Snake venoms are a mixture of pharmacologically active proteins and polypeptides that have led to the development of molecular probes and therapeutic agents. Here, we describe the structural and functional characterization of a novel neurotoxin, haditoxin, from the venom of Ophiophagus hannah (King cobra). Haditoxin exhibited novel pharmacology with antagonistic activity toward muscle (3). For example, the discovery of α-bungarotoxin, a postsynaptic neurotoxin from the venom of Bungarus multicinctus, led to the identification of the nicotinic acetylcholine receptor (nAChR), the first isolated receptor protein (4) as well as biochemically (5, 6). Subsequently, it was also used to characterize several other nAChRs (8–10).

Snake venom proteins can be broadly classified as enzymatic and non-enzymatic proteins. Three-finger toxins (3FTxs) are the largest group of non-enzymatic snake venom proteins (1, 11). They are most commonly found in the venoms of elapid and hydrophid snakes. Recently, our laboratory has also demonstrated the presence of 3FTxs from colubrid venoms (12, 13), and 3FTx transcripts have been found in the venom gland transcriptome of viperid snakes (14, 15). The proteins in this family of toxins share a common structural scaffold of three β-sheets loops emerging from a central core (11, 16). Despite the overall similarity in structure, these proteins have diverse functional properties. Members of this family include neurotoxins targeting the cholinergic system (7, 11, 16), cytotoxins/cardiotoxins interacting with the cell membranes (17), calcisepitene and related toxins that block the L-type Ca²⁺ channels (18), dendroaspin, which are antagonists of various cell adhesion processes (19), and β-cardiotxin antagonizing the β-adrenoceptors (20). The subtle variations in their structures, such as the presence of extra disulfide bonds, differences in size and overall conformation (twists and turns) of the loops, and longer C-terminal and/or N-terminal extensions (21), may contribute to the observed functional diversity as well as specificity of these toxins (22).
This family contains several types of neurotoxins that interact with different subtypes of nicotinic and muscarinic receptors involved in central and peripheral cholinergic transmission. Depending on the target receptors, these neurotoxins can be broadly divided into various groups. Curaremimetic or α-neurotoxins that target muscle (αβγδ or α- subtype) nAChRs (7, 16, 23) belong to short-chain and long-chain neurotoxins (classified based on size and number of disulfide bridges (24)). Long-chain neurotoxins, but not short-chain neurotoxins, also target neuronal α7-nAChRs associated with neurotransmission in the brain (25). α-Neurotoxins, such as κ-bungarotoxin (B. multicinctus), show specificity for other neuronal subtypes, αβ2- and αβ2- nAChRs (26, 27). Muscarinic 3FTxs, unlike many small molecule ligands, can distinguish between different types of muscarinic acetylcholine receptors (mAChRs) (for review, see Ref. 28) and hence are useful in the characterization of these receptor subtypes. Muscarinic toxin 1, isolated from the venom of Dendroaspis angusticeps, interacts with mAChR subtype 1 (M1) (29), whereas muscarinic toxin 3 (MT3), isolated from the same snake, interacts with M4 mAChRs (30). In recent years, new 3FTxs with distinct and novel receptor specificities have been characterized and added to this growing library (12, 13, 31–36), justifying the rational design of specific therapeutics.

All neurotoxins characterized to date exist as monomers with the exception of κ-neurotoxins from Bungarus sp. (37, 38), which is a non-covalently linked homodimer that binds neuronal (αβ2 and αβδ) but not muscle (αβγδ) nAChRs. More recently, we published the first report of a covalent heterodimeric neurotoxin, irditoxin, from the venom of Boiga sp., which was a uniquely irreversible inhibitor of muscle (αβγδ) nAChRs (13). Here, we report the purification, pharmacological characterization, and a high resolution crystal structure of a novel non-covalent homodimeric neurotoxin from the venom of Ophiophagus hannah (King cobra). Although its quaternary structure is similar to κ-neurotoxins, it exhibited novel pharmacology with potent blocking activity on muscle (αγδ) as well as neuronal (αγδ, α2β2, and α2β2) nAChRs. Based on the high resolution crystal structure (1.55 Å) we have explored its structural similarities with other neurotoxins. This new toxin was named haditoxin (O. hannah dimeric neurotoxin) and is the first homodimeric three-finger neurotoxin interacting with α7-nAChRs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lyophilized O. hannah crude venom was obtained from PT Venom Indo Persada (Jakarta, Indonesia) and Kentucky Reptile Zoo (Slade, KY). Reagents for N-terminal sequencing by Edman degradation are from Applied Biosystems (Foster City, CA). KCl, acetonitrile, and trifluoroacetic acid were from Merck KGaA, Darmstadt, Germany. Precision Plus protein standards, dual color (marker for SDS-PAGE), and bis(sulfosuccinimidyl) suberate (BS3) were purchased from Bio-Rad Laboratories and Pierce, respectively. Superdex 30 HiLoad (16/60) column and Jupiter C18 (5 μ, 300 Å, 4.6 × 150 mm) were purchased from GE Healthcare and Phenomenex (Torrance, CA), respectively. Crystal screening solution and accessories were obtained from Hampton Research (Aliso Viejo, CA). All other chemicals including α-bungarotoxin from B. multicinctus were purchased from Sigma-Aldrich. All the reagents were of the highest purity grade. Water was purified using a MilliQ system (Millipore, Billerica, MA).

