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ORIGINAL ARTICLE

Evolutionary Interpretations of Nicotinic Acetylcholine Receptor Targeting Venom Effects by a Clade of Asian Viperidae Snakes

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Abstract

Ecological variability among closely related species provides an opportunity for evolutionary comparative studies. Therefore, to investigate the origin and evolution of neurotoxicity in Asian viperid snakes, we tested the venoms of *Azemiops feae*, *Calloselasma rhodostoma*, *Deinagkistrodon acutus*, *Tropidolaeums subannulatus*, and *T. wagleri* for their relative specificity and potency upon the amphibian, lizard, bird, rodent, and human α-1 (neuromuscular) nicotinic acetylcholine receptors. We utilised a biolayer interferometry assay to test the binding affinity of these pit viper venoms to orthosteric mimotopes of nicotinic acetylcholine receptors binding region from a diversity of potential prey types. The *Tropidolaemus* venoms were much more potent than the other species tested, which is consistent with the greater prey escape potential in arboreal niches. Intriguingly, the venom of *C. rhodostoma* showed neurotoxic binding to the α-1 mimotopes, a feature not known previously for this species. The lack of prior knowledge of neurotoxicity in this species is consistent with our results due to the bias in rodent studies and human bite reports, whilst this venom had a greater binding affinity toward amphibian and diapsid α-1 targets. The other large terrestrial species, *D. acutus*, did not display any meaningful levels of neurotoxicity. These results demonstrate that whilst small peptide neurotoxins are a basal trait of these snakes, it has been independently amplified on two separate occasions, once in *Azemiops* and again in *Tropidolaemus*, and with *Calloselasma* representing a third possible amplification of this trait. These results also point to broader sources of novel neuroactive peptides with the potential for use as lead compounds in drug design and discovery.

Keywords Viperidae · Crotalinae · Pit vipers · Prey specificity · Evolution · Nicotinic acetylcholine receptor · Neurotoxins

Introduction

Within venomous snakes, a generalisation exists that the Elapidae family are neurotoxic, whilst those within the Viperidae family are coagulotoxic. However, as with any rule there are always exceptions. In the Asian vipers, two genera have been documented as being notably neurotoxic: *Azemiops* and *Tropidolaemus* (Hsiao et al. 1996; Lin et al. 1995; McArdle et al. 1999; Molles et al. 2002a, b; Tsai et al. 1995; Utkin et al. 2012b). As these genera are not sister to each other, this suggests two competing hypotheses: that neurotoxicity evolved convergently between the two lineages, or it was present in their last common ancestor but was independently amplified in each of the two genera. Supporting the latter hypothesis are the sequences of the neurotoxins themselves, which in both cases are small, proline-rich peptides that bind to the orthosteric sites of the nicotinic acetylcholine α-1 subunits at the neuromuscular junction (Hsiao et al. 1996; Lin et al. 1995; McArdle et al. 1999; Molles et al. 2002a, b; Tsai et al. 1995; Utkin et al. 2012b). These neurotoxic peptides are the result of de novo evolution from within the propeptide region of the C-type natriuretic peptide, whereby the ancestral gene not only expresses the natriuretic peptide gene but also expresses additional new peptides which are post-translationally liberated to exert their neurotoxic function (Brust et al. 2013; Debono et al. 2017).

There is evidence, however, of diversification in the neurotoxic peptides between the two lineages, with those of *Azemiops* (azemiospin peptide) remaining in the basal linear form, with the functional residues presented via proline bracketing, whilst those of *Tropidolaemus* (waglerin peptides) are more derived, possessing a newly evolved cysteine bond

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that present the functional residues as part of a constrained loop (Brust et al. 2013; Debono et al. 2017). Due to the small size of these viper neurotoxins, having simple structures that lack specific requirements to maintain critical folding that larger and more complex toxins require, they would be more easily subject to positive selection pressures to more potent forms (Schmidt et al. 1992) or for prey-specific targeting, as seen in some elapid and colubrid three-finger toxins (3FTxs) (da Silva Jr and Aird 2001; Pawlak et al. 2006; Pawlak et al. 2009). However, there have been few studies that have functionally tested the nAChR potency of these basal viper venoms to ascertain if prey-specific targeting of their neurotoxins occurs. Consequently, in both cases the relative potency against prey-specific lineages or humans is unknown, with testing only having been undertaken on a few taxonomical lineages such as birds or rodents (Hsiao et al. 1996; Lin et al. 1995; McArdle et al. 1999; Molles et al. 2002a, b; Tsai et al. 1995; Utkin et al. 2012b). Therefore, interpretations of these venoms in their evolutionary or clinical context have been lacking and their role in viper venom evolution is unclear as well as their hazard to humans.

