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Research paper

Identification and characterization of a taxon-specific three-finger toxin from the venom of the Green Vinesnake (*Oxybelis fulgidus*; family Colubridae)

William H. Heyborne [a,b,1], Stephen P. Mackessy [a,*]

[a] School of Biological Sciences, University of Northern Colorado, 501 20th St., CB 92, Greeley, CO 80639-0017, USA
[b] Department of Biology, Southern Utah University, 351 W University Blvd., Cedar City, UT 84720, USA

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ABSTRACT

Snake venoms contain a variety of protein and peptide toxins, and the three-finger toxins (3FTxs) are among the best characterized family of venom proteins. The compact nature and highly conserved molecular fold of 3FTxs, together with their abundance in many venoms, has contributed to their utility in structure-function studies. Although many target the nicotinic acetylcholine receptor of vertebrate skeletal muscle, often binding with nanomolar K_ds, several non-conventional 3FTxs show pronounced taxon-specific neurotoxic effects. Here we describe the purification and characterization of fulgimotoxin, a monomeric 3FTx from the venom of *Oxybelis fulgidus*, a neotropical rear-fanged snake. Fulgimotoxin retains the canonical 5 disulfides of the non-conventional 3FTxs and is highly neurotoxic to lizards; however, mice are unaffected, demonstrating that this toxin is taxon-specific in its effects. Analysis of structural features of fulgimotoxin and other colubrid venom 3FTxs indicate the presence of a "colubrid toxin motif" (CYTLY) and a second conserved segment (WAVK) found in *Boiga* and *Oxybelis* taxon-specific 3FTxs, both in loop II. Because specific residues in loop II conventional α-neurotoxic 3FTxs are intimately associated with receptor binding, we hypothesize that this loop, with its highly conserved substitutions, confers taxon-specific neurotoxicity. These findings underscore the importance of rear-fanged snake venoms for understanding the evolution of toxin molecules and demonstrate that even among well-characterized toxin families, novel structural and functional motifs may be found.

1. Introduction

The three-finger toxins (3FTx) are abundant and well-characterized members of the three-finger protein superfamily which are broadly distributed in venoms of the Elapidae and Colubridae [1–7]. These proteins represent a prime example of a highly conserved protein scaffold which supports myriad pharmacological functions, with biological roles as diverse as lethal prey immobilization [1], modulation of salamander courtship receptivity [8] and regulation of salamander limb regeneration [9,10]. The name is descriptive of the molecules' shared tertiary structure, which consists of three finger-like loops extending from a compact central core; both the core and the "fingers" are stabilized by disulfide bonds that are highly conserved among members of this protein family [11]. Within venoms, 3FTxs exhibit a similarly diverse array of biological activities, including multiple forms of neurotoxicity, enzyme inhibition, cardiotoxicity, cytotoxicity, ion channel blocking and anticoagulation [7, reviewed in 12,13].

Once known only from the venoms of the Elapidae, and thought to be unique to elapid snakes e.g. [14], 3FTxs are now known to be much more broadly distributed among advanced snakes than was previously suspected. Analysis of venom gland transcriptomes [5,6,15] has revealed that 3FTxs are also found among viperids, and cDNA sequences have been obtained for 3FTxs from several colubrid (*sensu stricto*) snakes (including *Dispholidus*, *Telescopus*, *Thrasops* and *Trimorphodon*) [16]. Additionally, many rear-fanged snake venoms contain proteins in the 6–9 kDa mass range, the size generally expected for 3FTxs [2,7,17–19], and subsequent analysis has demonstrated their occurrence in these venoms.

To date, only four three-finger toxins from the venoms of colubrid snakes have been isolated, sequenced and had biological activity characterized. α-colubritoxin, from the venom of *Coelognathus radiatus*, was the first purified colubrid venom toxin shown

* Corresponding author. Tel.: +1 970 351 2429; fax: +1 970 351 2335.
E-mail address: stephen.mackessy@unco.edu (S.P. Mackessy).
[1] Tel.: +1 435-865-8443; fax: +1 435-865-8605.

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to act post-synaptically at nicotinic acetylcholine receptors [18]. A second colubrid 3FTx, from the venom of *Boiga dendrophila* (boigatoxin-A) [20], is unusual among snake venom toxins in that it exhibits both weak postsynaptic neurotoxicity in skeletal muscle *and* pre-junctional neurotoxicity in smooth muscle. A second *B. dendrophila*-derived 3FTx, denmotoxin [3], exhibits irreversible postsynaptic neuromuscular activity in chick tissue preparations but is readily reversible in rodent tissue. This toxin was the first bird-specific toxin to be completely characterized and the first colubrid-derived three-finger toxin to be crystallized. More recently, irditoxin, a unique heterodimeric 3FTx, was purified as the dominant toxin in the venom of *Boiga irregularis*, and the complete sequence, crystal structure and highly specific biological activity were described [4]. All of these colubrid 3FTxs are considered 'non-conventional 3FTxs' [7], and those derived from *Boiga* venoms show pronounced taxon-specific toxicity [19].

Recently we noted that the venom from the Green Vinesnake (*Oxybelis fulgidus*) contains venom components in the 6–9 kDa size range, typical of the three-finger toxins (Heyborne and Mackessy, unpubl. data). Additionally, literature reports based on behavioral observations of this species suggests the production of a venom that is toxic to both endothermic [21–23] and ectothermic prey [24]. At present, no literature accounts exist with quantitative data regarding toxicity of *Oxybelis* venom components to specific prey types.

Although the venom from *O. fulgidus* is apparently toxic to the snake's native prey, it is non-toxic or only mildly toxic to humans [25–27]. These observations strongly suggested the presence of prey-specific toxins, as has been observed in venoms from several species of *Boiga* [3,4], but none have yet been documented among neotropical colubrid snakes. In this study, we demonstrate the occurrence of a taxon-specific 3FTx from the venom of *O. fulgidus* and present structural data for fulgimotoxin (*O. fulgidus* monomeric toxin), purified from *O. fulgidus* venom. This 3FTx, like the heterodimeric irditoxin [4], is a potent lethal toxin toward lizards, with little to no effect toward mammals.

2. Materials and methods

2.1. Reagents and supplies

Unless otherwise specified, all chemicals (analytical grade or better) were obtained from Sigma–Aldrich (St. Louis, MO). NuPAGE/Novex gels, accompanying buffers and Mark 12 molecular weight standards were obtained from Invitrogen (Carlsbad, CA). An IEC CM-825 cation-exchange column (8 μm, 500 Å, 8 × 75 mm) was purchased from Showa Denko America, Inc. (New York, NY) and a Vydak Protein C-18 column (5 μm, 300 Å, 4.6 × 250 mm) was from Western Analytical Products (Murrieta, CA). BioRad protein concentration assay kit was from BioRad Laboratories (Hercules, CA). $sPLA_2$ Assay Kit was from Cayman Chemical Co. (Ann Arbor, MI), and V8 and lysyl endopeptidases were from Wako Chemicals USA, Inc. (Richmond, VA). Sterile water for animal injection was from Butler Animal Health Supply (Dublin, OH) and ketamine-HCl was from Fort Dodge Animal Health (Ft. Dodge, IA).