**Animals**—Animals (Swiss albino mice and Sprague-Dawley rats) were acquired from the National University of Singapore Laboratory Animal Center and acclimatized to the Department Animal Holding Unit for at least 3 days before the experiments. They were housed, four per cage, with food and water available ad libitum in a light controlled room (12-h light/dark cycle, light on at 7:00 a.m.) at 23 °C and 60% relative humidity. Domestic chicks (Gallus gallus domesticus) were purchased from Chew’s Agricultural Farm, Singapore, and delivered on the day of experimentation. Animals were sacrificed by exposure to 100% carbon dioxide. All experiments were conducted according to the Protocol (021/07a) approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

**Purification of the Protein**—O. hannah crude venom (100 mg dissolved in 1 ml of MilliQ water and filtered) was loaded onto a Superdex 30 gel filtration column, equilibrated with 50 mM Tris-HCl buffer; pH 7.4, and eluted with the same buffer using an ÄKTA purifier system (GE Healthcare). Fractions containing the protein of interest were further subfractionated by reverse phase-high performance liquid chromatography (RP-HPLC) using a Jupiter C18 column, equilibrated with 0.1% (v/v) trifluoroacetic acid, and eluted with a linear gradient of 80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Elution was monitored at 280 and 215 nm. Fractions were directly injected into an API-300 liquid chromatography-tandem MS system (PerkinElmer Life Sciences) to determine the mass and homogeneity of the protein as described previously (20). Analyte software (PerkinElmer Life Sciences) was used to analyze and deconvolute the raw mass data. Fractions showing the expected molecular mass were pooled and lyophilized.

**Capillary Electrophoresis**—Capillary electrophoresis was performed on a BioFocus3000 system (Bio-Rad) to determine the homogeneity of the protein after RP-HPLC. The native protein (1 μg/μl) was injected to a 25 μm × 17 cm coated capillary using a pressure mode (5 p.s.i./s) and run in 0.1 m phosphate buffer (pH 2.5) under 18 kV at 20 °C for 7 min. The migration was monitored at 200 nm.

**N-terminal Sequencing**—N-terminal sequencing of the native protein was performed by automated Edman degradation using a Procise 494 pulsed liquid-phase protein sequencer (Applied Biosystems) with an on-line 785A phenylthiohydantoin derivative analyzer. The phenylthiohydantoin amino acids were sequentially identified by mapping the respective separation profiles with the standard chromatogram.

**CD Spectroscopy**—Far-UV CD spectra (260–190 nm) were recorded using a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan) as described previously (20). The protein samples (concentration range 0.25–1 mg/ml) were dissolved in MilliQ water.
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In Vivo Toxicity Study—Native protein (200 μl dissolved in 0.89% NaCl) was injected intraperitoneally using a 27-gauge 0.5-inch needle (BD Biosciences) into male Swiss albino mice (15 ± 2 g) at doses of 5, 10, and 25 mg/kg (n = 2). The symptoms of envenomation were observed, and in the event of death, the time of death was noted. The control group was injected with 200 μl of 0.89% NaCl. Postmortem examinations were conducted on all animals.

Ex Vivo Organ Bath Studies—Isolated tissue experiments were performed as described previously (13, 31) using a conventional organ bath (6 ml) containing Krebs solution of the following composition (in mM): 118 NaCl, 4.8 KCl, 1.2 KH2PO4, 2.5 CaCl2, 25 NaHCO3, 2.4 MgSO4, and 11 glucose; pH 7.4, at 37 °C. This is continuously aerated with carbogen (5% carbon dioxide in oxygen). The resting tension of the tissues was maintained at 1–2 g, and the preparations were allowed to equilibrate for 30–45 min. Electrical field stimulation was conducted on all animals.

