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Snake venomics of monocled cobra (*Naja kaouthia*) and investigation of human IgG response against venom toxins

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Highlights

- The venom proteome of the monocled cobra, *Naja kaouthia*, is presented
- Most abundant venom components are three-finger toxins (neurotoxins and cytotoxins) and phospholipases A_2
- Toxicity (LD_{50}) screening identified three-finger toxins as the most medically relevant of this venom
- IgG response from a self-immunized human was significant against several toxins, although weak against those with higher toxicity

Abstract

The venom proteome of the monocled cobra, *Naja kaouthia*, from Thailand, was characterized by RP-HPLC, SDS-PAGE, and MALDI-TOF-TOF analyses, yielding 38 different proteins that were either identified or assigned to families. Estimation of relative protein abundances revealed that venom is dominated by three-finger toxins (77.5%; including 24.3% cytotoxins and 53.2% neurotoxins) and phospholipases A_2 (13.5%). It also contains lower proportions of components belonging to nerve growth factor, ohanin/vespryn, cysteine-rich secretory protein, C-type lectin/lectin-like, nucleotidase, phosphodiesterase, metalloproteinase, L-amino acid oxidase, cobra venom factor, and cytidyltransferase protein families. Small amounts of three nucleosides were also evidenced: adenosine, guanosine, and inosine. The most relevant lethal components, categorized by means of a 'toxicity score', were α-neurotoxins, followed by cytotoxins/cardiotoxins. IgGs isolated from a person who had repeatedly self-immunized with a variety of snake venoms were immunoprofiled by ELISA against all venom fractions. Stronger responses against larger toxins, but lower against the most critical α-neurotoxins were obtained. As expected, no neutralization potential against *N. kaouthia* venom was therefore detected. Combined, our results display a high level of venom complexity, unveil the most relevant toxins to be neutralized, and provide prospects of discovering human IgGs with toxin neutralizing abilities through use of phage display screening.

(199 words)

1. Introduction

Snakebite is a serious medical condition affecting a large number of people worldwide; especially in impoverished rural areas of Asia, Africa and Latin America (Gutiérrez et al., 2006; Warrell, 2010a). Asia is the continent where the majority of these bites take place, and also where most deaths occur (Alirol et al., 2010; Chippaux, 1998; Kasturiratne et al., 2008). Currently there are 29 recognized extant species of terrestrial cobras assigned to the genus *Naja* (Utez and Hošek, 2015). Of these, 11 species are found in Asia, and 18 inhabit Africa (Utez and Hošek, 2015; Wallach et al., 2009). Among the cobras, *Naja kaouthia* (monocled cobra) is widespread in southern Asia and responsible for a significant part of the bites recorded (Viravan et al., 1986; Warrell, 1995; Kulkaew et al., 2009). A study in Bangladesh discovered records of 764 snakebites during 1988-1989, of which 34% of the 168 deaths (22% case fatality) were attributed to the cobras *Naja naja* and *N. kaouthia* (Warrell, 2010b). *N. kaouthia* occurs from north-eastern India, Bangladesh and Bhutan, across southern China, southward to northern Peninsular Malaysia (Prakash et al., 2012; Wüster, 1996) and is common in most of its range (Stuart and Wogan, 2012). *N. kaouthia* is adapted to a broad range of habitats, including agricultural land, human settlements, and bigger cities. However, its natural habitat includes paddy fields, swamps, mangroves, grasslands, scrublands and forest (Stuart and Wogan, 2012). The diet of *N. kaouthia* covers a broad range of animals from frogs, snakes, small birds to mammals (Cox et al., 1998), and even fish (Kyi and Zug, 2003). *N. kaouthia* is assessed as Least Concern at IUCN Red List of Threatened Species because of its large distribution, tolerance of a broad range of modified habitats, and is reported to be in abundance (Stuart and Wogan, 2012). In Thailand it has been reported that many cases of hospitalization due to snakebite

were caused by *N. kaouthia* (Kulkeaw et al., 2009). Human envenomings by *N. kaothia* are predominantly characterized by neuromuscular paralysis which in severe cases ends up in respiratory paralysis, and by local tissue damage, i.e. swelling, necrosis and blistering (Warrell, 1995; Wongtongkam et al., 2005).

Enzymatic activities and toxicity of the venom of *N. kaouthia* have previously been studied (Pakmanee et al., 1998; Mukherjee and Maity, 2001; Das et al., 2013). Furthermore, a qualitative proteome of the venom has been reported showing the presence of 61 proteins belonging to 12 protein families (Kulkaew et al., 2007). The most prominent component is the long α-neurotoxin α-cobratoxin (Richard et al., 2013), constituting about 25% of the venom (Kulkaew et al., 2009) and belonging to the three-finger toxin family, of which *N. kaouthia* venom has several other members (Kulkaew et al., 2007). Other proteins that have been studied include phospholipases A_2 (PLA_2s) (Joubert and Taljaard, 1980a; Reali et al., 2003; Doley and Mukherjee, 2003), L-amino acid oxidases (Tan and Swaminathan, 1992; Sakurai et al., 2001), cardiotoxins (Joubert and Taljaard, 1980b; Fletcher et al., 1991; Debnath et al., 2010; Jamunaa et al., 2012), and nerve growth factors (Kukhtina et al., 2001). However, a proteomic analysis of this venom that integrates an estimation of relative protein abundances together with a detailed screening of the toxicity of its various components, is pending. An integrated functional and proteomic characterization of *N. kaouthia* venom is relevant not only for a deeper understanding of the composition of the venom and its relationships to toxicity, but also to establish a basic platform for antivenomic analyses.

Developing more potent antivenoms with better safety profiles is of medical relevance. Besides the traditional animal-derived antivenoms generated by immunization of horses with pools of snake venoms (Gutiérrez et al., 2011), various alternative approaches

have been pursued at the experimental level in order to generate more specific antibody-based therapies aimed at neutralizing the most important toxins in the venom. One approach has been to raise murine antibodies specific towards relevant *N. kaouthia* toxins (Charpentier et al., 1990; Masathien et al., 1994). Another approach for identifying potent antitoxins is phage display screening (Roncolato et al., 2015). By using this methodology, inhibitors against *N. kaouthia* toxins have been identified for both PLA_2s (Chavanayarn et al., 2012) and α-cobratoxin (Stewart et al., 2007; Kulkaew et al., 2009; Richard et al., 2013). These studies discovered inhibitors through screening of phage display libraries based on heavy chain fragments obtained from llama. Although promising, it could, however, be argued that llama-derived inhibitors, despite being much smaller than whole IgG antibodies and therefore likely to be less immunogenic, are still non-human. An alternative to overcome this problem is based on modern antibody humanization procedures (Safdari et al., 2013). In another study, inhibitors based on human single chain Fv (scFv) fragments from non-immunized donors were developed (Kulkaew et al., 2009). These scFv fragments were not effective in rescuing mice injected with venom, which is likely to be due to naïve origin of the gene library for these fragments, since none of the human donors had been exposed to snake venom. Thus, the search for novel approaches to generate human antibodies against cobra venom toxins remains a challenge.

With this study, we report for the first time a venomics-based quantitative estimation of the proteome and a full protein lethality profile for *N. kaouthia* venom, in order to lay the foundation for developing a recombinant antivenom by identifying the most relevant toxins present in this venom. Furthermore, we uncover the presence of human IgG antibodies specific to *N. kaouthia* venom in the serum obtained from a unique individual exposed to low doses of a wide variety of snake venoms over a period of more than 25

132 years. The binding ability of his antibodies, as well as of those from a person bitten twice
133 by the Southern Burrowing Asp, *Atractaspis bibronii*, was tested against the different
134 venom fractions from *N. kaouthia*, in comparison to a healthy volunteer unexposed to snake
135 venoms. Finally, the neutralizing ability against *N. kaouthia* venom of the purified human
136 IgG antibodies was evaluated in a mouse model.
137
138 **2. Materials and Methods**
139 *2.1 Snake venom*
140 *Naja kaouthia* venom was obtained from Latoxan SAS, Valence, France. The
141 venom is a pool collected from several specimens, originally from Thailand.
142
143 *2.2 Venom separation by reverse-phase HPLC and SDS-PAGE*
144 Following the 'snake venomics' analytical strategy (Calvete, 2011), crude venom
145 was fractionated by a combination of RP-HPLC and SDS-PAGE separation steps. Venom
146 (2 mg) was dissolved in 200 µL of water containing 0.1% trifluoroacetic acid (TFA;
147 solution A) and separated by RP-HPLC (Agilent 1200) on a C_{18} column (250 x 4.6 mm, 5
148 µm particle; Teknokroma). Elution was carried out at 1 mL/min by applying a gradient
149 towards solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–15% B over 10
150 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously
151 described (Lomonte et al., 2014a). Manually collected fractions were dried in a vacuum
152 centrifuge, redissolved in water, reduced with 5% β-mercaptoethanol at 100 °C for 5 min,
153 and further separated by SDS-PAGE in 12% gels (Bio-Rad). Proteins were stained with
154 colloidal Coomassie blue G-250, and gel images were acquired on a ChemiDoc® recorder
155 using ImageLab® software (Bio-Rad).