2.2. Extraction of venom

Venom was extracted from wild-caught *O. fulgidus* (no locality data – purchased from commercial importer), housed in the UNC Animal Facility (IACUC protocol 9204.1). The extraction protocol followed that reported by Hill and Mackessy [28]. Snakes were anesthetized using a 20–25 μg/g dose of ketamine-HCl, injected subcutaneously anterior to the snake's heart in the lateral body surface. After approximately 20 min, the snakes were injected with a 6.0 μg/g dose of pilocarpine-HCl (to stimulate oral secretion) on the opposite side of the body; injections were given in a bolus of 150 μL sterile saline. Venom was collected, centrifuged at 10k× *g*, lyophilized and stored at −20 °C until further use. Typically, at least six weeks elapsed between consecutive extractions.

2.3. Isolation of an 8.1 kDa protein from venom of O. fulgidus

Crude *O. fulgidus* venom was reconstituted in 20 mM sodium phosphate, pH 6.0. Following centrifugation and filtration (0.45 μm pore filter) to remove insoluble materials, the venom solution was applied to a Shodex IEC CM-825 cation-exchange column in the same buffer at a flow rate of 0.5 mL/min; flow rate was increased to 1.0 mL/min at 8 min. Bound proteins were eluted with a 0.5%/minute gradient (from 0 to 25%) followed by a secondary 2.5%/minute gradient (from 25 to 50%) of 20 mM sodium phosphate containing 1.0 M NaCl, pH 6.0. Fractions containing the 8.1 kDa protein were subsequently applied to a Vydak C-18 reversed-phase (RP) column in 0.1% TFA at a flow rate of 1.0 mL/min. Bound proteins were eluted with a 10%/minute gradient (from 0 to 100%) of 80% acetonitrile, 0.1% TFA. Fractions containing the 8.1 kDa protein (fulgimotoxin) were combined and lyophilized.

2.4. SDS-PAGE and mass spectrometry

Crude venom and HPLC fractions containing the 8.1 kDa protein were subjected to analysis by SDS-PAGE using NuPAGE 12% Bis-Tris gels and MES buffer, as described previously [29]. The purity and mass of isolated fulgimotoxin was also determined by MALDI-TOF-MS. Approximately 0.5 μg of reduced/alkylated (see Section 2.5) or native protein (1 μL) was comixed with 1 μL sinapinic acid (10 mg/mL 50% acetonitrile in 0.1% TFA) matrix, spotted onto a MALDI target plate, and spectra were obtained using a Voyager DE-Pro mass spectrometer (Global Peptides, Ft. Collins, CO) operating in linear mode, with delayed extraction and positive polarity.

2.5. Biological activity of fulgimotoxin

Fulgimotoxin was evaluated for lethal toxicity (24 h i.p. LD_{50}) using two lizard species (*Hemidactylus frenatus* and *Anolis carolinensis*) and neonate NSA mice (*Mus musculus*). Purified native fulgimotoxin was reconstituted in sterile water and total protein concentration was determined using the Bio-Rad protein concentration assay according to the manufacturer's instructions. In order to minimize the number of animals used, three animals were used per dose. All doses were body mass adjusted and administered intraperitoneally in 25–50 μL 0.9% saline to both lizards and mice. All procedures were reviewed and approved by the UNC-IACUC (protocol No. 9401).

2.6. Sequencing of fulgimotoxin

Lyophilized protein was reconstituted in 1 mL of 6 M urea with 0.5 M ammonium bicarbonate and 200 μL of 100 mM dithiothreitol was added. This solution was incubated at 50 °C for 60 min under argon. After cooling to room temperature, 100 uL of 500 mM iodoacetamide was added and the solution was incubated at room temperature for 30 min, again under argon. The reduced and alkylated protein was purified from reaction chemistry by RP-HPLC (Vydak C-18 column, 4.6 × 250 mm) using a gradient from 0.1% in Millipore-filtered water (buffer A) to 95% acetonitrile in 0.1% TFA (buffer B) at a flow rate of 1.0 mL/min. Bound protein was eluted with a 0.5%/minute gradient (from 25 to 40% buffer B), and

fractions containing the 8.1 kDa protein were combined and dried via a Speed Vac. Because "colubrid" three-finger toxins are typically N-terminally blocked with pyroglutamate, the alkylated protein was deblocked using gamma-glutaminase prior to sequencing. N-terminal sequencing (Edman degradation) was performed on an ABI Procise 494 protein sequencer (Protein Structure Core Facility, University of Nebraska Medical Center, Omaha, NE).

The reduced and alkylated protein (50 μg) was reconstituted in 1 mL of 50 mM sodium phosphate buffer (pH 7.8) with 1 μg of V8 endopeptidase. The sample was vortexed and centrifuged, then incubated at 25 °C for 1 h, followed by incubation at 37 °C for 1 h. Clean up and isolation of the digestion products was accomplished using RP-HPLC (Vydak C-18 column) as above, with a flow rate of 1.0 m, using a gradient of 5–50% buffer B. Fractions containing peptide peaks were dried via a Speed Vac and were then subjected to N-terminal sequencing as described above. Elucidation of the complete protein sequence necessitated a second endopeptidase digestion (lysyl [Lys-C] endopeptidase, 1 μg) of the reduced and alkylated protein (50 μg), following the procedure used with V8 endopeptidase but in 50 mM HEPES buffer pH 7.4.

2.7. Cladistic analysis of fulgimotoxin

Fulgimotoxin was subjected to a BLAST search (Basic Local Alignment Search Tool) using the non-redundant protein sequences (pr) database at the NCBI web site (http://www.ncbi.nlm.nih.gov/); results clearly indicated that it is a member of the snake venom three-finger toxin family. Sequences with greater than 50% identity were included in an alignment using ClustalX 1.81 [30]. Sequence similarities were evaluated using the bootstrapped neighbor-joining algorithm (1000 bootstrap trials), also in ClustalX, and two 3FTx-like protein sequences (Ly-6) from mammalian sources (GenBank number Q9WVC2, mouse; Q9BZG9, human) were used to root the tree. Trees were then drawn using FigTree 1.3.1 (available at http://tree.bio.ed.ac.uk/software/figtree/) in order to evaluate the relationship of fulgimotoxin sequence to other venom 3FTxs.