RESULTS

Five novel 3FTxs were identified from the cDNA library of the venom gland tissue of O. hannah, and one of them, named β-cardiotoxin, has been characterized previously (20). Here, we describe the characterization of the second novel toxin, identified previously as an MTLP-3 homolog based on sequence homology (20) (Fig. 1). The liquid chromatography/MS profile of O. hannah venom (44) showed the presence of a 7,535.67 ± 0.60 Da protein, similar to the expected molecular mass of the protein being characterized. This protein was purified from the crude venom using a two-step chromatographic approach. Firstly, the venom components were separated based on their sizes into five peaks using gel filtration chromatography (Fig. 2A). Subsequently, each peak was fractionated by RP-HPLC, and the fractions were analyzed by ESI-MS to identify the presence of the protein of interest (Fig. 2A, black bar). Further, these
fractions were pooled and separated by RP-HPLC (Fig. 2B). The ESI-MS of fraction 2a (Fig. 2C, black arrow) showed three peaks with mass/charge (m/z) ratios ranging from +4 to +6 charges (Fig. 2C), and the final reconstructed mass spectrum showed a molecular mass of 7,535.67 ± 1.25 Da, which matched the calculated mass of 7,534.42 Da (Fig. 2C, inset). The secondary structural elements of haditoxin were analyzed using far-UV CD spectroscopy. The spectrum shows maxima at 230 and 198–200 nm and a minimum at 215 nm (Fig. 2D). Thus, haditoxin was found to be composed of β-sheeted structure similar to all other 3FTxs (11, 16). The presence of a single protein peak in the electropherogram (Fig. 2E) indicates the homogeneity of the protein, ensuring the absence of contaminants, especially other α-neurotoxins and cytotoxins present in the venom.

Identification was further confirmed by N-terminal sequencing of the first 36 residues, which matched the cDNA sequence of the MTLP-3 homolog (20). This protein was found to be a homodimer (see below) and hence was renamed as haditoxin (O. hannah dimeric neurotoxin) following the nomenclature of dimeric 3FTxs (12, 13).

Investigation of Haditoxin for Muscarinic Effects—As detailed in Fig. 1, A and B, haditoxin showed high similarity (80–83%) with muscarinic toxin homologs (MTLP and MTLP-3) as well as similarity with muscarinic toxins (MT-α, MT7, and MT3) (51–52%). As such, we examined the effects of haditoxin on in vitro smooth muscle preparations, the rat ileum and rat anococcygeus muscle, pharmacologically characterized to represent M2 (45) and M3 mAChRs (46), respectively. In both preparations, the protein had no effect on the contractile response of the muscle to exogenously applied ACh or electrical field stimulation, suggesting that haditoxin does not interact with M2 and M3 mAChRs (supplemental Fig. 1). Therefore, it is likely that the observed sequence similarity with muscarinic toxin homologs is probably coincidental due to either phylogeny or structure, including the presence of the core disulfide bridges, and not the function. This merits further investigation, including electrophysiological studies and/or binding assays on other subtypes of mAChRs.

In Vivo Toxicity of Haditoxin—In preliminary experiments to observe the biological effects of haditoxin, all mice injected with the toxin (5, 10, and 25 mg/kg) showed typical symptoms of peripheral neurotoxicity, such as paralysis of hind limbs and labored breathing, and finally died, presumably due to respiratory paralysis (47, 48). The time of death was recorded for each animal, with the average calculated to be 94, 32.5, and 20 min, respectively, for the 5, 10, and 25 mg/kg doses. On postmortem, no gross changes in the internal organs, notably hemorrhage, were observed.

Ex Vivo Neurotoxic Effects of Haditoxin—The observed peripheral neurotoxic symptoms produced by haditoxin in vivo warranted detailed pharmacological characterization on neuromuscular transmission using CBCM and RHD preparations. Haditoxin (1.5 μM) produced a reproducible time- and dose-dependent neuromuscular blockade in both preparations (Fig. 3, A and C). In the CBCM, it completely inhibited the contractile response to exogenous agonists (ACh and carbachol (CCh)), whereas response to exogenous KCl and twitches evoked by direct muscle stimulation were not inhibited, indicating a postsynaptic neuromuscular blockade and an absence of direct myotoxicity.

The IC₅₀ of haditoxin on CBCM and RHD was 0.27 ± 0.07 and 1.85 ± 0.39 μM, respectively (Fig. 3E) (considering the fact...
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that the protein exists as dimer in solution; see below). When compared with α-bungarotoxin (IC_{50} on CBCM 12.1 ± 5.4 nM and RHD 100.5 ± 22.5 nM) (Fig. 3E), haditoxin was about 50 times less potent on both avian (CBCM) and mammalian (RHD) neuromuscular junctions.

