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Drosotoxin, a selective inhibitor of tetrodotoxin-resistant sodium channels

Shunyi Zhu [a,*], Bin Gao [a,1], Meichun Deng [b,1], Yuzhe Yuan [a,2], Lan Luo [a], Steve Peigneur [c], Yucheng Xiao [b], Songping Liang [b], Jan Tytgat [c]

[a] Group of Animal Innate Immunity, State Key Laboratory of Integrated Management of Pest Insects & Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China
[b] Key Laboratory of Protein Chemistry and Developmental Biology of the Ministry of Education, College of Life Sciences, Hunan Normal University, Changsha, Hunan 410081, China
[c] Laboratory of Toxicology, University of Leuven, O&N 2, Herestraat 49, P.O. Box 922, 3000 Leuven, Belgium

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ABSTRACT

The design of animal toxins with high target selectivity has long been a goal in protein engineering. Based on evolutionary relationship between the *Drosophila* antifungal defensin (drosomycin) and scorpion depressant Na^+ channel toxins, we exploited a strategy to create a novel chimeric molecule (named drosotoxin) with high selectivity for channel subtypes, which was achieved by using drosomycin to substitute the structural core of BmKITc, a depressant toxin acting on both insect and mammalian Na^+ channels. Recombinant drosotoxin selectively inhibited tetrodotoxin-resistant (TTX-R) Na^+ channels in rat dorsal root ganglion (DRG) neurons with a 50% inhibitory concentration (IC_{50}) of 2.6 ± 0.5 μM. This chimeric peptide showed no activity on K^+, Ca^{2+} and TTX-sensitive (TTX-S) Na^+ channels in rat DRG neurons and *Drosophila* para/tipE channels at micromolar concentrations. Drosotoxin represents the first chimeric toxin and example of a non-toxic core scaffold with high selectivity on mammalian TTX-R Na^+ channels.

1. Introduction

Venomous animals evolved a variety of peptide toxins to assist acquiring prey and defend against predators. These toxic molecules primarily function as modulators or blockers of ion channels in excitable cells by binding to distinct receptor sites to change the gated properties or ion permeability of the channels [1–4]. These unique features make them valuable pharmacological tools to study the structure-function relationship of related ion channels. For example, MacKinnon and his co-workers used the scorpion toxin AgTx2 as a molecular probe to reveal the architecture of a K^+ channel pore [5] and to highlight structural conservation in prokaryotic and eukaryotic potassium channels [6]. In addition, animal toxins also constitute potential molecular leads for the development of novel therapeutic agents for human diseases associated with altered states of neuronal excitability or pesticides targeting insect ion channels [7–11]. Despite intensive efforts, most of animal toxin-derived agents cannot meet expectations, primarily due to low channel selectivity resulting in side effects or environmental contamination. From an evolutionary viewpoint, this is not surprising because low channel selectivity (i.e. multiple targets) of a toxin formed in evolution likely contributes to a higher efficiency for prey and defense of venomous animals. This seriously challenges drug discovery from naturally occurring toxins. Therefore, novel ideas and strategies are needed.

Scorpion toxins affecting Na^+ channels [2,12–15] comprise an evolutionarily related peptide family that share highest structural similarity with antimicrobial defensins from diverse origin [16,17]. All these peptides adopt a common cysteine-stabilized α-helical and β-sheet (CSαβ) folding architecture [16]. In a previous study based on combined data, we proposed a hypothesis that scorpion depressant Na^+ channel toxins could originate from ancestral antifungal defensins [17]. In the work presented here, we tested this hypothesis by mimicking natural evolutionary processes in the laboratory (*ex vivo* studies). By substituting the structural core of BmKITc, a scorpion depressant Na^+ channel toxin acting on a broad spectrum of channel targets (*Drosophila* para/tipE Na^+ channels, mammalian DRG TTX-S and TTX-R Na^+ channels) [18] using an evolutionarily related non-toxic *Drosophila* antifungal drosomycin [19], we obtained a chimeric peptide (drosotoxin) that selectively inhibits rat DRG TTX-R Na^+ channels. The toxin had no effect on K^+, Ca^{2+} and TTX-S

* Corresponding author. Tel.: +86 010 64807112; fax: +86 010 64807099.
E-mail address: Zhusy@ioz.ac.cn (S. Zhu).
[1] These authors equally contributed to this work.
[2] Current addresses: Institute of Blood Transfusion, Chinese Academy of Medical Sciences, Chengdu, Sichuan Province, China.

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Na^+ channels in DRG neurons and the *Drosophila* para/tipE channels. This selectivity, through application of the strategy presented here, offers a new approach for drug design.

2. Materials and methods

2.1. Reagents

Glutathione-Sepharose 4B was obtained from GE Healthcare (Shanghai, China); Enterokinase (EK) and dithiothreitol (DTT) were respectively purchased from Sinobio Biotech Co. Ltd (Shanghai, China) and Sbsbio (Beijing, China); Tetrodotoxin (TTX), trypsin (type III), collagenase (IA), trypsin inhibitor (type II-S), Dulbecco's modified Eagle's medium (DMEM) and tetraethylammonium chloride (TEA-Cl) were purchased from Sigma–Aldrich (Shanghai, China). All these reagents are of analytical or cell culture grade.