2.8. Molecular modeling of fulgimotoxin

Three dimensional structure of fulgimotoxin was modeled from the primary structure using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [31,32]. Several colubrid-derived neurotoxin structures for which crystal structures are known were identified by I-TASSER as suitable model construction templates (irditoxin B: Protein Database Accession Number 2H7Z_B; irditoxin A: 2H7Z_A; denmotoxin A: 2H5F_A). Figures were created using Discovery Studio Visualizer v3.1.1.11157 (Accelrys Software Inc., San Diego, CA). Data files for crystal structures of toxins used for comparisons in figures (irditoxin A & B, denmotoxin, cobrotoxin: 1COD, and α-cobratoxin: 1CTX) were obtained from the Protein Database (http://www.rcsb.org/pdb/home/home.do).

3. Results

3.1. Isolation of fulgimotoxin from venom of O. fulgidus

Cation exchange chromatography (QA 825) yielded a nearly pure 8 kDa protein (Fig. 1A) which eluted at approximately 53 min (22% B). This peak makes up approximately 32% of the total crude venom protein (based on HPLC-derived % peak area). To desalt the sample and ensure purity, the protein was subjected to RP-HPLC, which yielded a single 8 kDa peak at 14.5 min (94% B) (Fig. 1B). SDS-PAGE confirmed the purity and size of fulgimotoxin (Fig. 2A). MALDI-TOF mass spectrometry was used to determine precise mass and to verify the purity of the RP-HPLC product, which showed a single peak with a mass of 8146.06 Da (Fig. 2B). Following reduction and alkylation with IAA, the protein showed a mass of 8726.23 Da (Fig. 2C). This mass differential (580.17 Da) demonstrated the presence of ten cysteine residues, as the reducing reagent (iodoacetamide) increased the mass by approximately 58 Da per cysteine residue. This 8 kDa protein from *O. fulgidus* was named fulgimotoxin (*O. fulgidus* monomeric toxin), to indicate its origin and monomeric state [cf. [4]]. The monomeric state of fulgimotoxin was

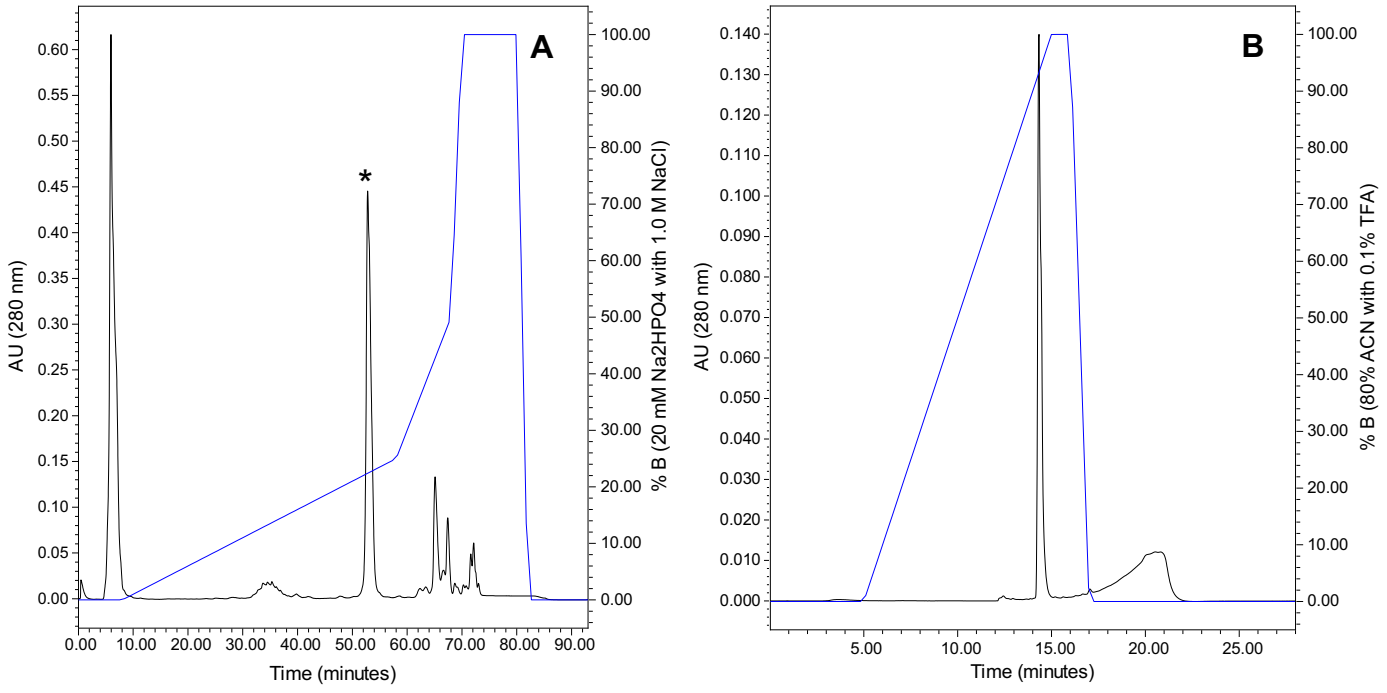

Fig. 1. Purification of fulgimotoxin. A. HPLC cation exchange (Shodex IEC CM-825) chromatogram of crude *Oxybelis fulgidus* venom (0.5 mg); fulgimotoxin (*) is the major retained protein. B. Reversed phase HPLC (Vydak C-18) chromatogram of fulgimotoxin peak from cation exchange.