The Viperidae family of snakes contains many species of medical importance and consists of three subfamilies; Crotalinae (pit vipers), Viperinae (true vipers), and Azemiopinae. Species within the Crotalinae subfamily inhabit numerous habitats across large ranges globally, from Asia through to the Americas (Alencar et al. 2016), and as such possess vast variability regarding their morphology and ecology. For example, a basal clade within Crotalinae consists of both terrestrial (*Calloselasma*, *Deinagkistrodon*, and *Hypnale* spp.) and arboreal (*Tropidolaemus* spp.) Asian genera. In contrast to the neurotoxicity of *Azemiops* and *Tropidolaemus* (Debono et al. 2017; Lin et al. 1995; Schmidt and Weinstein 1995; Schmidt et al. 1992; Tan et al. 2017; Utkin et al. 2012a, b), the venoms of Asian Viperidae species are predominantly coagulotoxic (Debono et al. 2019; Nielsen 2016; Nielsen and Frank 2018; Tang et al. 2016; Withana et al. 2014). In alignment with variable ecology and morphology within this group, one study observed significant inter-genus differences in venom coagulation within the basal clade (Debono et al. 2019). Such diversity in niche partitioning among closely related genera provides an ideal opportunity for evolutionary comparison studies. The closely related monotypic sister taxa Azemiopinae, consisting of the semi-fossorial *Azemiops feae*, can also provide a further point of evolutionary comparison, as it is thought to be an intermediate between the basal Viperinae and the derived Crotalinae.

Using a range of taxon-specific mimotopes on a biolayer interferometry (BLI) assay, we determined the prey specificity of crude venoms from species within a basal pit viper clade to the orthosteric site of α-1 nAChRs. Understanding the unique viper neurotoxicity within this clade will help answer fundamental questions about the ecology and evolution of this basal clade regarding their unique neurotoxins.

Results and Discussion

Results from the functional testing of these venoms support previous data indicating that waglerins are a more potently derived form of the azemiopsin peptide (Debono et al. 2017). This is indicated by the extreme increase in relative binding affinity from *A. feae* to both *T. wagleri* and *T. subannulatus* (Fig. 1), and is likely due to the addition of the cysteines in waglerins presenting the bioactive residues in a more stable conformation than in the linear forms present in *Azemiops* venom (Debono et al. 2017). The greater prey escape potential in an arboreal niche, such as occupied by the *Tropidolaemus* genus, would provide a strong selection pressure for faster acting and more potent toxins. This further supports the idea that toxins can evolve under positive selection pressures to a more potent form (Fry et al. 2003).

With further regard to the waglerins, the waglerin 1 and 3 peptides from *T. wagleri* share a very similar homology to waglerin 2 and 4 as well as the waglerin-Ts-1 from *T. subannulatus* (Debono et al. 2017). However, they differ by small amino acid changes from Y to H (tyrosine to histidine), with waglerin 1/3 having a -CHPPC- motif whilst waglerin 2/4 and waglerin-Ts-1 have a -CYPPC- (Debono et al. 2017). This amino acid change led other studies to suggest that the -CYPPC- motif is the plesiotypic form and that the waglerin 1/3 toxins of *T. wagleri* have evolved under positive selection pressures to increase the potency of these toxins (Fry 2005). Furthermore, this amino acid change has been shown to increase the functional neurotoxic activity, causing a greater LD_{50} (Schmidt et al. 1992). Thus, our data also support this finding, as *T. wagleri* indeed displayed an overall greater binding affinity to all mimotopes than that of *T. subannulatus* (Fig. 1). Other small nAChR targeting toxins such as 3FTxs have been suggested to act under similar selection pressures (Dashevsky and Fry 2018; Jackson et al. 2013). Future work should endeavour to examine the structure-function relationship of waglerin peptides and their binding activity to the mimotopes using synthetic waglerin analogues with amino acid changes at key positions.