156

157 *2.3 Protein identification by tandem mass spectrometry of tryptic peptides*

158 Protein bands were excised from the polyacrylamide gels and subjected to reduction
159 (10 mM dithiothreitol), alkylation (50 mM iodoacetamide), and overnight in-gel digestion
160 with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37°C. The
161 resulting tryptic peptides were extracted with 50% acetonitrile containing 1% TFA, and
162 analyzed by MALDI-TOF-TOF on an AB4800-Plus Proteomics Analyzer (Applied
163 Biosystems). Peptides were mixed with an equal volume of saturated α-cyano-
164 hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), and spotted (1 μL) onto an Opti-
165 TOF 384-well plate, dried, and analyzed in positive reflector mode. TOF spectra were
166 acquired using 1500 shots and a laser intensity of 3000. The ten most intense precursor ions
167 were automatically selected and their TOF/TOF fragmentation spectra were acquired using
168 500 shots at a laser intensity of 3900. External calibration in each run was performed with
169 CalMix® standards (ABSciex) spotted onto the same plate. For protein identification,
170 resulting spectra were searched against the UniProt/SwissProt database using ProteinPilot®
171 v.4 and the Paragon® algorithm (ABSciex) at ≥ 95% confidence, or manually interpreted.
172 Few peptide sequences with lower confidence scores were manually searched using
173 BLAST (http://blast.ncbi.nlm.nih.gov) for protein similarity and protein family assignment.
174 RP-HPLC fractions corresponding to small molecules, eluting in the initial peaks of
175 the chromatogram, were analyzed by ESI-MS/MS on a Q-Trap® 3200 instrument (Applied
176 Biosystems). Samples (10 μL) were loaded into metal-coated capillary tips (Proxeon) and
177 directly infused into a nano-ESI source (Protana) operated at 1300 V. Spectra were
178 acquired in positive Enhanced Resolution mode. nESI-MS performed in this instrument
179 was also used to determine the isotope-averaged mass of intact proteins in selected peaks

180 from the RP-HPLC separation. For this purpose, mass spectra were acquired in Enhanced
181 Multicharge mode in the m/z range 700-1700, and deconvoluted with the aid of the
182 Analyst® v.1.5 software (ABSciex).
183
184 *2.4 Relative protein abundance estimations*
185 The relative abundances of the venom proteins identified were estimated by
186 integrating the areas of their chromatographic peaks at 215 nm, using the ChemStation®
187 software (Agilent), which correlates with peptide bond abundance (Calvete, 2011). For
188 HPLC peaks containing several electrophoretic bands, their percentage distributions were
189 assigned by densitometry, using ImageLab® (Bio-Rad).
190
191 *2.5 Nucleoside and FAD analysis*
192 The presence of selected nucleosides (adenosine, inosine, guanosine), and flavine
193 adenine dinucleotide (FAD) was determined by spiking a sample of 1 mg of venom with 10
194 µg of each nucleoside or FAD, respectively, and separating it by reverse-phase HPLC as
195 described in section 2.2. If the nucleoside or FAD peak coincided with a peak already
196 present in a crude venom sample (as judged by the increment in the height of the peak), and
197 if this venom peak showed an ESI-MS spectrum similar to that of the nucleoside or FAD,
198 the identity of venom component was judged to be the same as the nucleoside or FAD.
199 Further confirmation of the molecular identities of the nucleosides was obtained by
200 acquiring their collision-induced dissociation MS/MS spectra in positive mode, using the
201 Enhanced Product Ion tool of the Analyst v1.5 in the QTrap 3200 mass spectrometer as
202 described (Laustsen et al., 2015). The abundance for nucleosides given in Table 1 is only
203 indicative of an approximate value, due to differences in absorbance at 215 nm between

204 nucleosides and proteins.

205

206 *2.6 In vitro enzymatic activities*

207 *2.6.1. Phospholipase A_2 activity*

208 PLA_2 activity was assayed on the monodisperse synthetic chromogenic substrate 4-
209 nitro-3-octanoyloxybenzoic acid (NOBA) (Holzer and Mackessy, 1996). Twenty-five µL
210 containing various amounts of venom were mixed with 200 µL of 10 mM Tris, 10 mM
211 $CaCl_2$, 0.1 M NaCl, pH 8.0, and 25 µL of NOBA to achieve a final substrate concentration
212 of 0.32 mM. Plates were incubated at 37 °C for 60 min, and absorbances were recorded at
213 405 nm in a microplate reader. Samples were assayed in triplicates.

214

215 *2.6.3 Proteinase activity*

216 Variable amounts of venom (10 to 40 µg) were added to 100 µL of azocasein (10
217 mg/mL in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM $CaCl_2$ buffer, pH 8.0), and incubated for
218 90 min at 37 °C. The reaction was stopped by addition of 200 µL of 5% trichloroacetic
219 acid, and after centrifugation (5 min, 13,000 rpm), 150 µL of supernatants were mixed with
220 100 µL of 0.5 M NaOH, and absorbances were recorded at 450 nm. The absorbance of
221 azocasein incubated with distilled water alone was used as a blank, being subtracted from
222 all readings (Wang et al. 2004). Samples were assayed in triplicates.

223

224 *2.7 Toxicological profiling*

225 *2.7.1 Animals*

226 *In vivo* assays were conducted in CD-1 mice, supplied by Instituto Clodomiro
227 Picado, following protocols approved by the Institutional Committee for the Use and Care

228 of Animals (CICUA), University of Costa Rica. Mice were housed in cages for groups of
229 4–8, and were provided food and water *ad libitum*.
230
231 *2.7.2 Toxicity of crude venom and isolated toxins*
232 The lethality of the whole venom and fractions or isolated toxins was tested by
233 intravenous (i.v.) injection in groups of four mice (18–20 g body weight). Various amounts
234 of venom or fractions/toxins were dissolved in phosphate-buffered saline (PBS; 0.12 M
235 NaCl, 0.04 M sodium phosphate buffer, pH 7.2), and injected in the caudal vein, using an
236 injection volume of 100 µL. Then, deaths occurring within 24 hr were recorded. Median
237 lethal dose (LD_{50}) was calculated by probits (Finney, 1971), using the BioStat® software
238 (AnalySoft).
239 The toxicity of venom fractions was initially screened by selecting a dose based on
240 fraction abundance. The dose was selected to assess whether the fraction would score above
241 or below 5 according to the Toxicity Score defined by Laustsen et al. (2015), which
242 represents the ratio of protein fraction abundance (%) in the venom divided by its estimated
243 LD_{50} in CD-1 mice by i.v. injection. Fractions that were not lethal at this dose (yielding a
244 Toxicity Score < 5) were considered as having insignificant toxicity. All fractions that were
245 lethal at this level, and some fractions killing mice at a Toxicity Score level between 1-5
246 were further evaluated, and precise LD_{50} values were further determined for them. Groups
247 of 3-5 mice per dose were used in order to minimize the use of experimental animals.
248
249 *2.8 Preparation of human IgGs and human serum*
250 *2.8.1 Donor 1*

This donor has over a period of 25 year injected himself with sub-lethal amounts of venom from the following snakes: *Viridovipera* (formerly *Trimeresurus*) *stejnegeri*, *Trimeresurus nebularis*, *T. macrops*, *T. popeorum*, *T. trigonocephalus*, *T. albolabris*, *T. venustus*, *Bothriechis schlegelii*, *Crotalus enyo enyo*, *Naja kaouthia*, *N. naja*, *N. siamensis*, *Agkistrodon contortrix*, *A. bilineatus*, *Bothrops asper*, *Crotalus oreganus*, *Crotalus oreganus helleri*, *Cerastes cerastes*, and *Micrurus fulvius*. In the last 5 years, this individual has been injecting himself with venom from *Naja kaouthia* on an average of every 2 weeks. Brian Lohse has a written consent from the Danish scientific ethical council (H-3-2013-FSP60) to use the blood sample from this donor according to the law of the ethical committee § 2.1. After having provided written and oral information about the project to this donor, written consent was given for obtaining blood samples and for performing the experiments included in the present work. The donor requested the inclusion of his name to be acknowledged in publications.

2.8.2 Donor 2

Donor 2 is an entomologist, who was unfortunate to be bitten twice by the snake *Atractaspis bibronii* (Southern Burrowing Asp) during fieldwork in August 2013. After having provided written and oral information about the project to this donor, written consent was given for obtaining blood samples and for performing the experiments here included. The donor requested the inclusion of his name to be acknowledged in publications.

2.8.3 Blood sampling for obtaining IgGs

200 ml of blood was sampled from each donor in Vacuette® tubes of 4 mL, containing Z Serum Clot Activator (Greiner bio-one). Every tube was gently tilted up and down ten times as soon as it was filled with blood to ensure thorough mixing with the coagulation factor. The tubes were kept at room temperature until full coagulation (30 min). Then, tubes were centrifuged at 3000 g for 10 min, and the serum was transferred to cryotubes and stored at -20 °C.

2.8.4 Protein A purification of IgG antibodies

After filtration of serum through a 0.45 μm membrane, the procedure accompanying the Protein A Antibody Purification Kit (PURE1A, Sigma-Aldrich) was followed in order to obtain human IgG antibodies in solution at physiological pH. IgG antibodies were stored at 4 °C.

2.8.5 Preparation of human serum from healthy volunteer

Blood was obtained from a healthy donor to serve as a negative control. After clotting, serum was separated by centrifugation, and stored at – 20 °C.

2.9 Immunoreactivity of human IgGs against venom fractions by ELISA

Wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 0.6 μg of each HPLC venom fraction dissolved in 100 μL PBS. Then, wells were blocked by adding 100 μL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma) and leaving the plates on a mixer at room temperature for 1 h. Plates were then washed five times with PBS. A dilution of each human IgG pool in PBS + 2% BSA was prepared such that the concentration of IgG proteins was 86 μg/mL (as measured by their absorbance at

280 nm on a NanoDrop® 2000c instrument, Thermo Scientific). Then, 100 µL of these IgG solutions were added to the wells, after which the plates were incubated for 2 h, followed by five additional washings with PBS. Then, 100 µL of a 1:2000 dilution of affinity-purified goat anti-human IgG (gamma-chain specific) antibodies conjugated to alkaline phosphatase (Cappel, Organon Teknika) in PBS + 2% BSA was added to each well. The plates were incubated for 2 h, and then washed five times with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 µM $ZnCl_2$, 1 mM $MgCl_2$, pH 7.4). Color development was achieved by the addition of 100 µL *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8). The absorbances at 405 nm were recorded (Multiskan FC, Thermo Scientific) at several time intervals.

2.10 Neutralization studies with human IgGs

After purification according to section 2.8.4, the human IgGs were kept refrigerated. Six months later, 3 mL of IgGs were dialyzed against 2 L of PBS overnight and lyophilized. The IgGs were reconstituted in 500 µL PBS and the protein concentration was measured on NanoDrop to be 14 mg/ml. IgG was mixed with venom at a ratio of 78 mg IgG/mg venom and incubated at 37 ºC for 30 min. Aliquots of 120 µL of this mixture, containing 4 LD_{50}s of venom, were then injected i.v. into a group of four mice (18-20 g) and deaths occurring within 24 hr were recorded.