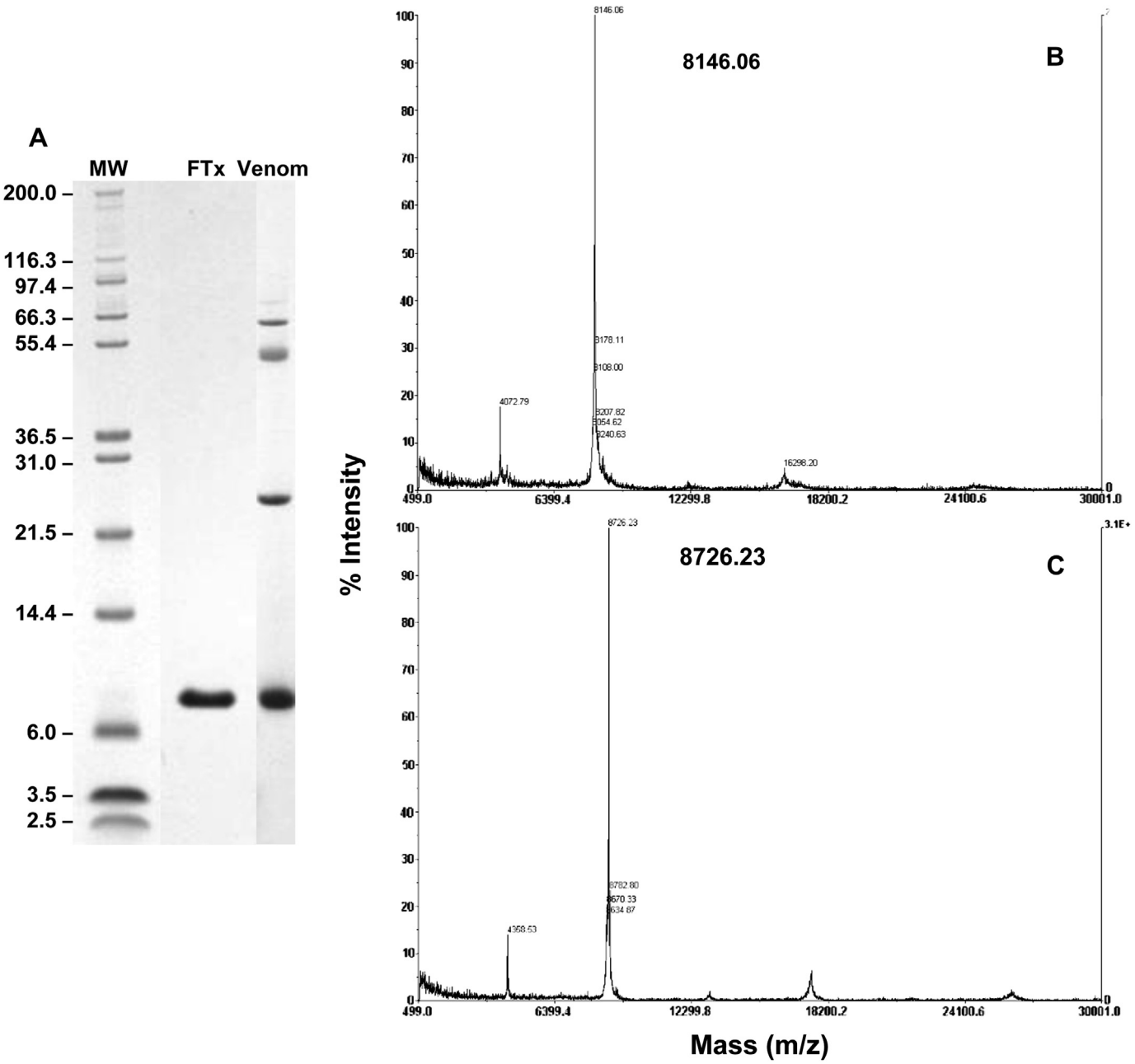

Fig. 2. Purity and mass of fulgimotoxin. A. Reducing SDS-PAGE revealed a single band with a mass of 8 kDa. B. MALDI-TOF-MS of native fulgimotoxin. C. MALDI-TOF-MS of reduced/alkylated fulgimotoxin. The mass difference between A and B (580.17) after reduction with iodoacetamide indicates the presence of five disulfides.

also demonstrated by size exclusion HPLC on a Tosohaas TSK G2000 SWxl column (data not shown).

3.2. Sequencing of fulgimotoxin

Direct N-terminal sequencing of purified fulgimotoxin, after deblocking by incubation with gamma-glutaminase, revealed a forty-four amino acid fragment: AIGPPYGLCFQCNQKTSSDCF-NAKxCxPxxxTCYTLxKxxGGEE (Fig. 3). Endopeptidase V8 digestion of purified reduced and alkylated fulgimotoxin resulted in nine dominant peaks and several additional minor peaks (Supplemental Fig. 1). Peaks two and five were chosen due to their apparent purity (as determined by MALDI-TOF MS) and were N-terminally sequenced, yielding an additional 28 and 18 residues respectively (Fig. 3). Visual alignment of the three fragments yielded a protein very similar in length and sequence to the other known colubrid neurotoxins, but the C-terminus remained unresolved. A second digestion using endopeptidase Lys-C resulted in >20 peaks (data not shown). Fractions 14, 18, 19 and 36 were sequenced, yielding several fragments which had previously been sequenced as well as the C terminal fragment (Fig. 3).

A BLAST search of the NCBI database using the complete fulgimotoxin protein sequence identified several colubrid-derived three-finger toxins with high sequence identity, including colubritoxin, irditoxin A and B, and denmotoxin (Fig. 4). The highest identity (67%) was seen with α-colubritoxin [18], a three-finger toxin from the venom of *C. radiatus*, an Asian colubrid snake. Observed in the fulgimotoxin sequence was a defining feature of the three-finger toxin family, the highly conserved 8–10 cysteine residues which participate in disulfide formation and constrain

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Complete FTx: QAIGPPYGLCFQCNQKTSSDCFNAKRCPPFHRTCYTLYKPDGGEEWAVKGCAKGCPTAGPDERVKCCHTPRCNN
N-term. Seq.: QAIGPPYGLCFQCNQKTSSDCFNAKxCxPxxxTCYTLxKxxGGEE
   V8 Peak 2:                                          WAVKGCAKGCPTAGPDERVKCCHTPRCN
   V8 Peak 5:                          RCPPFHRTCYTLYKPDGGE
Lys-C Fraction 14:                                                    CCHTPRCNN
Lys-C Fraction 18:           TSSDCFNAK
Lys-C Fraction 19:                                             GCPTAGPDERVK
Lys-C Fraction 36:                                   PDGGEEWAVK
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Fig. 3. Complete amino acid sequence of fulgimotoxin, deduced from N-terminal sequence, and V8 proteinase- and Lys-C proteinase-generated overlapping fragments.

molecular topology (Figs. 4 and 6). The theoretical molecular mass of the 74 amino acid fulgimotoxin, assuming oxidation of W46, was 8140.63 Da (within 0.1% of the experimentally determined mass; see Fig. 2), with a sequence-based predicted pI of 8.58.

3.3. Biological activity of fulgimotoxin

Purified fulgimotoxin was lethal to lizards at low doses but did not affect mice (Table 1). *Anolis* appeared to be most sensitive to the effects of fulgimotoxin (LD_{50} was approximately 0.28 μg/g), while *Hemidactylus* were less sensitive (LD_{50} approx. 0.6 μg/g). Many of the animals exhibited signs of toxin-induced stress at doses below the LD_{50}. At low doses (<0.2 mg/kg), *Hemidactylus* appeared unaffected throughout the monitoring period (24 h), while at the same doses some of the *Anolis* appeared lethargic, and two-thirds of the animals became immobile by ninety min post-injection but later recovered. At higher doses (>0.35 μg/g), the *Hemidactylus* became largely immobile by forty-five min post-injection but later recovered, while at this same dosage and time, all the *Anolis* were dead. Neonate mice (*M. musculus*) received doses up to 5 μg/g; higher doses were not attempted due to the limited availability of purified fulgimotoxin. No observable signs of distress or incapacitation were observed in treated mice, and no mortality resulted in any treatment groups.