Our data also suggests that *Calloselasma rhodostoma* may contain peptides similar to that of the plesiotypic azemiopsin peptide, by showing low binding affinity similar to *A. feae* (Fig. 1). The most parsimonious explanation here is that these neurotoxic peptides evolved in an earlier ancestor of *Azemiops*/*Calloselasma*, yet no such peptides have been isolated from *C. rhodostoma* venom (Ali et al. 2013; Tang et al. 2016). One explanation for this discrepancy may be that the proteomic analyses conducted might not have be able to detect or identify these small azemiopsin- or waglerin-like peptides

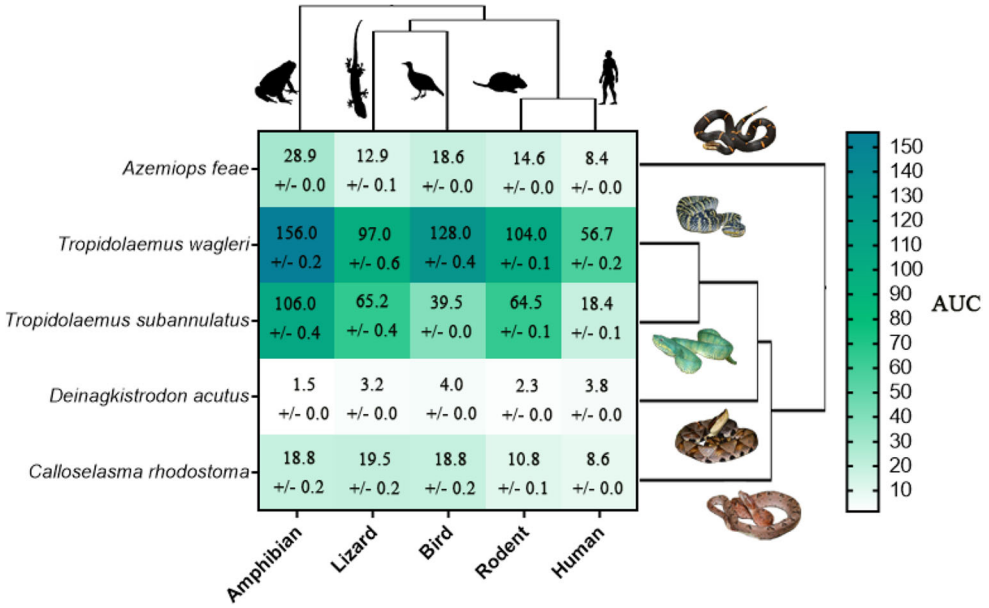

Fig. 1 A heatmap comparison of basal viper species *Tropidolaemus wagleri*, *T. subannulatus*, *Deinagkistrodon acutus*, and *Calloselasma rhodostoma* including the closely related monotypic sister taxa *Azemiops feae*. The heat map is based on the relative potency (AUC) ranking system—derived from Ka (binding rate) curves in triplicate—with darker green indicating greater affinity and vice versa. Values are AUC ± SEM with $N = 3$. Image credits: Tom Charlton—ecoanimalencounters.co.uk (*A. feae*); Rushden—(thainationalparks.com) via flikr.com CC BY-SA 2.0 (*T. wagleri* and *C. rhodostoma*); Bernard Dupont via flickr.com CC BY-SA 2.0 (*T. subannulatus*); Alex White via flickr.com CC BY-NC-SA 2.0 (*D. acutus*). Blacked-out images are public domain CC0 1.0 via phylopic.org

on 1D or 2D gels (Ali et al. 2013) or chromatography studies may not have returned significant database search hits due to the lack of a *C. rhodostoma* transcriptome. Thus, future analyses should aim to further isolate, characterise, and assess what toxins within *C. rhodostoma* venom are binding to the nAChR mimotopes. Such investigations would aid conclusions regarding the evolutionary history of the toxins within this basal clade.

Interestingly, *D. acutus* showed no binding to the mimotopes, as is evidenced by the small AUC values (Fig. 1) and the flat-line wavelength data paralleling both negative controls (Fig. 2). *Crotalus horridus* was chosen as a negative control because there is no evidence that this species utilises nAChR targeting neurotoxins, but also to give a comparison of a venom that is rich in other non-binding toxin types (e.g. PLA_2s, SVSPs and SVMPs) (Rokyta et al. 2013; Rokyta et al. 2015). We suspect that the small AUC values compared with water/glycerol negative control are due to large non-binding toxins such as PLA_2s having negligible steric interferences with the mimotopes (Fig. 2). Thus, these data suggest that *D. acutus* has subsequently lost the ancestral neurotoxic peptide after branching off from *C. rhodostoma*. Alternatively, a less parsimonious explanation is that selection pressures have evolved the toxin to become an allosteric binder of nAChRs, which would not be detected in this assay (Zdenek et al. 2019). Another explanation may be that the toxins have evolved to target other nAChR subunits rather than the α-1. However, since the α-1 is the only subunit located at the neuromuscular junction with all other subunits being neuronal and mostly located within the central nervous system (CNS) (Gotti and Clementi 2004; Le Novere and Changeux 1995), it is unlikely that the toxins have been evolutionarily selected to target any other nAChR subunit. This is due to the strict biochemical and physiological mechanisms of the blood-brain barrier controlling the passage of endo- and exogenous substances across (Abbott et al. 2010); thus, unlikely venom toxins can reach the CNS. Therefore, the α-1 seems the most likely intended evolutionary target since it is easily reached via the blood stream and located at the neuromuscular junction where other nAChR targeting neurotoxins have been shown to target, causing flaccid paralysis (Barber et al. 2013; Nirthanan and Gwee 2004). Future work including functional testing on chick biventer cervicis muscle preparations would be necessary to ascertain fully the lack of nAChR binding (regardless of orthosteric vs. allosteric binding).