3.0 Results and Discussion

3.1 Venomics

A proteomic characterization of *N. kaouthia* venom coupled with an estimation of its protein relative abundances was, with this study, carried out for the first time. Twenty-

322 eight peaks from the venom were resolved by RP-HPLC. The first three fractions eluting
323 from the column were non-peptidic, while the remaining 25 peaks were further resolved
324 into 50 protein bands by SDS-PAGE separation (Fig.1). In-gel digestion and MALDI-TOF-
325 TOF analysis of these yielded a total of 38 identified proteins, while only 4 remained
326 unknown. Altogether, the unidentified venom components represent less than 1% of the
327 total venom protein content, while the rest were either identified or assigned to protein
328 families (Table 1). A previous qualitative proteomic profile was reported for this venom,
329 describing matches to 61 orthologous proteins classified into 12 groups (Kulkaew et al.,
330 2007). Of these, oxoglutarate dehydrogenase complex was identified on the basis of a
331 single peptide that matched such enzyme from a prokaryote (*Burkholderia cenocepacia*),
332 while serum albumin likely corresponds to contaminating traces derived from slight trauma
333 that may occur during venom collection (see for example Lomonte et al., 2014b). In
334 addition, the previous study considered cardiotoxins and cytotoxins as two different protein
335 groups, in spite of these two names being used to refer to the same type of toxins,
336 structurally classified within the three-finger toxin family (Kini and Doley, 2010). Our
337 results are concordant with the remaining eight protein families detected by Kulkaew et al.
338 (2007), but further expand the proteomic profile of *N. kaouthia* venom with the addition of
339 members belonging to the nerve growth factor, ohanin-like/vespryn, C-type lectin/lectin-
340 like, nucleotidase, phosphodiesterase, L-amino acid oxidase, and cytidyltransferase protein
341 families (Table 1 and Fig.2), and moreover, providing a quantitative estimation of their
342 relative abundances in the venom.

343 Peaks 1 to 3 were analyzed by direct infusion using nESI-MS/MS, since they did
344 not show proteins by gel electrophoresis. Peaks 2 and 3 had molecular masses of 268 Da
345 and 283 Da, respectively, and the nucleoside analysis by HPLC (Fig.3) revealed their

346 identities as adenosine and guanosine, respectively. Also, a trace of inosine between peaks
347 2 and 3 was detected by the nucleoside analysis by HPLC (Fig.3).

348 All peptidic peaks were tested *in vivo* for acute toxicity, except for peaks 23 and 26,
349 which yielded very low amounts of proteins (Table 2), and peaks 24, 27, and 28, which
350 correspond to metalloproteinases, enzymes known to be inactivated by the RP-HPLC
351 separation procedure. In Fig.2, the overall protein composition of the venom of *N. kaouthia*,
352 expressed as percentage of total protein and nucleoside content, is represented. Two main
353 families predominate in the venom: Three-finger toxins (3FTx; 77.5%, of which 24.3% are
354 cytotoxins, the rest being neurotoxins) and PLA_2s (PLA_2; 13.5%). In partial agreement with
355 previous findings, the major single component of the three-finger toxins was determined to
356 be alpha-elapitoxin-Nk2a (α-cobratoxin), a long-chain neurotoxin. However, in our results,
357 this toxin represented 32.3% of the whole venom, which is significantly higher than the
358 reported values of 23-25% (Kulkaew et al., 2009). This increase might reflect either
359 geographical variations in venom composition or differences in methodology. As other α-
360 neurotoxins, alpha-elapitoxin-Nk2a is able to bind to nicotinic receptors at the motor end-
361 plate of muscle fibers, thus generating a flaccid paralysis leading to respiratory failure and
362 death (Ultsintong et al., 2009; Alkondon et al., 1990). This type of neurotoxin is
363 responsible for the life-threatening neuromuscular paralysis in human victims of neurotoxic
364 cobra bites (Warrell, 1995).

365 An interesting finding, discovered by the lack of high acute toxicity in the rodent
366 model (Table 2), and reinforced by the difference in human IgG binding in the ELISA
367 assays (Fig.6) was that the protein identified in peak 10 as alpha-elapitoxin-Nk2a appears to
368 correspond to a closely related homolog. In order to validate this, both peak 10 and peak 6

(known to contain alpha-elapitoxin-Nk2a) were analyzed by nESI-MS/MS (Fig.5), according to which their masses were 7619 and 7826 Da, respectively (alpha-elapitoxin-Nk2a has a reported mass of 7831 Da according to: http://www.uniprot.org/uniprot/P01391). Thus, peak 10 should be a homolog of alpha-elapitoxin-Nk2a sharing peptide sequences (Table 1), but different by 207 Da in total mass. Since the toxicity of this protein is markedly lower in comparison to alpha-elapitoxin-Nk2a, further studies to determine its complete amino acid sequence and bioactivities could be of interest, as it represents nearly 10% of the venom proteome.

In addition to α-neurotoxins, other representatives of the three-finger toxin family detected in *N. kaouthia* venom proteome are the so-called 'cytotoxins' (Table 1). These toxins are known to interact with and disrupt the integrity of plasma membrane of various cell types, thus inducing irreversible cell damage (Konshina et al., 2012). These cytotoxins are responsible for the local necrotic effect characteristic of envenomings by *N. kaouthia* (Warrell, 1995). Also, PLA_2 is commonly an abundant component in the majority of elapid venom proteomes characterized so far, and it was detected in our proteomic analysis in *N. kaouthia* venom. PLA_2 activity was confirmed for *N. kaouthia* venom *in vitro*, although it was significantly lower than for *M. nigrocinctus*, a Central American elapid species (Fig.4A). Elapid PLA_2s have been shown to exert a number of toxicological effects, such as myonecrosis (Bhat and Gowda, 1989; Harris, 1991), anticoagulation (Kini, 2005; Doley and Mukherjee, 2003), inhibition of platelet aggregation (Clemetson et al., 2007), and edema (Wang and Teng, 1990). In the case of *N. kaouthia* venom, PLA_2s might contribute to the local inflammatory and necrotizing effects observed in human patients. Furthermore, cardiotoxins and PLA_2s act synergistically to induce cell membrane damage (Harvey et al., 1983), thus potentiating the tissue damaging effect of the venom. Altogether, a high

proportion of the components of *N. kaouthia* venom belong to these two toxin families, i.e. three-finger toxins and PLA_2s. This further supports the concept that high diversity within just a few toxin families may be a general theme within elapid venoms (Calvete, 2013; Laustsen et al., 2015). Such idea is supported also by findings of Vonk et al. (2013) on the genome of the elapid *Ophiophagus hannah* (king cobra), in which the three-finger toxins family was found to be highly diversified.

In amounts each representing <2% of the total venom content, the venom also contains members of the nerve growth factor (NGF), Ohanin/vespryn (OHA), Cysteine-rich secretory protein (CRISP), C-type lectin/lectin-like (CTL), nucleotidase (NUCL), phosphodiesterase (PDE), metalloproteinase (MP), L-amino oxidase (LAO), Cobra venom factor (CVF), and Cytidyltransferase (CTT) families. The low content of proteinases is further supported by the negligible proteinase activity of the venom upon a general substrate such as azocasein (Fig.4B). Also, regarding non-peptidic material, small amounts were identified to be adenosine, inosine, and guanosine, which have also been detected in other snake venoms (Aird, 2002; Laustsen et al., 2015).

The results of toxicity testing of the fractions are summarized in Table 2. Unsurprisingly, the lethality of the venom stems from the presence of potent α-neurotoxins and cytotoxins, with PLA_2s playing a secondary role leading to local tissue damage at the bite wound. A Toxicity Score is shown in Table 2, which takes into account both potency and abundance. Toxins with a higher score, as estimated in mice, are expected to be of higher medical relevance. These are abundant and/or very potent, while toxins with a low score are less medically relevant. Based on this score, it is clear that alpha-elapitoxin-Nk2a is by far the toxin that is most clinically relevant to neutralize, with a value of 326. Some toxins have very low LD_{50}s (particularly fractions 4 and 5), but due to their lower

417 abundance their Toxicity Scores are much lower. Others have intermediate LD_{50} potencies,
418 but become relevant due to their high abundance (e.g. fraction 16). The cytotoxins present
419 in the venom act by a different mechanism than the α-neurotoxins, providing *N. kaouthia*
420 venom with a dual strategy for prey subduction. It is interesting to note that the
421 accumulated Toxicity Scores for all toxins amount to the same value as the Toxicity Score
422 for the whole venom. This is not the case for the venom from the related elapid,
423 *Dendroaspis polylepis*, which shows evidence of synergism, and where the accumulated
424 Toxicity Scores is considerably lower than the Toxicity Score of whole venom (indicating
425 synergism between the toxins and not simply additive effect) (Laustsen et al., 2015). One
426 interpretation of this finding could be that *N. kaouthia* venom does not exert significant
427 synergism, but instead relies on two complementary strategies for acute toxicity.

428 In terms of therapeutic targets, based on their Toxicity Score, the most relevant
429 toxins in *N. kaouthia* venom that should be neutralized by an effective antivenom are (in
430 order of importance): alpha-elapitoxin-Nk2a (P01391), cobrotoxin-c (P59276) and
431 cobrotoxin-b (P59275), and the homolog of three-finger toxin from *Naja atra (*E2IU03),
432 while the cytotoxins are of less importance in terms of overall lethality, although they are
433 likely to play a relevant role in local necrosis, and hence should be neutralized by
434 antivenoms. In addition, neutralization of PLA_2s should also be considered a priority in
435 order to limit local tissue damage from envenomings. Thus, antivenomic studies with *N.*
436 *kaouthia* venom should establish whether antivenoms are able to bind these toxins.

437

438 *3.2 Immunoreactivity of human IgGs against venom fractions*

439 The isolated human IgGs from the unique individual who had self-injected small
440 amounts of venom from various different snakes, including *N. kaouthia*, showed significant

441 binding ability to isolated venom fractions, clearly above the background established by
442 serum of a healthy donor, or by IgGs from the donor bitten twice by a different snake
443 species (Fig.6). It is evident that the antibody response of the individual exposed to *N.*
444 *kaouthia* venom was higher against larger toxins present in later eluting peaks, while the
445 response was limited against the most important α-neurotoxins (peaks 4, 5, and 6), which
446 could be due to the low immunogenicity of small toxins (Fernández et al., 2011). Therefore,
447 it was not surprising that IgGs were unable to neutralize the lethality of whole venom when
448 mice were challenged with 4 LD_{50}s mixed with IgGs at a level of 0.013 mg toxin per mg of
449 IgGs. Nevertheless, it could be particularly interesting to further investigate these
450 antibodies via phage display screening. In llamas, it has been shown that using naïve
451 libraries for phage display screening did not yield high affinity inhibitors to snake toxins,
452 while excellent inhibitors of alpha-elapitoxin-Nk2a were discovered this way, when a
453 library from an immunized llama was used (Richard et al., 2013). Kulkeaw et al. (2009)
454 were the first to report drug discovery effort using a human phage display library. However,
455 they similarly did not find strong inhibitors of snake toxins, most likely given the fact that
456 their library was naïve. Therefore, using the antibody genes from the unique donor to
457 construct a phage display library, and subsequently screening this library, has the potential
458 to yield toxin inhibitors of therapeutic value. Further studies of the donor's immune
459 response against toxins from other snake species are warranted and may help direct phage
460 display screening efforts towards the generation of new antibodies of human origin.
461
462 **4.0 Concluding remarks and outlook**
463 Differing from previous proteomic studies on *N. kaouthia* venom, the present work
464 combined the cataloguing of venom proteins with an estimation of their relative

abundances, together with an assessment of their lethal toxicity for mice. This approach uncovered that the most abundant group of proteins was three-finger toxins, followed by PLA_2s. Based on an evaluation of the accumulation of Toxicity Scores and knowledge about toxin functions, the venom seems to have a dual strategy for prey subduction elicited by α-neurotoxins and cytotoxins, between which pharmacological interaction is speculated only to be of additive value rather than synergism.