3.4. Relationship of fulgimotoxin to other 3FTxs

Fulgimotoxin shared 48–67% sequence identity with other "colubrid" 3FTxs, but it showed <35% identity with elapid 3FTxs and a viperid 3FTx, in spite of the highly conserved structural residues in all toxins (Fig. 4). Analysis of sequences for possible motif similarities indicated only two short canonical motifs: CYTLY, the "colubrid" motif, comprising fulgimotoxin residues 34–38, and WAVK (residues 46–49), which are unique to all known taxon-specific toxins; both are within loop II (Fig. 4). One other colubrid toxin (3FTx_Tel4) also shows these sequences, and several other colubrid toxins show very similar sequences in these regions; however, at present these other colubrid toxins are known only from cDNA sequences.

The bootstrapped 3FTx tree largely followed generally accepted phylogenetic relationships (family level) among advanced snakes

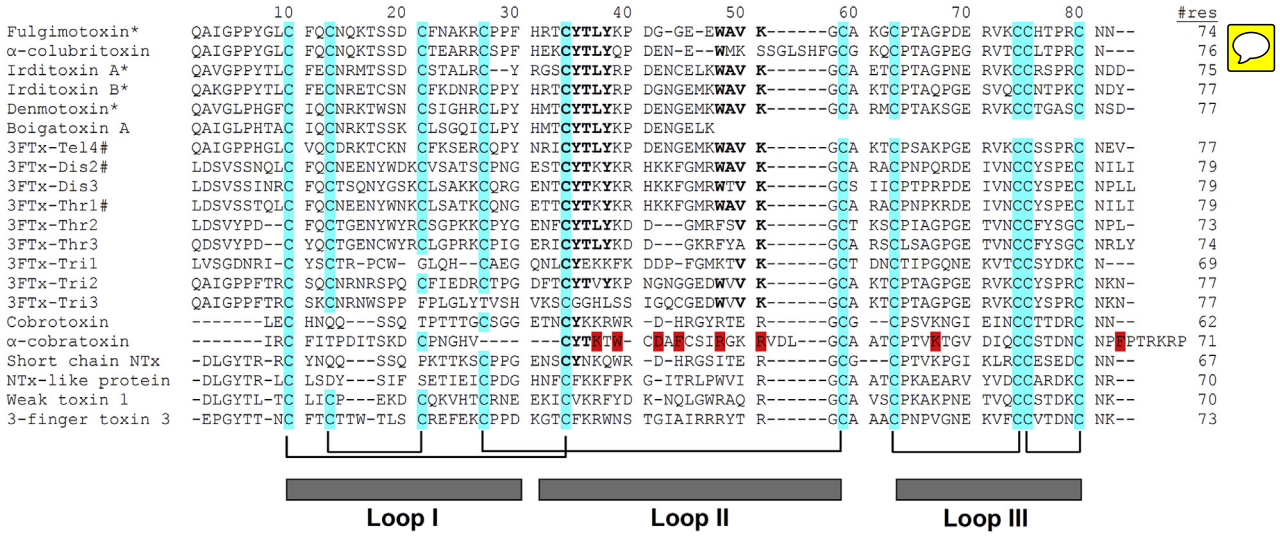

Fig. 4. Alignment of select venom-derived 3-finger toxin protein sequences with fulgimotoxin. Sequences selected represent diversity of toxins as well as diversity of taxa and include all isolated colubrid 3FTxs: fulgimotoxin (family Colubridae); α-colubritoxin, from *Coelognathus radiatus* (Colubridae; GenBank/EBI accession number P83490); irditoxin subunit A, from *Boiga irregularis* (Colubridae; ABC17853.1); irditoxin subunit B, from *B. irregularis* (Colubridae; ABC17854.1); denmotoxin (ABC95749.1) and boigatoxin A (P0C603.1; partial sequence), from *Boiga dendrophila* (Colubridae); 3FTx-Tel4, from *Telescopus dhara* (Colubridae; EU029686.1); 3FTx-Dis2 (EU029681.1) and 3FTx-Dis3 (EU029683.1), from *Dispholidus typus* (Colubridae); 3FTx-Thr1 (EU029682.1), 3FTx-Thr2 (EU029684.1), and 3FTx-Thr3 (EU029685.1), from *Thrasops jacksoni* (Colubridae); 3FTx-Tri1 (EU029675.1), 3FTx-Tri2 (EU029677.1), and 3FTx-Tri3 (EU029678.1), from *Trimorphodon biscutatus* (Colubridae); cobrotoxin, from *Naja* (*naja*) *atra* (Q9PTT0.1); α-cobratoxin, from *Naja* (*naja*) *kaouthia*, a long chain 3FTx with an additional disulfide in loop II (Elapidae; P01391.1), short chain neurotoxin, from *Laticauda colubrina* (Elapidae; BAA75757.1); neurotoxin-like protein NTL2, from *Naja naja* (Elapidae; Q9W717); weak toxin 1, from *Bungarus candidus* (Elapidae; AAL30059.1); and three finger toxin 3, from *Sistrurus catenatus edwardsii* (Viperidae; DQ464283.1). Numbering is optimized for fulgimotoxin but incorporates the insertion found in α-colubritoxin, and total number of residues (#res) follows each sequence. Sequences were aligned using ClustalX (Thompson et al., 1997). Conserved cysteine residues participating in intramolecular disulfide formation are highlighted in blue and unpaired cysteines in red. Residues demonstrated to be critical to muscle nicotinic acetylcholine receptor binding (for α-cobratoxin [42];) are highlighted in red. The residues highly conserved in colubrid 3FTxs (CYTLY) and the putative specificity-conferring residues (WAVK) are shown **bolded** for fulgimotoxin; other toxins which have at least two of these residues consecutively per motif are also bolded. Asterisks (*) indicate proteins for which taxon-specific toxicity has been empirically determined; (#) indicates cDNA-based sequences for proteins predicted to show taxon-specific toxicity.

Table 1
Toxicity of fulgimotoxin to lizards and mice.