Venom from both species of *Tropidolaemus* showed a stronger affinity toward the amphibian than other mimotopes (Figs. 1 and 2). This result is surprising in that the literature reports that adults of both *Tropidolaemus* species feed primarily on rodents and birds (Das and Charles 2015; Orlov et al. 2002). Although bird and rodent mimotopes were still potently targeted (Figs. 1 and 2), the literature only suggests amphibians as main prey items for *T. wagleri* in juveniles and small males (Orlov et al. 2002), and no amphibians noted in the diet for *T. subannulatus* (Das and Charles 2015). Thus, other toxins with different functions within the venom (e.g.

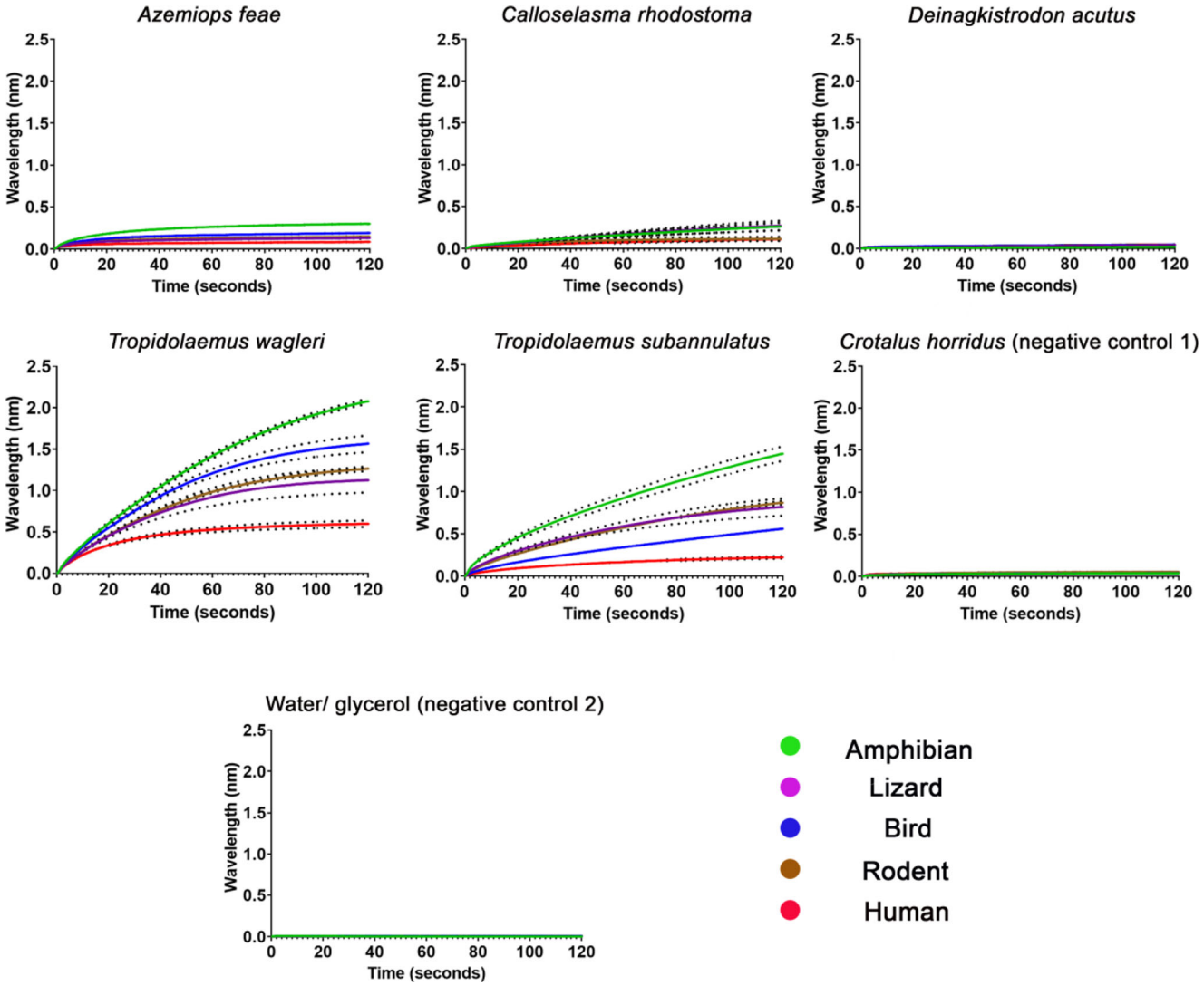

Fig. 2 A comparison of the wavelength (nm) curves of the association step (ka binding step) over a 120-s assay run period of *Azemiops feae*, *Calloselasma rhodostoma*, *Deinagkistrodon acutus*, *Tropidolaemus wagleri*, *T. subannulatus*, *Crotalus horridus* (negative control 1) and water/glycerol (negative control 2). All venoms were tested against amphibian, lizard, bird, and rodent mimotopes in triplicate. The dots surrounding the curve lines are error bars based on SEM values with $N = 3$

coagulotoxic and myotoxic PLA_2s) are likely to play an equally vital role in prey immobilisation of different taxa types, which has been suggested in other studies (Harris et al. 2020; Lyons et al. 2020), with a certain prey types possibly being immobilised more effectively by different pathophysiological functions and molecular constituents of the venom.

Azemiops feae showed its highest binding preference toward amphibian mimotope, although with much less disparity between the taxa mimotopes than what both *Tropidolaemus* species displayed. This result was interesting as the azemiopsin peptide is thought to be a plesiotypic form of the derived waglerin peptides (Debono et al. 2017), thus further supporting the idea that toxins can evolve under selection pressures toward a greater potency and possibly prey specificity since the *Tropidolaemus* venoms were more potent and target specific toward amphibian than the *A. feae* venom (Figs. 1 and 2). The low levels of neurotoxicity for *C. rhodostoma* and lack of postsynaptic neurotoxicity in *D. acutus* would suggest their venom is more suited to targeting other pathophysiological functions such as the blood coagulation of prey and that *C. rhodostoma* is likely evolving to lose the nAChR targeting components of its venom and shifting to a more coagulotoxin-rich composition (Ali et al. 2013; Debono et al. 2019). Not surprisingly, functional studies on the coagulation of *C. rhodostoma* and *D. acutus* venom have indicated that they display strong coagulotoxic effects: with *C. rhodostoma* being procoagulant through Factor X activation whilst conversely *D. acutus* has a pseudo-