Reversibility of the neuromuscular blockade was tested for both preparations with intermittent washing (Fig. 3, D and F, black arrows). Partial recovery of the contractile response (60% recovery in 2 h) was observed in the CBCM (Fig. 3B) but not in the RHD (Fig. 3D). These results indicate that unlike typical α-neurotoxins such as α-bungarotoxin, haditoxin exhibits partial reversibility in action, at least in the CBCM.

Effect of Haditoxin on Human nAChRs—Because haditoxin blocked the muscle activity of the CBCM and RHD, we examined its activity on human αβδε-nAChRs. Haditoxin completely inhibited the ACh-induced αβδε currents at a concentration of 10 μM (Fig. 4A) with an IC_{50} value of 550 nM (n = 13) (Fig. 4B). This inhibition was practically irreversible within 8 min of washout. This result is in good agreement with the findings on ex vivo studies with RHD as discussed earlier. Next, we tested the activity of haditoxin on α_7- and α_2β_2-nAChRs. On α_7-nAChRs, an irreversible block was observed at 10 μM concentration of haditoxin (Fig. 4C) with an IC_{50} value was 180 nM (n = 4) (Fig. 4D). As shown in Fig. 4E, 10 μM haditoxin fully blocked the response of α_2β_2-nAChRs, with an IC_{50} value of 500 nM (n = 4) (Fig. 4F). Notably, the blockade at α_2β_2 was fully reversible, whereas long-lasting blockade was observed at α_2β_2-nAChRs. This suggests that the K_{Off} value at the α_2 receptor is much smaller than at α_2β_2-nAChRs. As these two receptors display about equivalent IC_{50} values, this indicates that their respective K_{On} values are probably significantly different. However, the experimental protocol used herein prevents the detailed analysis of the K_{On} and K_{Off} values. An additional difference between these two receptors resides in their structural composition. Although it is thought that α_2-nAChRs display five identical ligand binding sites, only two binding sites are proposed for the α_2β_2-nAChRs. The difference in number of binding sites and effects on competitive blockade was previously discussed for α_7- and α_2β_2-nAChRs, showing significant functional outcomes (49). Interestingly, haditoxin was almost 3-fold more potent to block ACh-induced responses mediated by α_7-nAChRs (IC_{50} = 180 nM, n = 4) when compared with

FIGURE 2. Purification of haditoxin from the venom of O. hannah. A, gel filtration chromatogram of crude venom. Crude venom (100 mg/ml) was fractionated using a Superdex 30 HiLoad (16/60) column. The column was pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.4). Proteins were eluted at a flow rate of 1 ml/min using the same buffer. A black bar at Peak 2 indicates the fractions containing haditoxin. B, RP-HPLC profile of the gel filtration fractions containing haditoxin. Jupiter C18 (5 μ, 300 Å, 4.5 × 250 mm) analytical column was equilibrated with 0.1% (v/v) trifluoroacetic acid. Protein of interest was eluted from the column with a flow rate of 1 ml/min with a gradient of 23–49% buffer B (80% acetonitrile in 0.1% trifluoroacetic acid). The dotted line indicates the gradient of the buffer B. The downward arrow at Peak 2a indicates fractions containing haditoxin. C, ESI-MS profile of the RP-HPLC fraction containing haditoxin. The protein was dissolved in MilliQ water (0.5 mg/ml), and the CD spectra were recorded using a 0.1-cm path length cuvette. D, far-UV CD spectrum of haditoxin; ESI-MS profile of the RP-HPLC fraction containing haditoxin. The spectrum shows a series of multiply charged ions, corresponding to a single, homogenous peptide with a molecular mass of 7,555.88 Da. Inset, reconstructed mass spectrum of haditoxin; CPS = counts/s; amu = atomic mass units. D, far-UV CD spectrum of haditoxin. The protein was dissolved in MilliQ water (0.5 mg/ml), and the CD spectra were recorded using a 0.1-cm path length cuvette. E, electropherogram of haditoxin. The sample was injected using pressure mode 5 p.s.i./s, and electrophoresis runs were carried out using a coated capillary (17 cm × 25 μm) at 18 kV, with 0.1 M phosphate buffer (pH 2.5) at 20 °C for 7 min.
and αβδε- and αβδ-nAChRs. There was no recovery after application of haditoxin. Finally, we tested the effect of haditoxin on αβδ-nAChRs. Application of 10 μM haditoxin blocked only 70% of the current with partial reversibility (Fig. 4G). The IC50 value of the blockade is in the micromolar range (IC50 = 2.6 μM, n = 3) (Fig. 4H). However, further experiments will be necessary to discriminate between the different mechanisms of blockade and recovery. These results show that haditoxin had a higher potency for αβδ-nAChRs than for the other nAChRs. IC50 values for αβδε- and αβδ-nAChRs were in the same nanomolar range, whereas for αβδ-nAChRs, it was in the micromolar range.