Species	LD_{50} (μg/g)
Anolis carolinensis	0.28
Hemidactylus frenatus	0.6
Mus musculus	>5.0

(Fig. 5); two minor deviations from this pattern were observed. The 3FTx from *Sistrurus catenatus* (SCE3) clustered deep in the "colubrid" group, and two "colubrid"-derived toxins (from *Enhydris polylepis* and *Liophis poecilogyrus*) grouped with two elapid-derived toxins (from *Dendroaspis jamesoni* and *Bungarus candidus*) to form a distinct group. Fulgimotoxin clustered most closely with α-colubritoxin from *C. radiatus*, within a slightly larger group including 3FTx-Tel4 toxin from *Telescopus dhara* and the three *Boiga*-derived toxins described above.

Fig. 5. Cladogram of relationship of fulgimotoxin (FTx_*Oxybelus fulgidus*) to other 3FTxs, using the mammalian 3FTx-like Ly-6 proteins (from mouse and human) as outgroups. The bootstrapped neighbor-joining tree was constructed initially using ClustalX and then optimized in FigTree 1.3.1. Note that the general topology follows broad phylogenetic patterns (traditional family level, with viperids basal), with the exception of a small basal clade containing two colubrid (EU029668, EU029670) and two elapid (P25682, P81782) sequences; the viperid 3FTx SCE3 (DQ464283) also clustered within colubrid sequences. Blue clades, mammalian sequences; green, "colubrid" sequences; red, elapid sequences; black, viperid sequences. Numbers represent GenBank accession numbers.

3.5. Molecular modeling of fulgimotoxin

The three-dimensional conformation of fulgimotoxin clearly showed the highly conserved three-finger folds attached to a globular central core, a consequence of the constraining influence of the four core disulfide bridges (Fig. 6A). Like other 3FTxs, the three loops are stabilized by antiparallel beta sheets, and the position of the colubrid-specific motif and the putative taxon-specific toxin motif are indicated on loop II (Fig. 6B). The surface of the molecule is dominated by positive charges, as expected of this basic protein (pI = 8.58) and also similar to most snake venom 3FTxs (Fig. 6C and D). Fulgimotoxin is also predicted to share very similar three dimensional conformation with denmotoxin, another monomeric colubrid 3FTx, and with cobrotoxin (Fig. 7A), an elapid 3FTx, in spite of the fact that only the elapid toxin is lethal to mammal models (including humans) and it shows only 32% sequence identity with fulgimotoxin. This basic fold is shared with other elapid lethal toxins, such as α-cobratoxin (B); residues of loop II which are critical to *Torpedo* acetylcholine receptor binding are indicated. The colubrid-specific motif and the putative taxon-specific toxin motif occur in a similar region of loop II as the elapid critical binding residues, and in all three 3FTxs for which taxon-specific toxicity has been experimentally confirmed, these motifs occur in precisely the same region of loop II (Fig. 7C–E).

4. Discussion

4.1. Fulgimotoxin, a new colubrid venom three-finger toxin

Three-finger toxins are broadly distributed among colubroid snake venoms [2,13,16,17], and as more venoms are investigated, the pharmacological diversity of these toxins will likely increase.

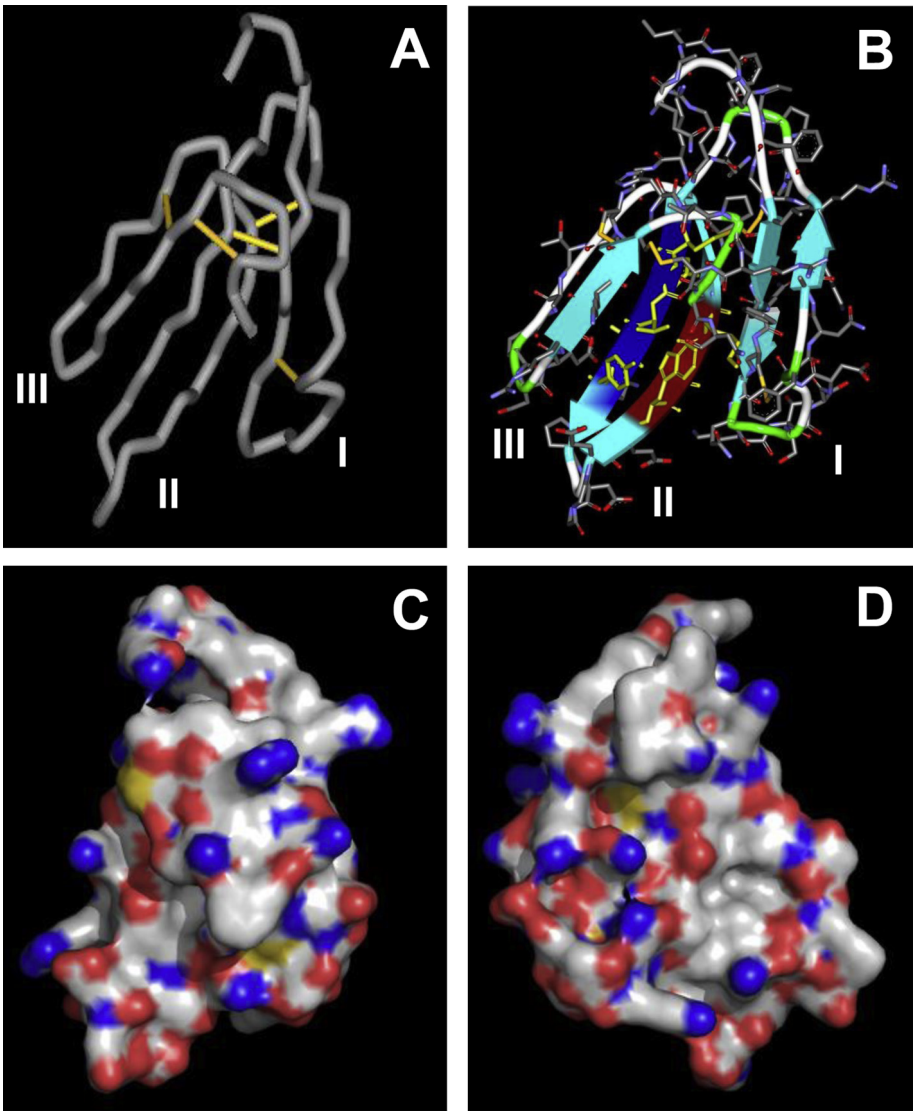

Fig. 6. Structural models of fulgimotoxin, a three-finger toxin from *Oxybelis fulgidus* venom. A. Stick diagram showing the position of the five disulfide bonds (gold); the fifth disulfide of loop one is a characteristic of non-conventional 3FTxs, and the three "fingers" defined by the conserved disulfides are apparent. B. Solid ribbon/stick model, showing the antiparallel beta sheets characteristic of loops one and two of 3FTxs. Dark blue sheet includes residues CYTLY, the highly conserved colubrid toxin residues, and the red sheet includes residues WAVK, the putative specificity-conferring residues. Note that both are found in loop II. C. Space-filling with electrostatic potential of the surface, same perspective as in A & B. D. 180° rotation from C. For C and D, the positively and negatively charged residues are shown in blue and red colors, respectively, and the hydrophobic residues are shown in white. Modeling from the primary structure was done using the I-TASSER server (based on colubritoxin and irditoxin b); figures were constructed using Discovery Studio Visualizer v3.1.1.11157 (Accelrys Software Inc., San Diego, CA).