ELISA immunoprofiling of human IgGs isolated from a human donor, who had repeatedly injected himself with snake venom from various different species, revealed presence of antibodies with high binding ability to several of the isolated venom fractions, although not against the medically most relevant toxins of *N. kaouthia*. Still, the presence of these antibodies opens the prospect of discovering inhibitors of therapeutic value.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest related to this study. Sources that provided financial support were not involved in the collection, analysis, or interpretation of data, nor in writing the report and submitting it for publication.

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677

678 **Figure legends**

679

680 **Figure 1:** Separation of *N. kaouthia* **(A)** venom proteins by RP-HPLC **(C)**, followed by
681 SDS-PAGE **(B)**. Two mg of venom were fractionated on a C_{18} column and eluted with an
682 acetonitrile gradient (dashed line), as described in Methods**.** Fractions were further
683 separated by SDS-PAGE under reducing conditions. Molecular weight markers (Mw) are
684 indicated in kDa. Coomassie-stained bands were excised, in-gel digested with trypsin, and
685 subjected to MALDI-TOF/TOF analysis for assignment to protein families, as shown in
686 Table 1.

687

688 **Figure 2:** Composition of *N. kaouthia* venom according to protein families, expressed as
689 percentages of the total protein content. 3FTx: three-finger toxin; PLA_2: phospholipase A_2;
690 NGF: nerve growth factor; OHA: Ohanin/vespryn; CRISP: Cysteine-rich secretory protein;
691 CTL: C-type lectin/lectin-like; NUCL: nucleotidase; PDE: phosphodiesterase; MP:
692 metalloproteinase; LAO: L-amino oxidase; CVF: Cobra venom factor; CTT:
693 Cytidyltransferase; UNK: Unidentified proteins; NP: non-protein components. A division
694 between cytotoxins (CYT) and neurotoxins (NTX) is given for the three-finger toxins.

695

696 **Figure 3:** Presence of selected nucleosides and FAD in *Naja kaouthia* venom shown by
697 spiking crude venom with 10 µg of nucleosides (adenosine, inosine, guanosine) or FAD and
698 separating the venom components by reverse-phase HPLC. If a the peak of a nucleoside
699 coincides with the peak of a venom component, and if mass determination yielded the same
700 mass for the venom component as calculated for the nucleoside, the venom component was

judged to consist of the corresponding nucleoside. *N*. *kaouthia* venom contains small amounts of adenosine, guanosine, and traces of inosine, but no FAD.

Figure 4: (A) Comparison of the phospholipase A_2 activity between the venoms of *Naja kaouthia* and *Micrurus nigrocinctus*. *N*. *kaouthia* displays some enzymatic activity, although significantly less than *M*. *nigrocinctus*. **(B)** Comparison of the proteolytic activity between the venoms of *N*. *kaouthia*, *M*. *nigrocinctus*, and *Bothrops asper*, evaluated on azocasein. *N*. *kaouthia* shows negligible proteinase activity.

Figure 5: Electrospray ionization-mass spectrometry analysis of *N*. *kaouthia* venom fractions 6 and 10 (see Fig.1 and Table 1). Samples were analyzed in enhanced multi-charge positive mode as described in Methods. Fraction 6 (Nk-6) presented a homogeneous multi-charged series **(A)** which deconvoluted to an isotope-averaged mass (M_{av}) of 7826.0 ± 0.8 Da **(B)**. The multi-charged series of fraction 10 (Nk-10) **(C)** showed a main protein with M_{av} 7619.0 ± 1.3 Da and a less abundant protein of 7656.0 ± 0.7 Da **(D)**, thus demonstrating difference between fractions 6 and 10.

Figure 6: ELISA-based immunoprofiling of human antibodies (**Donor 1 IgG**: protein A-purified IgG antibodies from Donor 1, **Donor 2**: protein A-purified IgG antibodies from Donor 2, **Human negative:** normal human serum from healthy volunteer, never bitten by a snake) to the fractions of *Naja kaouthia* venom separated by RP-HPLC (see Materials and Methods for details). Toxins with the highest Toxicity Score (4, 5, and 6) are marked with a skull symbol.

Table 1: Assignment of the RP-HPLC isolated fractions of *Naja kaouthia* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

Peak	%	Mass (kDa)▼	Peptide ion		MS/MS-derived or N-terminal (Nt) sequence	Conf (%)	Sc	Protein family; ~ related protein *
			m/z	z				
1	0.2	-	-	1	non-peptidic	-	-	unknown
2	0.4	-	-	1	non-peptidic (Adenosine)	-	-	non-peptidic (Nucleosides)
3	0.5	-	-	1	non-peptidic (Guanosine)	-	-	non-peptidic (Nucleosides)
4	1.2	13	2945.2	1	XECHNBBSSBTPTTTGCSGGETNCYK	99	25	**3FTx** three-finger toxin *Naja atra*, ~E2IU03
5a	0.3	67	1475.7	1	SXDXXBBWEEBS(R^{lo})	99	11	**Cytidyltransferase** choline-phosphate cytidylyltransferase A isoform X2 *Python bivittatus*, ~XP_007440684
5b	0.4	54	-	-	-	-	-	unknown
5c	3.3	10	1728.7	1	XECHNBBSSBAPTTK	99	15	**3FTx**
			1014.5	1	BWWSDHR	99	8	cobrotoxin-c *Naja kaouthia,* ~P59276
			1316.6	1	VBPGVNXNCCR	99	11	
			1453.6	1	(N^{da})GXEXNCCTTDR	99	12	three-finger toxin *Naja atra,* ~E2IU03
			2945.2	1	XECHNBBSSBTPTTTGCSGGETNCYK	99	28	
			1758.8	1	XECHNBBSSBTPTTK	99	13	cobrotoxin-b *Naja kaouthia,* ~P59275
6a	13.5	13	1315.5	1	TWCDAFCSXR	99	13	**3FTx**
			2241.9	1	TGVDXBCCSTDNCNPFPTR	99	16	alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391
6b	18.8	10	1547.7	1	RVDXGCAATCPTVK	99	13	**3FTx**
			2184.9	1	TGVDXBCCSTDNCNPFPTR	99	13	alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391
			1315.5	1	TWCDAFCSXR	99	14	
			2241.9	1	TGVDXBCCSTDNCNPFPTR	99	24	
7	0.9	10	1315.5	1	TWCDAFCSXR	99	13	**3FTx**
			2241.9	1	TGVDXBCCSTDNCNPFPTR	99	21	alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391
			2187.9	1	SXFGVTTEDCPDGBNXCFK	99	23	muscarinic toxin-like prot.2 *Naja kaouthia*,
			1011.5	1	WH(M^{ox})XVPGR	82.8	8	~P82463
8	4.1	10	1629.6	1	XTCXNCPEMFCGK	99	16	**3FTx**
			2578.0	1	GCADTCPVGBPYEMXECCSTDK	99	28	weak tryptophan-containing neurotoxin, *Naja*
			2391.0	1	XTCXNCPEMFCG(K^{ca})FBXCR	99	11	*kaouthia,* ~P82935

9a	0.3	10	1415.6	1	CBNPNPEPSGCR	99	10	**Nerve growth factor**
			1363.6	1	AXTMEGNBASWR	99	18	NGF *Naja kaouthia*, ~P61899
			2603.1	1	EDHPVHNXGEHSVCDSVSAWVTK	99	29	
			2260.9	1	GXDSSHWNSYCTETDTFXK	99	27	
			962.4	1	BYFFETK	99	11	NGF *Naja sputatrix*, ~Q5YF89
9b	0.4	10	1177.5	1	TXCYNHXTR	99	7	**3FTx**
			2186.8	1	TSETTEXCPDSWYFCYK	99	17	muscarinic toxin-like prot.3 *Naja kaouthia*, ~P82464
			2241.9	1	TGVDXBCCSTDNCNPFPTR	99	11	
			1315.5	1	TWCDAFCSXR	98.8	10	alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391
			1629.6	1	XTCXNCPEMFCGK	99	14	weak neurotoxin NNAM2 *Naja atra*, ~Q9YGI4
10	10.2	10	1315.5	1	TWCDAFCSXR	99	13	**3FTx**
			2241.9	1	TGVDXBCCSTDNCNPFPTR	99	15	alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391
11a	4.9	15	1842.7	1	SWWDFADYGCYCGR	99	10	**Phospholipase A_2** acidic PLA_2 - 2 *Naja sagittifera*, ~P60044
11b	2.0	10	1826.6	1	CCBVHDNCYNEAEK	99	22	**Phospholipase A_2**
			1669.5	1	GDNDACAAAVCDCDR	99	20	acidic PLA_2 - 1 *Naja kaouthia*, ~P00596
			1232.5	1	NMXBCTVPNR	99	13	
			1697.6	1	TYSYECSBGTXTCK	99	16	
			1842.6	1	SWWDFADYGCYCGR	99	18	
			900.4	1	CWPYFK	98,2	9	
12a	2.1	15	1842.6	1	SWWDFADYGCYCGR	99	11	**Phospholipase A_2** acidic PLA_2 - 1 *Naja sputatrix*, ~Q9I900
12b	2.0	10	1610.5	1	GGNNACAAAVCDCDR	99	20	**Phospholipase A_2**
			1842.6	1	SWWDFADYGCYCGR	99	19	acidic PLA_2 - 1 *Naja atra*, ~P00598
			1697.6	1	TYSYECSBGTXTCK	99	14	
			1157.5	1	XSGCWPYFK	99	13	
			1826.6	1	CCBVHDNCYNEAEK	99	20	
			2356.0	1	XAAXCFAGAPYNNNNYNXDXK	99	22	
13a	2.5	22	1842.6	1	SWWDFADYGCYCGR	99	9	**Phospholipase A_2** acidic PLA_2 - D *Naja sputatrix*, ~Q9I900
13b	5	10	2829.3	1	TCPAGBNXCYBMFMVSNBTVPVBR	99	14	**3FTx**
			1087.4	1	YVCCNTDR	99	10	cytotoxin 3 *Naja kaouthia*, ~P01446
			1798.8	1	NSXXV(K^{ca})YVCCNTDR	99	15	
			1842.6	1	SWWDFADYGCYCGR	99	14	**Phospholipase A_2**