Haditoxin Is a Dimer—During the gel filtration of the crude venom, we observed that haditoxin eluted earlier when compared with other 3FTxs (Fig. 2A, most of the 3FTxs elutes in Peak 3). This led us to investigate the oligomeric states of this protein. Thus, we carried out analytical gel filtration experiments using a Superdex G-75 column. Protein, at concentrations (0.25–10 μM) covering the IC50 in CBCM (0.27 ± 0.07 μM) and RHD (1.85 ± 0.39 μM) preparations, was loaded onto the column. At all of these concentrations, the presence of a single peak corresponding to a relative molecular mass of 16.25 kDa was observed (Fig. 5A), supporting the existence of a dimeric species. To observe the effect of SDS on dimerization, we treated the protein (0.25–10 μM) with SDS and eluted using the same column. It eluted as a single peak with a Mr of 8.16 kDa (Fig. 5A), similar to the monomeric species. The dimerization was further confirmed by Tris-Tricine SDS-PAGE analysis in the presence and absence of a cross-linker, BS3 (Fig. 5B). In the presence of BS3, both the dimeric and the monomeric species were visualized (Fig. 5B, lane 1), whereas only the monomeric species were observed in its absence (Fig. 5B, lane 2). These results, together with MS data (showing monomeric mass, Fig. 2C), indicate the existence of

![Figure 3](image-url)

**FIGURE 3.** Pharmacological profile of haditoxin. A–D, a segment of tracing showing the effect of haditoxin (1.5 μM) on CBCM preparations (A), reversibility of CBCM preparation (B), RHD preparations (C), and reversibility of RHD preparation (D). Contractions were produced by exogenous ACh (300 μM), carbachol (CCCh; 10 μM), and KCl (30 mM). The black bar indicates the electrical field stimulation. The point of washing out the toxin with Krebs solution in reversibility studies is indicated by the abbreviation W. E, dose-response curve of haditoxin and α-bungarotoxin on CBCM and RHD. The block is calculated as a percentage of the control twitch responses of the muscle to supramaximal nerve stimulation. Each data point is the mean ± S.E. of at least three experiments. F, comparative reversibility profile of α-bungarotoxin, haditoxin, and candoxin.
haditoxin as a homodimer in solution at pharmacologically relevant concentrations, and the dimerization occurs through non-covalent interactions. Further, as haditoxin loses its β-sheeted structure and becomes random coil in the presence of SDS (as indicated by CD studies; data not shown), its overall conformation may play a critical role in the dimerization.

**Crystal Structure of Haditoxin**—To determine the three-dimensional structure of haditoxin, we used the x-ray crystallographic method. Diffraction quality crystals of haditoxin were obtained with 0.1 M Tris, pH 8.5, 20% v/v ethanol (Hampton Research crystal screen 2, condition 44). Diffraction up to 1.55 Å was observed, and the crystals belonged to the space group P2₁ (Table 1).

**Structure Determination and Refinement**—The structure of haditoxin was solved by the molecular replacement method (Molrep) (50). Initially, toxin-α, isolated from *Naja nigricollis* venom, was used as a search model (Protein Data Bank (PDB) code 1IQ9; sequence identity ~48%). The rotation and translation resulted in a correlation factor of 0.07 and R*cry*st of 0.57. Further minimization in Refmac (51) reduced the R factor to 0.42. An excellent quality electron density map was calculated at this stage, which allowed us to auto-build 90% of the haditoxin model with ARP/wARP (52). The resulting model with the electron density map was examined to manually build the rest of the model using the Coot program (53). After a few cycles of map fitting and refinement, we obtained an R factor of 0.194 (R*free = 0.225) for reflections I > σI within 20-1.55 Å resolution. Throughout the refinement (Table 1), no noncrystallographic symmetry restraint was employed. All 65 residues (considering one subunit) are well defined in the electron density map (Fig. 6A), and statistics for the Ramachandran plot using PROCHECK (54) showed the presence of 88.9% of non-glycine residues in the most favored region.
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loops protruding from a central core, tightened by four highly conserved disulfide bridges (Cys-3–Cys-24, Cys-17–Cys-41, Cys-45–Cys-57, and Cys-58–Cys-63) (Fig. 6, B and C), and are structurally similar to short-chain α-neurotoxins such as toxin-α and erabutoxins (Fig. 7B). Loop I forms a two-stranded β-sheet (Lys-2–Tyr-4 and Thr-14–Ile-16), whereas loops II and III form a three-stranded β-sheet (Glu-34–Thr-42, Phe-23–Asp-31, and Lys-53–Cys-58). The antiparallel β-strands of the β-sheet are stabilized by main chain-main chain hydrogen bonding.