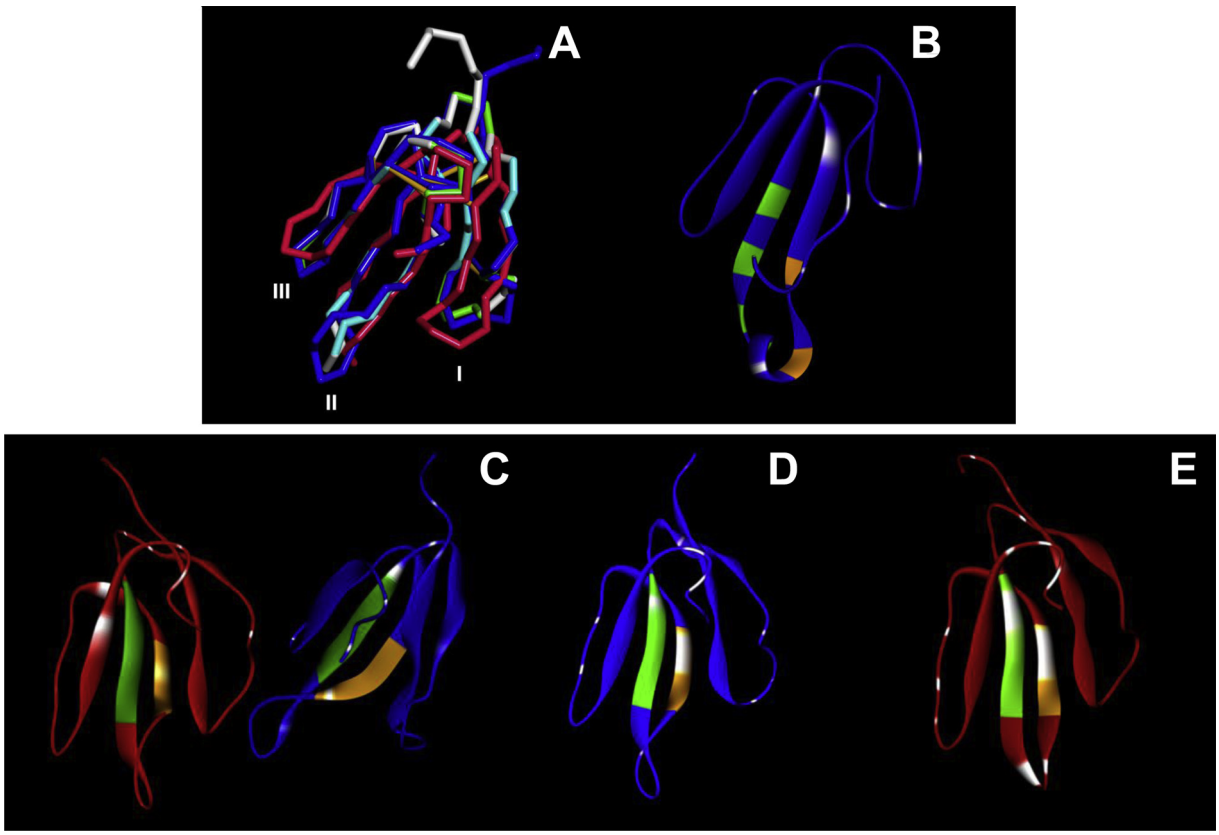

Fig. 7. Structural comparison of fulgimotoxin with other three-finger toxins. A. Backbone of fulgimotoxin (multicolors) overlain with those of cobrotoxin (red) and denmotoxin (blue). Note that although cobrotoxin lacks taxon-specific toxicity and has only 35% sequence identity with fulgimotoxin, all three toxins show very similar three dimensional configuration. B. Ribbon diagram for α-cobratoxin; residues in loop II which are important to receptor binding are shown in green and orange. C–E. Conservation of sequence motifs in loop II of taxon-specific 3FTxs. In all colubrid venom toxins, the portion of the beta sheet representing the CYTLY motif is green, and that for WAVK is orange; only irditoxin is a heterodimer. Coordinates for crystal structures of cobratoxin (A), α-cobratoxin (B), irditoxin (C) and denmotoxin (D) were obtained from the Protein Database; the structure of fulgimotoxin (E) is based on coordinates obtained from the I-TASSER server. All structures were drawn using Discovery Studio Visualizer v3.1.1.11157.

The presence of rapid acting, potently neurotoxic 3FTxs in colubrid venoms further supports the conclusion that colubrid venoms, like those from front-fanged species, have been subjected to positive selection and have evolved into mechanisms which facilitate prey acquisition and handling [17,33,34]. The taxon-specific activity of fulgimotoxin reported here provides additional evidence that a single molecular scaffold, common to the venoms of several advanced snake groups, may be subtly modified by selective pressure to elicit effects specific to a species and that species' unique biology [13].

4.2. Biological role of fulgimotoxin

Purified fulgimotoxin was quite toxic to *Anolis* (i.p. LD_{50} of 0.28 μg/g) yet was non-toxic to mice at mass-adjusted doses more than fifteen times this dose, showing taxon-specific toxicity comparable to the well-characterized colubrid 3FTxs denmotoxin and irditoxin [3,4]. Denmotoxin (not tested on lizards) was reported to be a bird-specific toxin, and because *O. fulgidus* feeds on lizards and also birds [35], it is hypothesized that both toxins would show high toxicity in both lizard and bird models, as is observed for irditoxin [4]. Like irditoxin, fulgimotoxin is highly toxic to lizards and accounts for a significant fraction of the crude venom protein (approximately 32%), so even the relatively low yields of this venom (~4–6 mg dry venom per extraction) are in excess of what is required to kill moderate sized prey quickly.