								acidic PLA_2 - D *Naja sputatrix*, ~Q9I900
14a	1.3	25	1087.4	1	YVCCNTDR	91.6	7	**3FTx** cytotoxin VC-1 *Naja oxiana*, ~Q9PS33
14b	1.1	10	2829.3	1	TCPAGBNXCYBMFMVSNBTVPVBR	99	10	**3FTx**
			1087.4	1	YVCCNTDR	99	12	cytotoxin 2 *Naja kaouthia*, ~P01445
			1798.8	1	NSXXV(K^{ca})YVCCNTDR	99	15	
15	2.4	11	1412.6	1	MFMMSDXTXPVK	99	11	**3FTx**
			1798.8	1	NSXXV(K^{ca})YVCCNTDR	99	15	cytotoxin VC-1 *Naja oxiana*, ~Q9PS33
			1568.7	1	MFMMSDXTXPVBR	99	14	
			1087.4	1	YVCCNTDR	99	10	
			1420.6	1	CHNTBXPFXYK	99	16	cytotoxin 5V *Naja atra*, ~Q9W716
16a	5.0	21	1087.5	1	YVCCNTDR	man	man	**3FTx** three-finger toxin *Naja atra*, ~E2IU04
16b	3.7	10	2861.3	1	TCPAGBNXCYBMFMMSDXTXPVBR	99	11	**3FTx**
			1412.6	1	MFMMSDXTXPVK	99	11	three-finger toxin *Naja atra*, ~E2IU04
			1568.7	1	MFMMSDXTXPVBR	99	17	
			1798.7	1	NSXXV(K^{ca})YVCCNTDR	99	11	
			1087.4	1	YVCCNTDR	99	10	
17a	0.5	12	2141.9	1	ADVTFDSNTAFESXVVSPDK	99	15	**Ohanin/vespryn**
			2270.0	1	ADVTFDSNTAFESXVVSPDBK	99	17	thaicobrin *Naja kaouthia*, ~P82885
			930.4	1	EWAVGXAGK	99	13	
			913.4	1	SPPGNWBK	99	10	
			1810.8	1	TVENVGVSBVAPDNPER	99	19	
			1495.6	1	FDGSPCVXGSPGFR	99	19	
17b	1.2	10	1771.8	1	SSXXV(K^{ca})YVCCNTDR	99	14	**3FTx**
			2918.2	1	GCXDVCPBSSXXVBYVCCNTDRCN	83,4	7	cytotoxin 4N *Naja atra*, ~Q9W6W9
			1087.4	1	YVCCNTDR	99	11	
			1798.8	1	NSXXV(K^{ca})YVCCNTDR	99	16	cytotoxin 3a *Naja atra*, ~Q98959
18a	0.8	13	1315.5	1	TWCDAFCSXR	94.7	8	**3FTx** alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391
18b	4.6	10	1087.4	1	YVCCNTDR	99	10	**3FTx**
			948.4	1	GCXDVCPK	99	10	cytotoxin 8 *Naja atra*, ~Q91124
			924.4	1	MFMVATPK	99	10	
			2918.3	1	GCXDVCPBSSXXVBYVCCNTDRCN	99	11	
			1771.8	1	SSXXV(K^{ca})YVCCNTDR	99	14	
19	0.2	10	-	-	-	-	-	unknown

20a	0.1	57	-	-	-	-	-	unknown
20b	0.5	20	2102.9	1	XGCGENXFMSSBPYAWSR	99	16	**Cysteine-rich secretory protein**
			1409.7	1	VXBSWYDENBK	99	11	natrin-2 *Naja atra*, ~Q7ZZN8
20c	0.1	12	1810.9	1	TVENVGVSBVAPDNPER	99	17	**Ohanin/vespryn**
			2270.0	1	ADVTFDSNTAFESXVVSPDBK	99	10	thaicobrin *Naja kaouthia*, ~P82885
			1495.7	1	FDGSPCVXGSPGFR	99	12	
20d	0.1	10	-	-	-	-	-	unknown
21	0.3	11	1296.6	1	BYXWEWTDR	99	11	**C-type lectin/lectin-like**
			1168.5	1	YXWEWTDR	99	12	LP-Pse-6 CTL *Pseudonaja modesta*, ~R4G314
22a	1.1	21	2013.9	1	VXEGXBCGESXYMSSNAR	99	23	**Cysteine-rich secretory protein**
			1434.7	1	ZBEXVDXHNSXR	99	17	natrin-1 *Naja atra*, ~Q7T1K6
			1553.6	1	MEWYPEAASNAER	99	16	
			3174.5	1	NFVYGVGANPPGSVTGHYTBXVWYBTYR	99	15	
			1870.8	1	TWTEXXHXWHDEYK	99	21	
			1258.4	1	SNCPASCFCR	99	14	
			1784.8	1	WANTCSXNHSPDNXR	99	23	
22b	0.1	15	1553.6	1	MEWYPEAASNAER	99	13	**Cysteine-rich secretory protein**
			1784.7	1	WANTCSXNHSPDNXR	99	18	natrin-1 *Naja atra*, ~Q7T1K6
			1870.8	1	TWTEXXHXWHDEYK	99	15	
			2013.9	1	VXEGXBCGESXYMSSNAR	99	14	
22c	0.1	12	1168.5	1	YXWEWTDR	99	11	**C-type lectin/lectin-like**
			1296.6	1	BYXWEWTDR	97.6	8	LP-Pse-6 CTL *Pseudonaja modesta*, ~R4G314
22d	0.1	10	1168.5	1	YXWEWTDR	98.5	7	**C-type lectin/lectin-like**
								LP-Pse-6 CTL *Pseudonaja modesta*, ~R4G314
23a	0.1	56	1470.8	1	XPVYSAYVYNPGK	99	12	**Nucleotidase**
			1695.9	1	SSTFTXTNXVPBFXK	99	8	endonuclease domain-containing 1 prot *Micrurus*
			1635.8	1	GHXNPNGHBPDYSAK	99	13	*fulvius,* ~tr|U3FCT9
			2024.0	1	EVVDSFBDHCPBFFXR	99	9	
23b	0.2	26	2080.9	1	GEVVDSFBDHCPBFFXR	99	10	**Nucleotidase**
			2023.8	1	EVVDSFBDHCPBFFXR	99	14	endonuclease domain-containing 1 prot, *Micrurus*
			1695.8	1	SSTFTXTNXVPBFXK	96.9	8	*fulvius,* ~U3FCT9
24a	0.1	250	1430.8	1	YXEFYVXVDNR	99	10	**Metalloproteinase**
								kaouthiagin *Naja kaouthia*, P82942
24b	0.2	66	2689.1	1	HDCDXPEXCTGBSAECPTDSXBR	99	7	**Metalloproteinase**
			1099.6	1	DYBEYXXR	99	9	kaouthiagin *Naja kaouthia*, P82942
			1471.7	1	TAPAFBFSSCSXR	99	17	

			1430.8	1	YXEFYVXVDNR	99	15	
24c	0.2	57	1099.5	1	DYBEYXXR	99	10	**Metalloproteinase**
			2689.1	1	HDCDXPEXCTGBSAECPTDSXBR	99	13	kaouthiagin *Naja kaouthia*, P82942
			1471.7	1	TAPAFBFSSCSXR	99	14	
			1430.7	1	YXEFYVXVDNR	99	15	
24d	0.7	40	2354.1	1	CPTXTNBCXAXXGPHFTVSPK	99	25	**Metalloproteinase**
			1471.6	1	TAPAFBFSSCSXR	99	17	kaouthiagin *Naja kaouthia*, ~P82942
			1912.6	1	(N^{da})GHPCBNNBGYCY(N^{da})GK	99	23	
			1012.4	1	GCFDXNMR	99	11	
			1430.7	1	YXEFYVXVDNR	99	13	
			2689.0	1	HDCDXPEXCTGBSAECPTDSXBR	99	25	
			1099.5	1	DYBEYXXR	99	12	
25a	0.1	139	1434.7	1	DFYTFDSEAXVK	99	8	**Phosphodiesterase**
			1244.7	1	SP(N^{da})NXWVEER	99	9	phosphodiesterase 1 *Micrurus fulvius*, ~U3FAB3
			2106.1	1	RPDFSTXYXEEPDTTGHK	99	11	
25b	0.3	108	1434.7	1	DFYTFDSEAXVK	99	16	**Phosphodiesterase**
			1244.7	1	SP(N^{da})NXWVEER	99	15	phosphodiesterase 1 *Micrurus fulvius*, ~U3FAB3
			2106.1	1	RPDFSTXYXEEPDTTGHK	99	20	
			1634.7	1	YCSGGTHGYDNEFK	99	14	
			1518.9	1	XWNYFHSTXXPK	99	11	
			1355.7	1	AATYFWPGSEVK	99	9	
25c	0.1	61	1449.8	1	VVSXNVXCTECR	99	8	**5'-nucleotidase**
			2421.3	1	FHECNXGNXXCDAVVYNNXR	99	9	ecto-5'-nucleotidase 1 *Micrurus fulvius*, ~U3FYP9
			1719.0	1	ETPVXSNPGPYXEFR	99	10	
			1459.8	1	YXGYXNVXFDDK	93,5	7	
26a	0.1	250	1339.7	1	EGWYVNMGPMR	99	9	**L-amino acid oxidase**
			1718.9	1	TFVTADYVXVCSTSR	99	15	L-amino-acid oxidase *Naja atra*, ~A8QL58
			2517.2	1	SPXEECFBBNDYEEFXEXAR	99	20	L-amino-acid oxidase *Naja oxiana*, ~P0DI91
26b	0.1	70	2030.0	1	FFYXDGNENFHVSXTAR	99	12	**Cobra venom factor**
			1386.7	1	BXDXFVHDFPR	99	10	CVF *Naja kaouthia*, Q91132
			1875.1	1	XXXNXPXNABSXPXTVR	99	13	
26c	0.3	55	2673.3	1	RSPXEECFBBNDYEEFXEXAR	99	8	**L-amino acid oxidase**
			2922.5	1	FGXBXNEFFBENENAWYYXNNXR	99	10	L-amino-acid oxidase *Naja oxiana*, ~P0DI91
			2517.2	1	SPXEECFBBNDYEEFXEXAR	99	17	
27	0.3	46	1603.8	1	VYEMVNAXNTMYR	99	11	**Metalloproteinase**
			1235.7	1	VTXDXFGEWR	99	12	mocarhagin, *Naja mossambica,* ~Q10749
28	0.9	44	2713.2	1	DDCDXPEXCTGBSAECPTDVFBR	99	11	**Metalloproteinase**