procoagulant mechanism resulting in a net anticoagulant state due to fibrinogen being cleaved to form weak, unstable clots (Debono et al. 2019).

In regard to human effects, proportionally larger amounts of venom would be required to produce neurotoxic symptoms in humans than in prey animals; this is indicated by human being the lowest of all binding across the mimotopes (Figs. 1 and 2). Thus, this emphasises that using human bite reports to predict prey effects is a poor methodological strategy to ascertain ecological and evolutionary inferences (Davies and Arbuckle 2019) as prey-specific effects may not be captured in clinical observations of envenomation to a much larger organism who are less sensitive to a particular venom effect than would be native prey animal. Thus, evolutionary interpretations regarding venom diversification patterns can only be elucidated through evidence obtained by testing on prey-relevant assay platforms and not clinical human envenomations.

This research has highlighted many important and previously unknown facets about the neurotoxins of this basal viper group. Our results corroborate previous research suggesting that the *Tropidolaemus* waglerin neurotoxins are a more potent and derived form of the Azemiopsin peptide from *Azemiops feae* (Debono et al. 2017). We also show that the venom of *C. rhodostoma* seems to contain nAChR targeting neurotoxins, whilst *D. acutus* lack this neurotoxic action, both of which have never been tested for functional neurotoxic activity. Further to this, we showed that *T. wagleri* and *T. subannulatus* venom was more selective toward the amphibian mimotope than the other taxa mimotopes tested, which seems inconsistent with their preferred prey types; thus, it would seem that other venom functions will likely play an equally vital role in prey immobilisation of different taxa types. Future research should endeavour to understand the evolutionary history of this basal group in terms of venom composition, function, and evolutionary ecology.

Methods

Venom Collection and Preparation

Venoms were obtained from pooled snake venom extractions of multiple individuals (captive and wild-caught) from either the long-term cryogenic collection of the Venom Evolution Laboratory.