Dimeric Interface—The dimeric interface was analyzed using the Protein Interfaces, Surfaces and Assemblies (PISA) server (56). It is mainly formed by loop III of each subunit. Strands D, C, E, E′, C′, and D′ form a six β-sheeted pleated sheet with an overall right-handed twist (Fig. 6B) in the dimer. Approximately 565 Å² (or 12% of the total) surface areas and 17 residues of each monomer contribute to the dimerization. The close contacts between the monomers are maintained by 14 hydrogen bonds (<3.2 Å) and extensive hydrophobic interactions (Table 2). Six main chain-main chain hydrogen-bonding contacts exist across the interface involving strand E of monomer A and E′ of B (Table 2, Fig. 7C). Four are observed between the main chain amide hydrogen and carbonyl oxygen of Val-55 and Cys-57 from monomer A and Val-55′ and Cys-57′ from monomer B, and the remaining two exist between the carbonyl oxygen of Lys-53 (and Lys-53′) and the amide hydrogen of Arg-59 (and Arg-59′). In addition, there are another eight hydrogen-bonding contacts mediated through the side chains of Thr-44, Cys-3–Cys-24, Cys-17–Cys-41, Lys-2–Tyr-4, and Thr-14–Ile-16, whereas loops II and III form a three-stranded β-sheet (Glu-34–Thr-42, Phe-23–Asp-31, and Lys-53–Cys-58). Two hydrophobic clusters further stabilize the dimeric structure. The side chains of Phe-23 and Leu-48 from both monomers form one cluster, whereas the disulfide bridge between Cys-45–Cys-57 and Val-55 of both monomers form the other. These observations strongly suggest the existence of haditoxin as non-covalent homodimeric species.

**DISCUSSION**

Nonenzymatic neurotoxins from snake venom belonging to the 3FTx family consist of closely related polypeptides with a molecular mass range of 6,500–8,000 Da. Functionally, most interfere with cholinergic neurotransmission and are highly specific for different subtypes of muscarinic or nicotinic cholinergic receptors (for details, see the Introduction). This underscores their immense potential as lead molecules in drug discovery and as research tools in the characterization of receptor subtypes.

Here, we have described the purification and characterization of a novel neurotoxin, haditoxin, from the venom of *O. hannah*. It is a non-covalent homodimer that produces potent postsynaptic neuromuscular blockade of the mammalian muscle (IC₅₀ = 1.85 ± 0.39 μM) and avian muscle (IC₅₀ = 0.27 ± 0.07 μM) (αβγδ)
nAChRs (Fig. 3, A and C). In electrophysiological studies, it was an antagonist of muscle (αβδε) (IC$_{50}$ = 0.55 μM) as well as neuronal αγ (IC$_{50}$ = 0.18 μM), αβδε (IC$_{50}$ = 0.50 μM), and αβδε (IC$_{50}$ = 2.60 μM) nAChRs (Fig. 4). Interestingly, haditoxin exhibited a novel pharmacology with combined blocking activity on muscle (αβγδ) as well as neuronal (αγ, αβ, and αβγδ) nAChRs but with the highest potency on αγ-nAChRs, which is recognized by neither short-chain α-neurotoxins nor κ-neurotoxins.
The reversibility of this neuromuscular blockade was taxa-specific; it is partially reversible by washing in the chick neuromuscular junction, whereas it was almost irreversible in the rat neuromuscular junction. Earlier, we reported taxa-specific neurotoxicity of dennotoxin from *Boiga dendrophila* (12) and irditoxin from *Boiga irregularis* (13). Taxa specificity manifests the natural targeting of the venom toxins toward their prey (13, 57–59). Snakes from the *Boiga* sp. mainly feed on the non-mammalian prey such as birds (12, 13, 60), whereas elapids, including the king cobra, mainly prey on snakes and rodents and only occasionally and opportunistically on birds (61, 62). Venom compositions of snakes are known to be dependent on prey specificity to ensure efficiency in their capture and killing (58). Therefore, the taxa-specific reversibility of the neuromuscular blockade produced by haditoxin is likely due to the natural species specificity of the king cobra venom and not because of low toxicity.