1207.6	1	ATXDXFGEWR	99	10	cobrin *Naja kaouthia*, ~Q9PVK7
1807.9	1	YXEFY(M^{dt})VVDNXMYR	99	12	
2983.4	1	AABDDCDXPEXCTGBSAECPTDVFBR	99	10	

* Cysteine residues are carbamidomethylated, unless underlined. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot® (ABSciex) ▼: estimated mass by SDS-PAGE under reducing conditions, in kDa. ∇: observed isotope-averaged masses as determined by nESI-MS of selected RP-HPLC peaks, in Da. X: Leu/Ile; B: Lys/Gln; Z: pyroglutamate (2-oxo-pyrrolidone carboxylic acid). Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: ^{ox}: oxidized; ^{da}: deamidated; ^{ca}: carbamyl; $^{am:}$ amide; $^{na:}$ Na cation; $^{al:}$ ammonia loss; $^{dl:}$ delta^{H2C2}; man: manually interpreted MS/MS spectrum.

Table 2: Median lethal doses (LD_{50}s) of *Naja kaouthiua* venom and the RP-HPLC isolated fractions

Peak	%	Protein family	LD_{50} (95% C.L.)	Reported LD_{50} (mg/kg)	Toxicity Score[1] % / LD_{50} (kg/mg)
Whole venom			0.24 (0.12-0.41)	0.115*	423.2
4	1.2	**3FTx** three-finger toxin *Naja atra*, ~E2IU03	0.24 (0.12-0.41)		15.4
5	4.0 (3:4:33 mix[2])	**Cytidyltransferase (7.5%)** choline-phosphate cytidylyltransferase A isoform X2 *Python bivittatus*, ~XP_007440684 **Unknown (10%)** **3FTx (82.5%)** cobrotoxin-c *Naja kaouthia,* ~P59276 three-finger toxin *Naja atra,* ~E2IU03 cobrotoxin-b *Naja kaouthia,* ~P59275	0.07 (0.001-0.19)	80 (cobrotoxin-c), 400 (cobrotoxin-b) [Meng et al., 2002]	41.3
6	32.3	**3FTx** alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391	0.10 (0.02-0.18)	0.1 [Karlsson, 1973]	326
7	0.9	**3FTx** alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391 muscarinic toxin-like prot.2 *Naja kaouthia*, ~P82463	0.36 (0.17-0.72)		2.5
8	8.0	**3FTx** weak tryptophan-containing neurotoxin, *Naja kaouthia,* ~P82935	~2		~4
9	0.7 (3:4 mix[2])	**Nerve growth factor (43%)** NGF *Naja kaouthia*, ~P61899 NGF *Naja sputatrix*, ~Q5YF89 **3FTx (57%)** muscarinic toxin-like prot.3 *Naja kaouthia*, ~P82464 alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391 weak neurotoxin NNAM2 *Naja atra*, ~Q9YGI4	>1		<1

10	10.2	**3FTx** alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391	>10		<1
11	6.9 (49:20 mix[2])	**Phospholipase A_2** acidic PLA_2 - 2 *Naja sagittifera*, ~P60044 (71%) acidic PLA_2 - 1 *Naja kaouthia*, ~P00596 (29%)	5.38 (2.01-55.94)	10 [Chuman et al., 2000]	1.3
12	4.1 (51:49 mix[2])	**Phospholipase A_2** acidic PLA_2 - 1 *Naja sputatrix*, ~Q9I900 (51%) acidic PLA_2 - 1 *Naja atra*, ~P00598 (49%)	3.74 (2.17-8.75)		1.1
13	7.5 (1:2 mix[2])	**Phospholipase A_2 (33%)** acidic PLA_2 - D *Naja sputatrix*, ~Q9I900 **3FTx (66%)** cytotoxin 3 *Naja kaouthia*, ~P01446	0.9 (0.09-1.29)		8.3
14	2.4 (27:23 mix[2])	**3FTx** cytotoxin VC-1 *Naja oxiana*, ~Q9PS33 (54%) cytotoxin 2 *Naja kaouthia*, ~P01445 (46%)	1.60 (1.21-2.28)	1.2 (cytotoxin 2) [Joubert and Taljaard, 1980b]	1.57
15	2.4	**3FTx** cytotoxin VC-1 *Naja oxiana*, ~Q9PS33	1-2.5		<2.5
16	8.7	**3FTx** three-finger toxin *Naja atra*, ~E2IU04	0.75 (0.30-1.66)		11.6
17	1.7 (5:12 mix[2])	**Ohanin/vespryn (29%)** thaicobrin *Naja kaouthia*, ~P82885 **3FTx (71%)** cytotoxin 4N *Naja atra*, ~Q9W6W9 cytotoxin 3a *Naja atra*, ~Q98959	1.36 (0.62-2.03)		1.25
18	5.4 (4:23 mix[2])	**3FTx (15%)** alpha-elapitoxin-Nk2a *Naja kaouthia*, ~P01391 **3FTx (85%)** cytotoxin 8 *Naja atra*, ~Q91124	1.00 (0.43-2.93)		5.4
19	0.2	**Unknown**	>0.25		<1

20	0.8 (2:5:1 mix[2])	**Unknown (25%)** **Cysteine-rich secretory protein (62.5%)** natrin-2 *Naja atra*, ~Q7ZZN8 **Ohanin/vespryn (12.5%)** thaicobrin *Naja kaouthia*, ~P82885	>1	<1
21	0.3	**C-type lectin/lectin-like** LP-Pse-6 CTL *Pseudonaja modesta*, ~R4G314	>0.5	<1
22	1.4 (6:1 mix[2])	**Cysteine-rich secretory protein (86%)** natrin-1 *Naja atra*, ~Q7T1K6 **C-type lectin/lectin-like (14%)** LP-Pse-6 CTL *Pseudonaja modesta*, ~R4G314	>1.5	<1
23	0.3	**Nucleotidase** endonuclease domain-containing 1 prot *Micrurus fulvius,* ~tr|U3FCT9		N.t.
24	1.2	**Metalloproteinase** kaouthiagin *Naja kaouthia*, P82942		N.t.
25	0.5 (4:1 mix[2])	**Phosphodiesterase (80%)** phosphodiesterase 1 *Micrurus fulvius*, ~U3FAB3 **5'-nucleotidase (20%)** ecto-5'-nucleotidase 1 *Micrurus fulvius*, ~U3FYP9	>0.25	<2
26	0.5 (1:1:3 mix[2])	**L-amino acid oxidase (20%)** L-amino-acid oxidase *Naja atra*, ~A8QL58 L-amino-acid oxidase *Naja oxiana*, ~P0DI91 **Cobra venom factor (20%)** CVF *Naja kaouthia*, Q91132 **L-amino acid oxidase (60%)** L-amino-acid oxidase *Naja oxiana*, ~P0DI91		N.t.
27	0.3	**Metalloproteinase** mocarhagin, *Naja mossambica,* ~Q10749		N.t.
28	0.9	**Metalloproteinase** cobrin *Naja kaouthia*, ~Q9PVK7		N.t.

*http://snakedatabase.org/pages/LD50.php#legendAndDefinitions

[1] Toxicity Score was defined as the ratio of protein fraction abundance (%) in the venom divided by its estimated median lethal dose (LD_{50}) for CD-1 mice by i.v. injection.

[2] Mix indicates that the fraction did not contain a pure, isolated toxin, but instead a mixture of 2-4 different toxins in variable ratios indicated in the table.

N.t. : not tested

Figure 1
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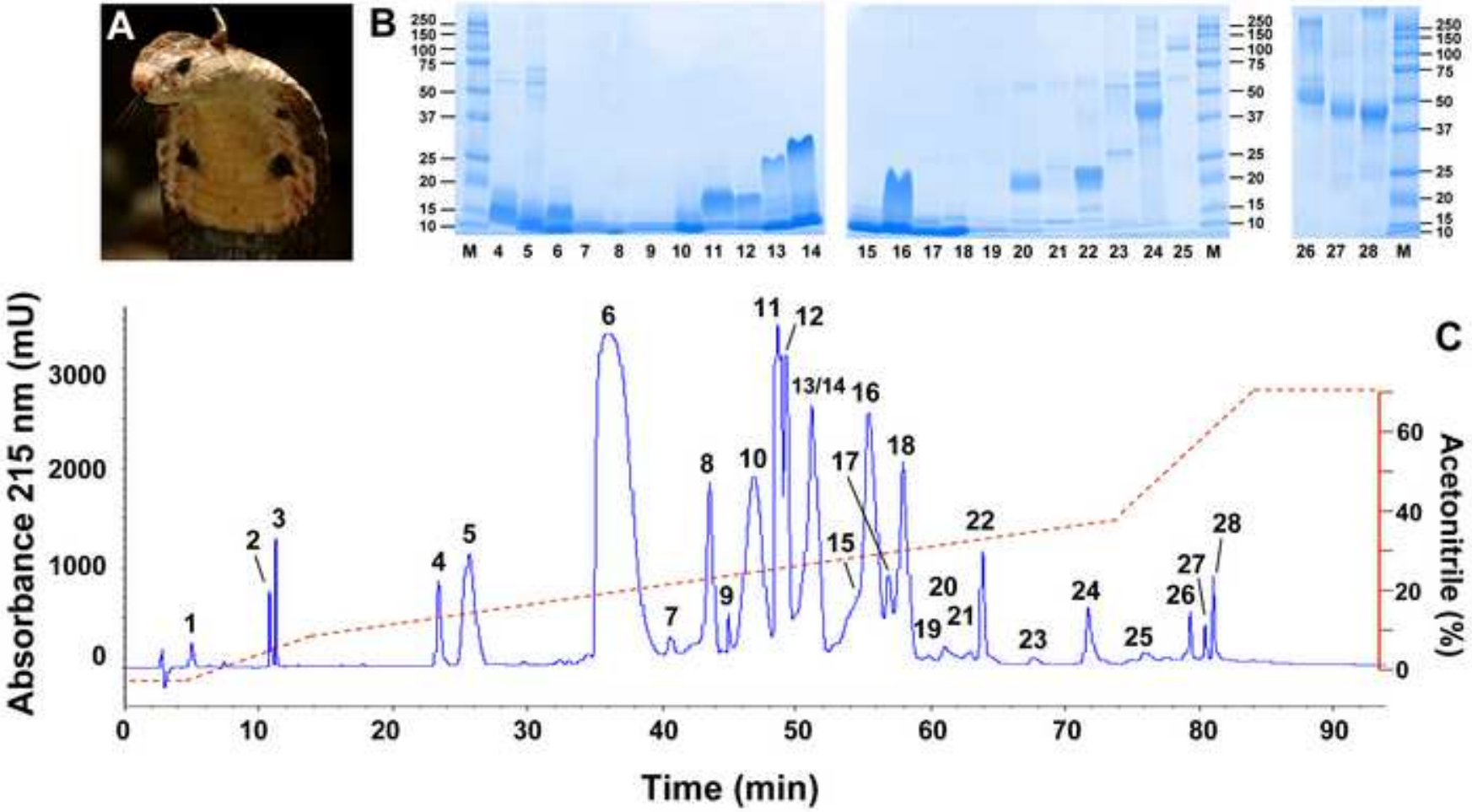