All venom samples were lyophilised and reconstituted in double deionised water (ddH_2O) then centrifuged (4 °C, 10 min at 14,000 RCF). The supernatant was then made into a working stock (1 mg/mL) in 50% glycerol to prevent freezing at − 20 °C. The concentrations of working stocks were determined in triplicate using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermofisher, Sydney, NSW, Australia) at an absorbance wavelength of 280 nm.

Mimotope Production and Preparation

Expanding upon previous research (Bracci et al. 2001; Bracci et al. 2002; Chiappinelli et al. 1996; Katchalski-Katzir et al. 2002) a 13–14 amino acid mimotope of ACh orthosteric site of vertebrate α-1 nAChR subunit was developed by GenicBio Ltd. (Shanghai, China) designed upon specifications which were adapted from publicly available sequences of cholinergic receptors (Chrna1) from GenBank.

The amino acid sequences for the α-1 orthosteric site for each taxon were obtained with the following accession codes: amphibian α-1 (uniprot F6RLA9), lizard α-1 (genbank XM_015426640), avian α-1 (uniprot E1BT92), rodent α-1 (uniprot P25108), human α-1 (uniprot G5E9G9).

The Cys-Cys of the native mimotope is replaced during peptide synthesis with Ser-Ser to avoid uncontrolled postsynthetic thiol oxidation. The Cys-Cys bond in the nAChR binding region does not participate directly in analyte-ligand binding (McLane et al. 1994; McLane et al. 1991; Tzartos and Remoundos 1990); thus, replacement to Ser-Ser is not expected to have any effect on the analyte-ligand complex formation. However, the presence of the Cys-Cys bridge is key in the conformation of the interaction site of whole receptors (Testai et al. 2000). As such, we suggest direct comparisons of kinetics data, such as K_a or KD, between nAChR mimotopes and whole receptor testing should be avoided, or at least approached with caution. Mimotopes were further synthesised to a biotin linker bound to two aminohexanoic acid (Ahx) spacers, forming a 30 Å linker.

Mimotope dried stocks were solubilised in 100% dimethyl sulfoxide (DMSO) and diluted in deionised water at 1:10 dilution to create a working stock of 50 μg/mL. All stocks were stored at − 80 °C until use and limited to three freeze-thaw cycles.

Bio-layer Interferometry

The bio-layer interferometry (BLI) assay was performed on the Octet RED 96 system (ForteBio). The assay used, including all methodology and data analysis, was based upon a validated protocol (Zdenek et al. 2019).

Data Processing and Statistical Analyses

All data obtained from BLI on Octet RED 96 system (ForteBio) were processed in exact accordance to the validation of this assay (Zdenek et al. 2019). The association step data (in triplicate) were obtained in an Excel .csv file extracted from raw outputs of the Octet Red 96 system and then

imported into Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA) where area under the curve (AUC) calculations were made and graphs produced. Phylogenetic trees were obtained from timetree.org and then further manually recreated using Mesquite software (version 3.2).

Author Contributions Conceptualisation: BGF; methodology: RJH, CNZ, BGF; investigation: RJH, JD; resources: JD, DH, BGF; writing of first draft: RJH; editing subsequent drafts; RJH, CNZ, BGF; project administration: BGF.

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References

Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ (2010) Structure and function of the blood–brain barrier. Neurobiol Dis 37: 13–25

Alencar LR, Quental TB, Grazziotin FG, Alfaro ML, Martins M, Venzon M, Zaher H (2016) Diversification in vipers: phylogenetic relationships, time of divergence and shifts in speciation rates. Mol Phylogenet Evol 105:50–62

Ali SA et al (2013) Proteomic comparison of *Hypnale hypnale* (humpnosed pit-viper) and *Calloselasma rhodostoma* (Malayan pit-viper) venoms. J Proteome 91:338–343

Barber CM, Isbister GK, Hodgson WC (2013) Alpha neurotoxins. Toxicon 66:47–58

Bracci L, Lozzi L, Lelli B, Pini A, Neri P (2001) Mimotopes of the nicotinic receptor binding site selected by a combinatorial peptide library. Biochemistry 40:6611–6619

Bracci L et al (2002) A branched peptide mimotope of the nicotinic receptor binding site is a potent synthetic antidote against the snake neurotoxin α-bungarotoxin. Biochemistry 41:10194–10199

Brust A et al (2013) Differential evolution and neofunctionalization of snake venom metalloprotease domains. Mol Cell Probes 12:651–663

