advantage in terms of antibody screening throughput since it eliminates the requirement for an antibody purification step and subsequent concentration determination. Despite the ability to identify the same toxins in both analyses, notable differences were observed in the protein scores and sequence coverages for the identified toxins. The toxins captured by purified antibodies had an average coverage of 47%, while those bound by serum-free medium captured antibodies showed an average coverage of 27%. This decrease of 43% in sequence coverage was expected, given the significantly lower amount of antibodies present in the serum-free medium compared to the purified samples. Similarly, the average protein score for toxins found with purified antibodies was 496, whereas it was 182 for those identified with serum-free medium antibodies. This indicates a decrease of 63% in protein score. Nevertheless, a protein score of 182 still represents a very high probability (>99%) of the presence of the identified proteins in our study, a detailed explanation on the probability scoring and additional information on database searching using Mascot is found in the study by Perkins et al. (1999). Consequently, it can be inferred that conducting high-throughput screening of antibodies for their toxin binding capabilities is achievable through the use of serum-free media. This approach eliminates the necessity for antibody purification, enabling a significantly increased throughput capacity.

In most cases, the primary goal is to develop therapeutic antibodies targeting specific entities, such as individual peptides, proteins, glycoproteins, or even specific posttranslational modifications in a target protein. This is typically achieved by screening antibody repertoires from animals or humans, whether they are naïve, immune, or synthetic, against the desired target. However, when dealing with venoms, irrespective of the source (e.g., snakes, spiders, scorpions, jellyfish), the approach differs somewhat. Venoms consist of multiple components, including toxins, and the most effective therapeutic strategy is to provide comprehensive coverage and neutralisation of all these components. Instead of immunising against a single specific element, animals are immunised with the entire venom or toxoids derived from it. This approach offers advantages, as it eliminates the need for costly individual toxin purification, recombinant protein production, and numerous immunisation campaigns targeting individual components. The resulting antivenoms are mixtures of polyclonal antibodies or their fragments, each with varying neutralisation capabilities. Transitioning towards a defined formulation comprising a select few human monoclonal antibodies against the most critical and harmful toxin components would ensure consistent quality and neutralisation capacity in the final product on the market. This approach would also obviate the necessity for large-scale animal immunisations and mitigate potential adverse immune reactions to antibodies of animal origin in treated individuals.

4. Conclusion

A high-throughput bioanalytical methodology employing ligand fishing with antibodies that display affinity towards toxins present in the venom of *Calloselasma rhodostoma* venom has been established in this study. The methodology is capable of rapidly screening purified antibodies or antibodies that are present in serum free media for binding to and identification of toxins present in the snake venom. For this, a low-throughput immunoprecipitation method was first established and then successfully transferred to 384-well filter-plate format, resulting in an effective and high-throughput technique to investigate antibody-toxin binding from crude venoms. Since much of the procedure is performed in an automated fashion, hundreds of antibody-venom combinations can be screened in a single experiment. To obtain further information on the binding affinity of the antibodies against venom toxins from the *Calloselasma rhodostoma* venom, control experiments using peptide mass fingerprinting of crude venom could provide a more semi-quantitative reference point for the ligand fishing results. For this, comparing MS peak intensities of the relevant toxins measured in crude venom and those of toxin-fishing experiments could be compared to better assess the relative abundance of toxins bound to the antibodies under study. Also, subsequent Elisa experiments or affinity assays, using for example Forte Bio, would be essential to determine the relative binding affinity of the detected antibody-toxin complexes. For neutralisation assessment, bioassays are evidently required. For most toxins however, it is difficult to find the appropriate in vitro bioassay(s) as their exact functioning remains unknown or at best is based on sequence similarities with other toxins biochemically investigated in the past.

Ethical statement

Authors declare that international ethical guidelines for scientific papers were followed in the preparation of this manuscript.

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CRediT authorship contribution statement

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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

Supplementary data is attached

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxcx.2024.100185.

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