*Structurally Important Residues for Haditoxin*—Haditoxin contains all 8 conserved cysteine residues that are essential for the three-finger folding (24, 63). They form four disulfide bridges located in the core region of the molecule. In addition, this molecule possesses several other structurally invariant residues, responsible for the stability of the three-finger fold. For example, Tyr-25 (numbering of the residues is according to erabutoxin-a, unless stated otherwise), the crucial residue stabilizing the antiparallel β-sheet structure (64), is conserved in a similar three-dimensional orientation. Similarly, the structurally invariant Gly-40, involved in the tight packing of the three-dimensional fold by accommodating the bulky side chain of the Tyr-25 (24), is also conserved. The 2 proline residues Pro-44 and Pro-48, potentially associated with the formation of the β-turn (24), are conserved in haditoxin as Pro-46 and Pro-50. The salt bridge between the
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TABLE 2

| Hydrogen bonds       | Monomer A | Monomer B | Distance |
|----------------------|-----------|-----------|----------|
| Main chain-main chain|           |           |          |
| Cys-57 (O)           | Val-55 (N)| 2.93      |          |
| Val-55 (O)           | Cys-57 (N)| 2.85      |          |
| Cys-57 (N)           | Val-55 (O)| 2.86      |          |
| Val-55 (N)           | Cys-57 (O)| 2.90      |          |
| Lys-33 (O)           | Arg-59 (N)| 3.10      |          |
| Arg-59 (N)           | Lys-53 (O)| 3.40      |          |
| Side chain-side chain|           |           |          |
| Glu-47 (OE1)         | Thr-44 (OG1)| 2.63   |          |
| Glu-47 (OE2)         | Cys-45 (N)| 2.76      |          |
| Pro-50 (O)           | Arg-59 (NE)| 3.39     |          |
| Thr-49 (O)           | Arg-59 (NH2)| 3.37   |          |
| Thr-44 (OG1)         | Glu-47 (OE1)| 2.68   |          |
| Cys-45 (N)           | Glu-47 (OE2)| 2.81   |          |
| Arg-59 (NH2)         | Thr-49 (O)| 2.66      |          |
| Arg-59 (NE)          | Gly-52 (O)| 2.76      |          |

N-terminal amino group and the carboxyl group of Glu-58 as well as the C-terminal carboxyl group and the guanidinium group of Arg-39 in erabutoxin-a (24) is maintained by the N-terminal amino group and the carboxyl group of Asp-58 as well as the C-terminal carboxyl group and the guanidinium group of the Arg-39 in haditoxin. Thus, the presence of these structurally invariant residues contributes to the stable three-finger fold of haditoxin.

**Functionally Important Residues for Haditoxin—Extensive structure-function relationship studies on the short-chain α-neurotoxin, erabutoxin-a (24, 65, 66), and the long-chain α-neurotoxins, α-cobratoxin (67, 68) and α-bungarotoxin (69, 70), revealed the critical residues involved in the recognition of nAChRs by snake neurotoxins. The crucial residues for α-neurotoxins to bind to muscle (αβγδ) nAChRs are Lys-27, Trp-29, Arg-34, and Lys-35, which are strictly conserved in all nAChRs from snake venoms exist (13, 37, 72). Among them, the most well studied and characterized are the three-finger neurotoxins derived from snake venoms (13, 37, 72). Among them, the most well studied and characterized are the α-neurotoxins, known to be composed of two identical monomers held together by noncovalent interactions (77, 78). Haditoxin has Arg-33 in a homologous position, which may contribute in part to high affinity interaction with αβ2− (IC\textsubscript{50} = 0.5 μM) and αβ2− (IC\textsubscript{50} = 2.6 μM) nAChRs. Mutagenesis studies on κ-bungarotoxin revealed that the replacement of Pro-36 to an amino acid residue bearing a bulky, charged side chain, such as the Lys found in α-bungarotoxin, causes a 16-fold decrease in the efficacy of the toxin to block neurotransmission in the chick ciliary ganglion assay (76). Haditoxin bears an equivalent glycine residue, lacking a bulky, charged side chain, which can explain the high affinity of this toxin toward the neuronal (αβ2− and αβ2−) nAChRs.