Chiappinelli VA, Weaver WR, McLane KE, Conti-Fine BM, Fiordalisi JJ, Grant GA (1996) Binding of native κ-neurotoxins and site-directed mutants to nicotinic acetylcholine receptors. Toxicon 34:1243–1256

da Silva NJ Jr, Aird SD (2001) Prey specificity, comparative lethality and compositional differences of coral snake venoms. Comp Biochem Physiol, Part C: Toxicol Pharmacol 128:425–456

Das I, Charles JK (2015) Venomous snakes and envenomation in Brunei. In: Gopalakrishnakone P (ed) Clinical Toxinology in Asia Pacific and Africa. Springer Science, pp 103–114

Dashevsky D, Fry BG (2018) Ancient diversification of three-finger toxins in *Micrurus* coral snakes. J Mol Evol 86:58–67

Davies E-L, Arbuckle K (2019) Coevolution of snake venom toxic activities and diet: evidence that ecological generalism favours toxicological diversity. Toxins 11:711

Debono J, Xie B, Violette A, Fourmy R, Jaeger M, Fry BG (2017) Viper venom botox: the molecular origin and evolution of the waglerin peptides used in anti-wrinkle skin cream. J Mol Evol 84:8–11

Debono J, Bos MH, Coimbra F, Ge L, Frank N, Kwok HF, Fry BG (2019) Basal but divergent: clinical implications of differential coagulotoxicity in a clade of Asian vipers. Toxicol in Vitro 58: 195–206

Fry BG (2005) From genome to "venome": molecular origin and evolution of the snake venom proteome inferred from phylogenetic analysis of toxin sequences and related body proteins. Genome Res 15:403–420

Fry BG, Wüster W, Ryan Ramjan SF, Jackson T, Martelli P, Kini RM (2003) Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxinological implications. Rapid Commun Mass Spectrom 17:2047–2062

Gotti C, Clementi F (2004) Neuronal nicotinic receptors: from structure to pathology. Prog Neurobiol 74:363–396

Harris RJ, Zdenek CN, Harrich D, Frank N, Fry BG (2020) An appetite for destruction: Detecting prey-selective binding of α-neurotoxins in the venom of Afro-Asian elapids. Toxins 12(3):205

Hsiao Y-M, Chuang C-C, Chuang L-C, Yu H-M, Wang K-T, Chiou S-H, Wu S-H (1996) Protein engineering of venom toxins by synthetic approach and NMR dynamic simulation: status of basic amino acid residues in waglerin I. Biochem Biophys Res Commun 227:59–63

Jackson T et al (2013) Venom down under: dynamic evolution of Australian elapid snake toxins. Toxins 5:2621–2655

Katchalski-Katzir E, Kasher R, Balass M, Scherf T, Harel M, Fridkin M, Sussman JL, Fuchs S (2002) Design and synthesis of peptides that bind α-bungarotoxin with high affinity and mimic the three-dimensional structure of the binding-site of acetylcholine receptor. Biophys Chem 100:293–305

Le Novere N, Changeux J-P (1995) Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. J Mol Evol 40:155–172

Lin W, Smith L, Lee C (1995) A study on the cause of death due to waglerin-I, a toxin from *Trimeresurus wagleri*. Toxicon 33:111–114

Lyons K, Dugon MM, Healy K (2020) Diet breadth mediates the prey specificity of venom potency in snakes. Toxins 12(2):74

McArdle JJ, Lentz TL, Witzemann V, Schwarz H, Weinstein SA, Schmidt JJ (1999) Waglerin-1 selectively blocks the epsilon form of the muscle nicotinic acetylcholine receptor. J Pharmacol Exp Ther 289:543–550

McLane KE, Wu X, Diethelm B, Conti-Tronconi BM (1991) Structural determinants of α-bungarotoxin binding to the sequence segment 181-200 of the muscle nicotinic acetylcholine receptor. α-subunit: effects of cysteine/cystine modification and species-specific amino acid substitutions. Biochemistry 30:4925–4934

McLane KE, Wu X, Conti-Tronconi BM (1994) An α-bungarotoxin-binding sequence on the *Torpedo* nicotinic acetylcholine receptor α-subunit: conservative amino acid substitutions reveal side-chain specific interactions. Biochemistry 33:2576–2585

Molles BE, Rezai P, Kline EF, McArdle JJ, Sine SM, Taylor P (2002a) Identification of residues at the α and ε subunit interfaces mediating species selectivity of Waglerin-1 for nicotinic acetylcholine receptors. J Biol Chem 277:5433–5440

Molles BE, Tsigelny I, Nguyen PD, Gao SX, Sine SM, Taylor P (2002b) Residues in the ε subunit of the nicotinic acetylcholine receptor interact to confer selectivity of Waglerin-1 for the α− ε subunit interface site. Biochemistry 41:7895–7906