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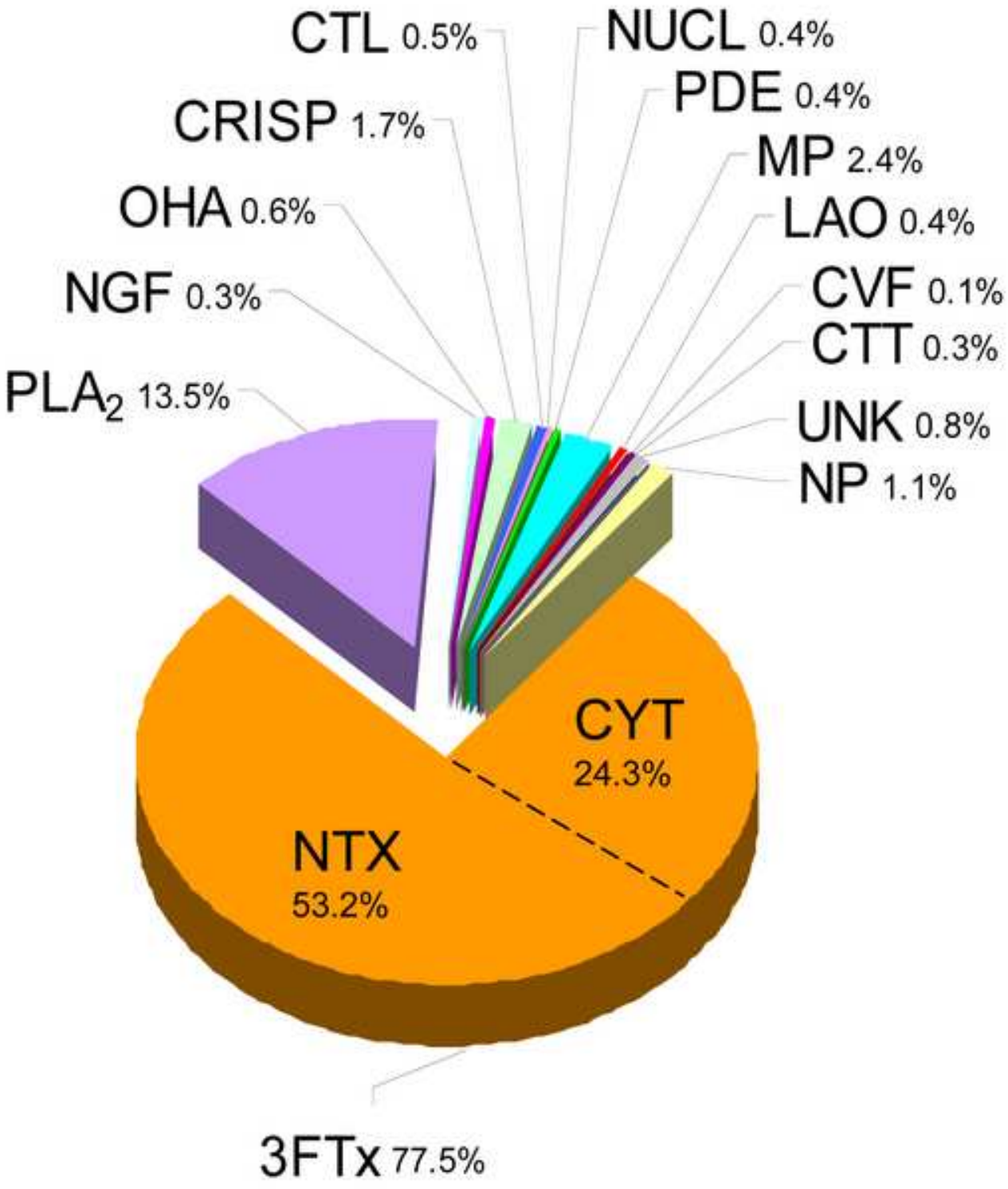

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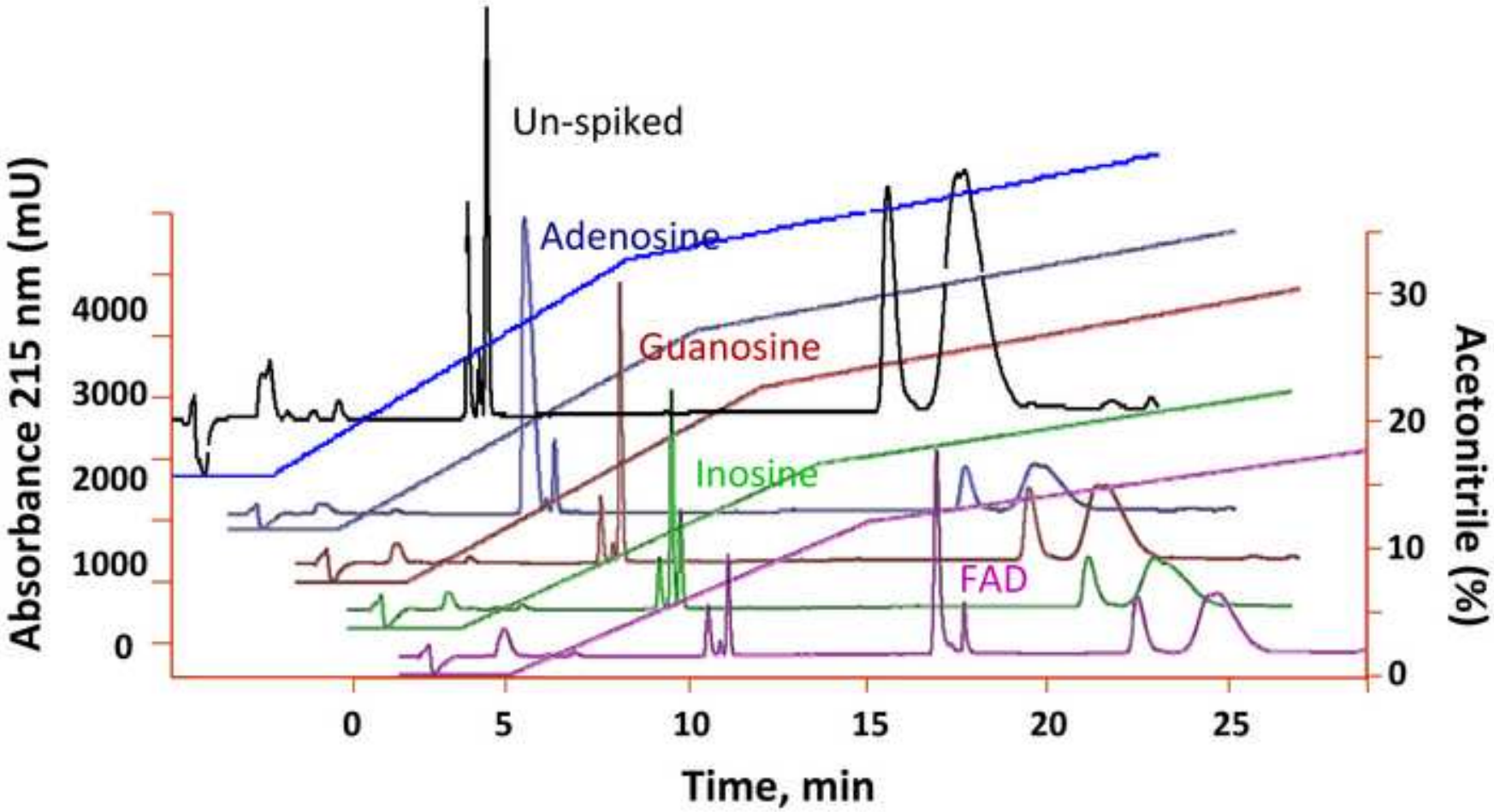