**Comparison of Haditoxin with Other Dimeric 3FTxs—** Few known examples of dimeric three-finger neurotoxins derived from snake venoms exist (13, 37, 72). Among them, the most well studied and characterized are the κ-neurotoxins, known to be composed of two identical monomers held together by noncovalent interactions (77, 78). The observed dimeric form of haditoxin, with the characteristic six β-pleated sheets, is similar to that formed by κ-bungarotoxin (77). Superposition of both molecules yielded an r.m.s.d. of 1.95 Å for 104 Cα atoms (Fig. 8A). The major deviations are located in the loops between the antiparallel β-strands. However, each monomer in κ-bungarotoxin is structurally homologous to long-chain α-neurotoxins unlike haditoxin, which resembles short-chain α-neurotoxins (Fig. 7C). The dimeric interface for both is maintained by six main chain-main chain hydrogen bonds (77). In addition, haditoxin has eight side chain hydrogen-bonding contacts between the monomers, whereas only three similar contacts were observed in κ-bungarotoxin (Fig. 7A) (77), suggesting that haditoxin forms tighter dimers than κ-bungarotoxin. The side chain interactions in κ-bungarotoxin are maintained by Phe-49, Leu-57, and Ile-20, which are strictly conserved in all κ-neurotoxins (77, 79). Mutagenesis studies have proven that replacing Phe-49 or Ile-20 with alanines renders a toxin with an apparent lack of ability to fold into the native structure, even as a monomer (79).

![FIGURE 7. Structural details of haditoxin. A, superimposition of both subunits of haditoxin. Subunits A and B are shown in blue and red, respectively. B, superimposition subunit A of haditoxin with short-chain α-neurotoxins. Subunit A is shown in blue, erabutoxin-a is shown in magenta, erabutoxin-b is shown in cyan, and toxin-α is shown in green. C, stereo diagram of comparison of dimer interface of haditoxin (top) and κ-bungarotoxin (bottom). The residues to form the hydrogen bonds are labeled. The main chain-main chain hydrogen bonds are shown in red, and the other hydrogen bonds are shown in yellow.](image)

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intact dimeric structure. The electrostatic surface potentials for both molecules were apparently similar (Fig. 8B) except for the tip of loop II, which revealed a strong positive patch for haditoxin when compared with κ-bungarotoxin (77).

Functionally, κ-neurotoxins interact with the neuronal (αβγδ and αβγδ) nAChRs with high affinity (26, 27), whereas haditoxin interacts with both muscle (αβγδ) and a variety of neuronal (αγ, αβγδ, and αβγδ) nAChRs. The crystal structure of the κ-bungarotoxin dimer showed that the guanidinium groups of the essential arginine residues, situated at the tip of the loop II, maintains nearly identical distance (44 Å) (77), like the acetylcholine binding sites in the pentameric receptor (30–50 Å) (80, 81). The κ-bungarotoxin dimer may both interact with the acetylcholine binding sites on a single neuronal receptor and physically block ion flow by spanning the channel (77, 79). However, this mode of interaction does not explain the inability of the κ-neurotoxins to block the muscle nAChRs. Haditoxin maintains a distance of ~52 Å between the guanidinium groups of the critical arginine residues present in the turn region of the second loop, which is almost similar to the acetylcholine binding sites in the pentameric receptor mentioned above. However, unlike the κ-neurotoxins, haditoxin interacts with both muscle and neuronal nAChRs. This supports the fact that the dimeric toxins may have a unique mode of interaction with the nAChRs, which demands further investigation.

More recently, other heterodimeric 3FTxs from elapid venoms have been reported, including covalently (disulfide) linked homodimers of a long-chain κ-neurotoxin (κ-cobratoxin) and heterodimers of κ-cobratoxin in combination with a variety of three-finger cytotoxins (72). Unlike haditoxin, all of these dimers are formed by covalent bonding (disulfide linkage) of the monomeric units. Functionally, the κ-cobratoxin-cytotoxin heterodimers were able to block neuronal (αγ) nAChRs, whereas the κ-cobratoxin homodimer exhibited blockade of
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both neuronal α- and αβ,αβ,αβ,αβ nACHRs, unlike monomeric α-cobratoxin, which interacts with muscle (αβγδ) and neuronal (αγ) nACHRs (72).

Our laboratory has also reported on a colubrid venom-derived covalently linked heterodimeric 3FTx, irditoxin (13), which was found to target muscle (αβγδ) nACHRs, in sharp contrast to the reported function of elapid dimeric toxins (72). Another distinguishing feature was that the subunits of irditoxin structurally resemble non-conventional 3FTxs (13). Haditoxin is both structurally and functionally distinct from the α-cobratoxin hetero/homodimers as well as irditoxin. Structurally, haditoxin is a non-covalently linked homodimer of the short-chain α-neurotoxin type, and functionally, it has a broad pharmacological profile with high affinity and selectivity for muscle (αβγδ) and neuronal (αγ, αβ, αβ, αβ) nACHRs. Haditoxin exhibits a unique structural and functional profile and is the first reported dimeric 3FTx interacting with the muscle (αβγδ) nACHR as well as the first short-chain type of α-neurotoxin to interact with neuronal αγ-nACHr with nanomolar affinity.

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