Nielsen VG (2016) Ancrod revisited: viscoelastic analyses of the effects of *Calloselasma rhodostoma* venom on plasma coagulation and fibrinolysis. J Thromb Thrombolysis 42:288–293

Nielsen VG, Frank N (2018) Differential heme-mediated modulation of *Deinagkistrodon*, *Dispholidus*, *Protobothrops* and *Pseudonaja* hemotoxic venom activity in human plasma. Biometals 31:951–959

Nirthanan S, Gwee MC (2004) Three-finger α-neurotoxins and the nicotinic acetylcholine receptor, forty years on. J Pharmacol Sci 94:1–17

Orlov N, Ananjeva N, Khalikov R (2002) Natural history of pitvipers in Eastern and Southeastern Asia. Biol Vipers 345–361

Pawlak J, Mackessy SP, Fry BG, Bhatia M, Mourier G, Fruchart-Gaillard C, Servent D, Ménez R, Stura E, Ménez A, Kini RM (2006) Denmotoxin, a three-finger toxin from the colubrid snake *Boiga dendrophila* (mangrove catsnake) with bird-specific activity. J Biol Chem 281:29030–29041

Pawlak J et al (2009) Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. FASEB J 23:534–545

Rokyta DR, Wray KP, Margres MJ (2013) The genesis of an exceptionally lethal venom in the timber rattlesnake (*Crotalus horridus*) revealed through comparative venom-gland transcriptomics. BMC Genomics 14:394

Rokyta DR, Wray KP, McGivern JJ, Margres MJ (2015) The transcriptomic and proteomic basis for the evolution of a novel venom phenotype within the timber rattlesnake (*Crotalus horridus*). Toxicon 98:34–48

Schmidt JJ, Weinstein SA (1995) Structure-function studies of waglerin I, a lethal peptide from the venom of Wagler's pit viper, *Trimeresurus wagleri*. Toxicon 33:1043–1049

Schmidt JJ, Weinstein SA, Smith LA (1992) Molecular properties and structure-function relationships of lethal peptides from venom of Wagler's pit viper, *Trimeresurus wagleri*. Toxicon 30:1027–1036

Tan CH, Tan KY, Yap MKK, Tan NH (2017) Venomics of *Tropidolaemus wagleri*, the sexually dimorphic temple pit viper: unveiling a deeply conserved atypical toxin arsenal. Sci Rep 7:43237

Tang ELH, Tan CH, Fung SY, Tan NH (2016) Venomics of *Calloselasma rhodostoma*, the Malayan pit viper: a complex toxin arsenal unraveled. J Proteome 148:44–56

Testai FD, Venera GD, Peña C, de Jiménez Bonino MJB (2000) Histidine 186 of the nicotinic acetylcholine receptor α subunit requires the presence of the 192–193 disulfide bridge to interact with α-bungarotoxin. Neurochem Int 36:27–33

Tsai M-C, Hsieh W, Smith L, Lee C (1995) Effects of waglerin-I on neuromuscular transmission of mouse nerve-muscle preparations. Toxicon 33:363–371

Tzartos S, Remoundos MS (1990) Fine localization of the major alpha-bungarotoxin binding site to residues alpha 189-195 of the *Torpedo* acetylcholine receptor. Residues 189, 190, and 195 are indispensable for binding. J Biol Chem 265:21462–21467

Utkin YN, Weise C, Anh HN, Kasheverov I, Starkov V, Tsetlin V (2012a) The new peptide from the Fea's viper *Azemiops feae* venom interacts with nicotinic acetylcholine receptors. In: Doklady biochemistry and biophysics, vol 442. vol 1. Springer Science, pp 33–35

Utkin YN, Weise C, Kasheverov IE, Andreeva TV, Kryukova EV, Zhmak MN, Starkov VG, Hoang NA, Bertrand D, Ramerstorfer J, Sieghart W, Thompson AJ, Lummis SCR, Tsetlin VI (2012b) Azemiopsin from *Azemiops feae* viper venom, a novel polypeptide ligand of nicotinic acetylcholine receptor. J Biol Chem 287:27079–27086

Withana M, Rodrigo C, Gnanathasan A, Gooneratne L (2014) Presumptive thrombotic thrombocytopenic purpura following a hump-nosed viper (*Hypnale hypnale*) bite: a case report. J Venomous Anim Toxins Incl Trop Dis 20:26

Zdenek CN et al (2019) A taxon-specific and high-throughput method for measuring ligand binding to nicotinic acetylcholine receptors. Toxins 11:600