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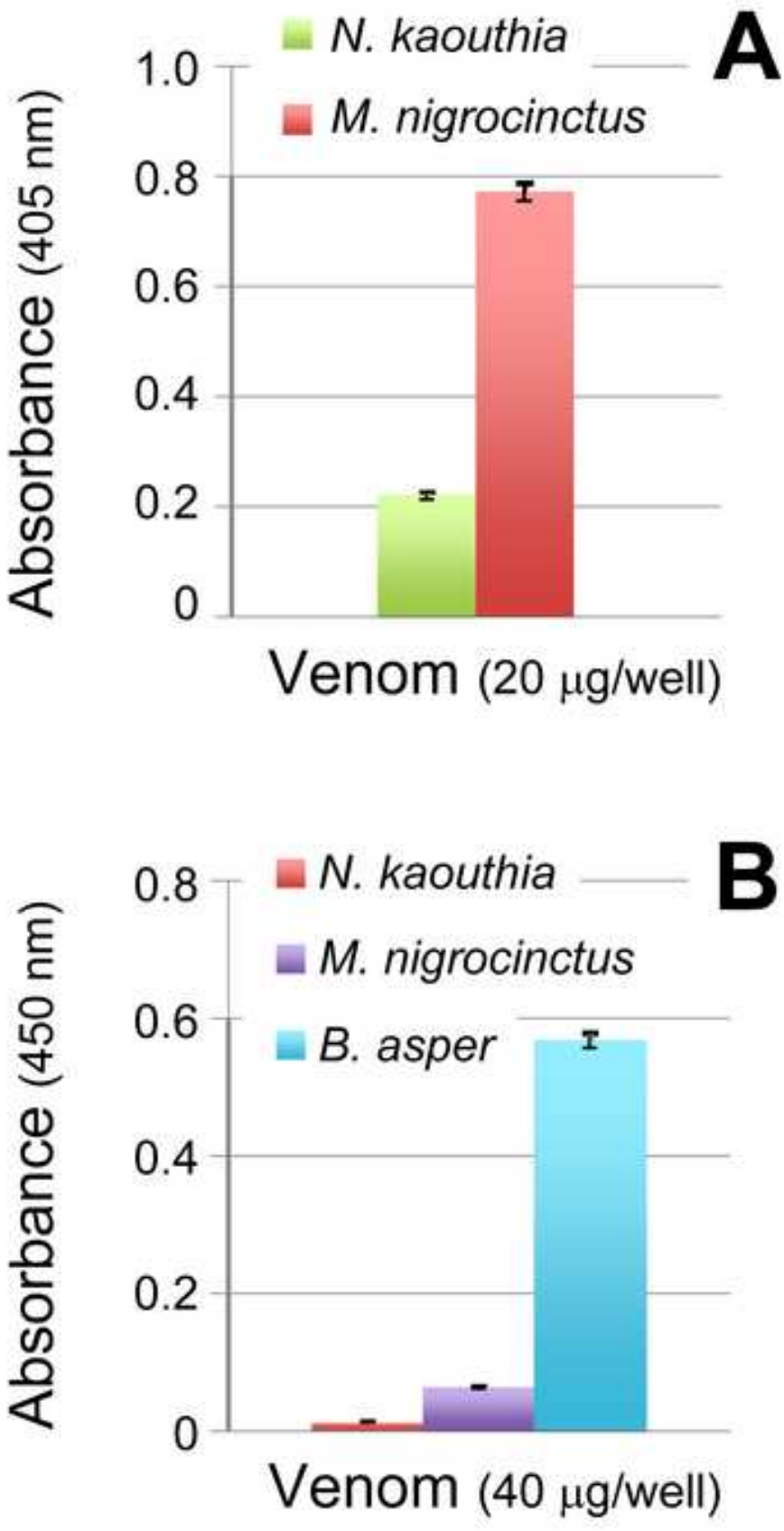

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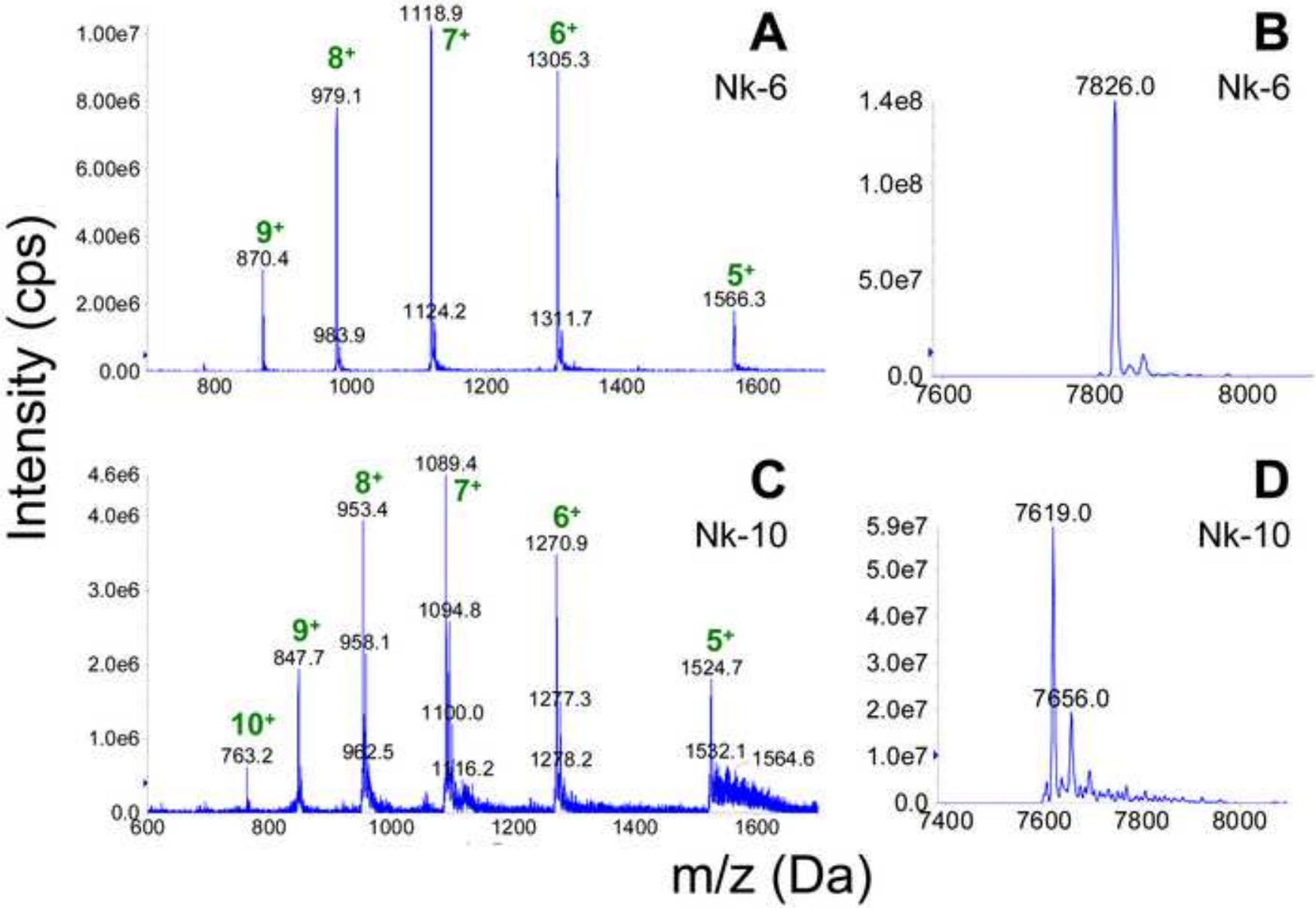

Figure 6

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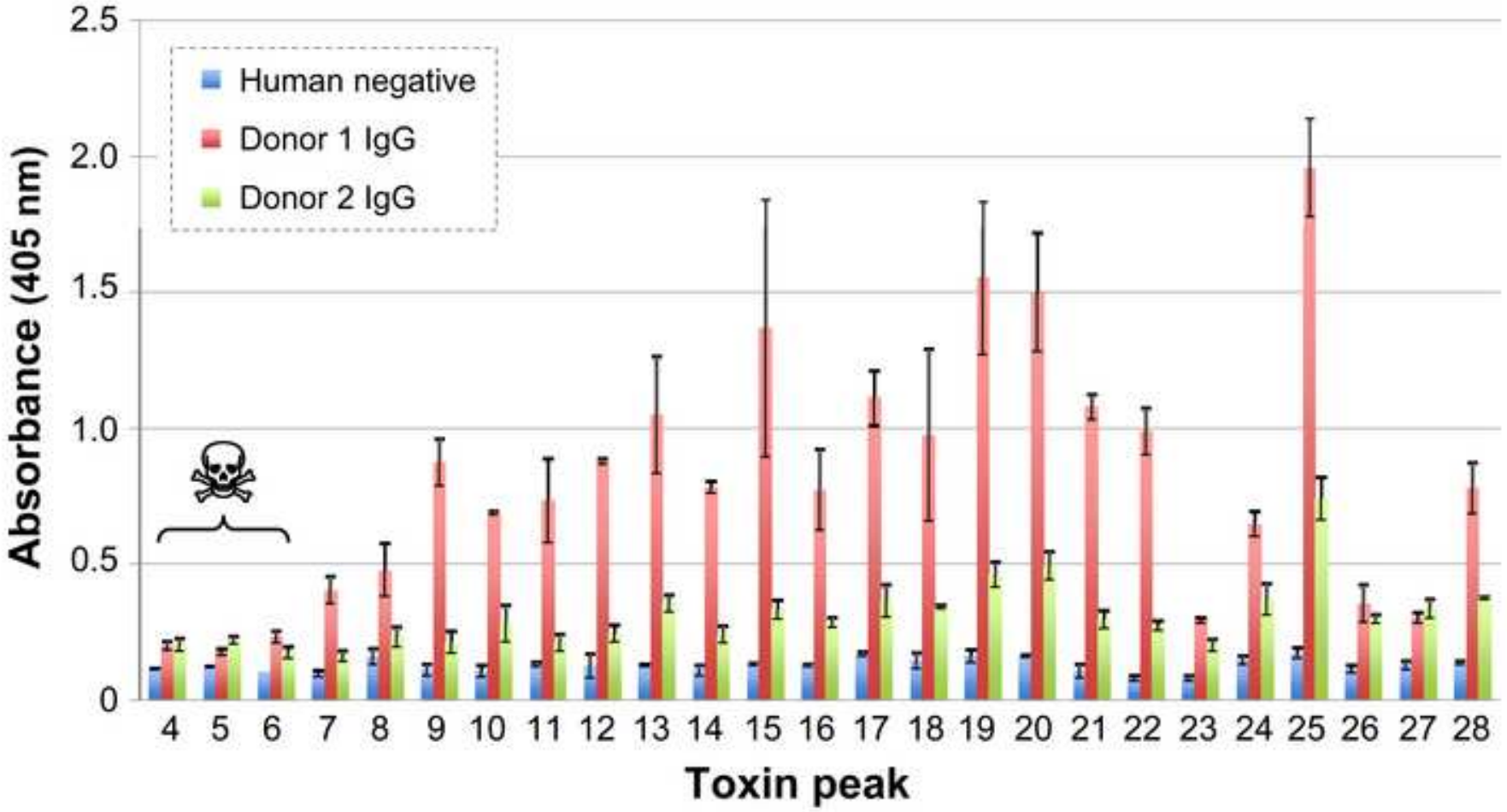

Graphical abstract

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