Whereas many 3FTxs from elapid venoms have post-synaptic neurotoxic effects toward vertebrates generally [7,36], the taxon-specific effect of fulgimotoxin is likely due to selective pressures favoring rapid-acting venoms against preferred prey, in this case lizard prey (similar to irditoxin from *B. irregularis* venom) [4]. Although not tested empirically, reports of feeding behavior of *O. fulgidus* from the wild clearly suggest toxicity to birds [21,22]. Of note is the report of Endo et al. [23], who reported the capture of a Black-fronted Nunbird (*Monasa nigrifrons*, approximately 280 mm in length), held near the head by the snake without constriction until dead. We suggest that given the average size of the very slender adult *O. fulgidus* (maximum 2 m), the capture, incapacitation and subsequent ingestion of a prey animal of this size would necessitate the use of a toxic venom component. Given the low complexity of the *O. fulgidus* venom proteome and the abundance of the toxin in crude venom, fulgimotoxin is almost certainly that component.

At first consideration, it seems enigmatic that various members of this subset of 3FTxs are highly toxic to lizards (ectotherms) and/or birds (endotherms) but are essentially harmless to mammals (also endotherms). However, morphological studies of both living [37] and fossil [38] birds have long linked them with the Reptilia. More recent molecular studies e.g. [39,40] have reaffirmed the close phylogenetic relationship of birds and reptiles, relative to the ancient common ancestry of birds and mammals. Based on phylogeny rather than endothermic physiology, it is therefore expected that many of the toxins being described from colubrid venoms should exhibit specific toxicity toward both lizards and birds, with low or no toxicity toward the more distantly related mammals.

4.3. Structural features of fulgimotoxin

As indicated by MALDI-TOF MS analysis of native and IAA-reduced fulgimotoxin, and confirmed by protein sequencing, the ten cysteine residues present in fulgimotoxin participate in disulfide formation, and five are concentrated in the last 25 amino acid residues. These ten cysteine residues are 100% conserved among most colubrid-derived members of this protein family and form five intramolecular disulfide bonds [3,4]. The position of four of the disulfides is a canonical feature of the 3FTx family [7]; a fifth disulfide is found in loop I of most non-conventional 3FTxs, including fulgimotoxin. Based on homology, we infer that the cysteines in fulgimotoxin share the same internal disulfide arrangements as those of irditoxin A and B, and denmotoxin (Fig. 4).

Two other highly conserved sequence regions were noted among the colubrid 3FTxs for which sequence and toxicity data is known: CYTLY, (residues 34–38, fulgimotoxin numbering) and WAVK (residues 46–49). Colubritoxin possesses the CYTLY motif, but is anomalous among sequenced colubrid 3FTxs in that it contains an apparently unique 6 residue insertion (SGLSHF) immediately following the WAVK motif region (WMKS in colubritoxin) and immediately prior to the second cysteine residue of loop II. Among these species, *C. radiatus* (produces colubritoxin) has the most generalized diet, consuming not only lizards and birds but also commonly mammals. As the other toxins are all produced by lizard and/or bird specialists, we hypothesize that the WAVK motif, in conjunction with the CYTLY motif, is involved with the taxon-specificity of these 3FTxs. The fact that both conserved sequence motifs occur in the second loop likely has functional significance, as this loop has been implicated in receptor specificity (for α7 nAChR) in some long chain neurotoxins [41]. Additionally, loop II of α-cobratoxin contains most of the residues which are critically important to binding to *Torpedo* nicotinic acetylcholine receptor [42]. Further, loop II of fasciculin, a 3FTx inhibitor of acetylcholinesterase, has been shown to be critical to binding to its target [43], demonstrating the structural importance of loop II of several pharmacologically-distinct 3FTxs to specific ligand interactions.

Among colubrid 3FTxs for which cDNA sequences are known (but for which functional data is lacking) [16], the CYTLY motif is seen in toxins of venoms from *T. dhara* 3FTx-Tel4 (A7X3V0), *Thrasops jacksoni* 3FTx-Thr2 (ABU68484) and 3FTx-Thr3 (ABU68485). A slightly modified form (CYTKY) is exhibited by *Dispholidus typus* 3FTx-Dis2 (ABU68481), 3FTx-Dis3 (ABU68483) and *T. jacksoni* 3FTx-Thr1 (ABU68482). Yet a third variant, CYTVY, occurs in cDNA from *T. dhara* 3FTx-Tel2 (ABU68479). The WAVK motif is also differentially expressed only in *T. dhara* 3FTx-Tel4 (A7X3V0), *D. typus* 3FTx-Dis2 (ABU68481) and *T. jacksoni* 3FTx-Thr1 (ABU68482). Slightly modified variants, WTVK and WVVK, are exhibited by *D. typus* 3FTx-Dis3 (ABU68483) and *Trimorphodon biscutatus* 3FTx-Tri2 (A7X3S0) and 3FTx-Tri3 (A7X3S2) respectively. Interestingly, like *B. dendrophila*, *B. irregularis* and *O. fulgidus*, *T. dhara*, *T. jacksoni* and *D. typus* also feed primarily on lizards and/or birds [44]. We hypothesize that several of these toxins of African colubrid venoms are also taxon-specific and that this limited-occurrence motif is involved in conferring taxon specificity to this group of 3FTxs. The absence of these motifs in the 3FTxs from both elapid and viperid snake venoms provides one last piece of evidence for these regions conferring the taxon-specific activity of the colubrid-derived toxins, which has not been reported from the other advanced snake groups.

Three finger toxins represent a fascinating family of venom proteins which have been subjected to positive selection, resulting in specific variants which show divergent pharmacologies while retaining a highly conserved molecular fold. For example, lethal toxicity (to mice) of 3FTxs in elapid venoms ranges from 0.07 μg/g to non-toxic [45]. Results presented here indicate that even among well-characterized toxin families, there are novel structure/function relationships which can be identified, particularly among lesser explored sources of toxins such as the rear-fanged colubrid snakes. Our recent work with disintegrins in rattlesnake venoms further illustrates that venom toxins may have unexpected and novel biological roles [46]. Because trophic specializations can lead to the production of highly derived toxin variants, aspects of natural history (i.e., dietary specialization) can be informative for selecting natural sources of toxins with novel activities, and current efforts are focused on characterizing venom toxins from highly specialized colubrid snakes.

5. Conclusions

We report the purification, characterization and protein sequence of a novel taxon-specific neurotoxin, fulgimotoxin, isolated from the venom of the American neotropical colubrid snake *O. fulgidus*. This toxin shows high lethal toxicity toward lizards but is non-toxic to mammal models. We identify specific limited-occurrence sequence motifs which are hypothesized to confer this specificity, and these sequences are absent from other characterized members of the 3FTx family of proteins isolated from snake venoms. This subset of 3FTxs demonstrates unequivocally the effect of directional positive evolution of toxins which provides defined selective advantages to snakes, namely the capacity to immobilize preferred prey rapidly and specifically. The conserved protein scaffold of 3FTxs illustrates that very fine structural variation can impart important functional specificity to these toxins which can be utilized to explore protein:protein interactions and to exploit in drug discovery efforts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2013.06.025.

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