2.2. Construction of the expression vector of pGEX-6P-1-drosotoxin

To generate the drosotoxin gene for expression in *E. coli*, we firstly transferred the C-tail of BmKITc to the drosomycin scaffold by two successive standard PCR amplifications, where pGEX-6P-1-drosomycin [20] was used as a template and DrW-F and DrNa-RM/DrNa-R1 as primers. The PCR product derived from the second amplification was ligated into pGEX-6P-1 and the resultant plasmid (pGEX-6P-1-Drosomycin + C) was used as a template for inverse PCR to introduce the N-turn by primers DrNa-F2 and DrNa-R2 (pGEX-6P-1-drosotoxin) according to the previously described method [21]. All recombinant plasmids were transformed into *E. coli* DH5α and their sequences were verified by DNA sequencing. The nucleotide sequence of drosotoxin has been deposited in the GenBank database under the accession number of GQ412336. Primers used include: DrW-F: ATGGATCCGATGACGATGACAAGGACTGCCTGTCCGGAAGA; DrNa-RM: ATATTTCCATTTTTCATTATC AGGAAGTCCTTCGCACCAGCACTT; DrNa-R1: ATGTCGACTTAGCTACCGCATGTATTACTTTCATATTTCCATTTTTCATTATC; DrNa-R2: pTCTTCCGGACAGGCCGTCCTTG TCA-3′; DrNa-F2: pAGTGACGGATGCTACAAGGGTCCCTGTGCCGTCTG ('p' in primers DrNa-R2 and DrNa-F2 represents phosphorization in their 5′-ends).

2.3. Expression, purification and identification of drosotoxin

The pGEX-6P-1-drosotoxin plasmid was transformed into *E. coli* BL21 (DE3) and its expression was carried out according to a previous method [22]. Briefly, the fusion protein (GST-drosotoxin), obtained from a sonication supernatant by affinity chromatography with glutathione-Sepharose 4B beads, was digested with EK at room temperature for 3 h. The released drosotoxin was separated from GST using RP-HPLC on a C18 column (Agilent Zorbax 300SB-C18, 4.6 mm × 150 mm, 5 μm) with an Agilent 1100 system. To prepare homogeneous drosotoxin as a single HPLC peak, we used low concentration of DTT to treat the HPLC-purified product and re-collected it by RP-HPLC under identical conditions as described above.

Molecular weights (MWs) of recombinant peptides were determined by MALDI-TOF mass spectra on a Kratos PC Axima CFR plus (Shimadzu Co., Ltd., Kyoto). The automated Edman degradation by ABI Procise 492cLC protein sequencer was used for sequencing amino-termini of recombinant peptides (Shanghai GeneCore BioTechnologies Co., Ltd., Shanghai, China).

2.4. Antifungal assay

Inhibition zone assay was used to compare antifungal activity of peptides against *N. crassa* according to the previously described method [22].

2.5. Electrophysiological assays for DRG neurons

Dorsal root ganglion (DRG) cells were acutely dissociated from adult (4 weeks old) Sprague–Dawley rats and maintained in short-term primary culture using the method described by Xiao et al. [23]. Briefly, rats of either sex were killed by decapitation without anesthetization, the dorsal root ganglia were removed quickly from the spinal cord, and after having been cut into as small tissues as possible, they were transferred into DMEM containing trypsin (0.15 mg/ml, type III), collagenase (0.3 mg/ml, type IA) to incubate at 34 °C for 20 min. Trypsin inhibitor (1 mg/ml, type II-S) was added to terminate enzyme treatment. After transferring into 35-mm culture dishes containing 90% DMEM, 10% newborn calf serum, 3.7 g/l $NaHCO_3$ and penicillin–streptomycin (100 units/ml each), the DRG cells were incubated in CO_2 incubator (5% CO_2, 95% air, 37 °C) for 1–4 h before patch-clamping.

DRG cells with a large diameter (around 50 pF in slow capacitance) and those with a relatively small diameter (around 20 pF in slow capacitance) were chosen for study of TTX-S and TTX-R sodium currents, respectively. In addition, TTX (final concentration at 200 nM) was used to separate the TTX-R sodium current from the TTX-S sodium current. Sodium currents, which were filtered at 10 kHz and digitized at 3 kHz with an EPC-9 patch-clamp amplifier (HEKA Electronics, Germany), were recorded at room temperature (20–25 °C). Micropipettes (2–3 μm diameter) were pulled from borosilicate glass capillary tubing by using a two-step vertical puller (PC-10, Narishige, Olypmus). The resistances of micropipettes were 1–2 MΩ after filled with internal solution. For sodium current recordings, the pipette internal solution contained (in mM): CsF 135, NaCl 10 and HEPES 5 at pH 7.0 with CsOH. The external bathing solution contained (in mM): NaCl 30, KCl 5, CsCl 5, D-glucose 25, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5 and TEA-Cl 90 at pH 7.4. Sodium currents were elicited by depolarization to −10 mV from a holding potential of −80 mV. Calcium channel currents were measured using Ba^{2+} as a charge carrier. For these experiments, external solution contains (in mM): triethanolamine-Cl 160, HEPES 10, $BaCl_2$ 2, glucoses 10, and tetrodotoxin 200 nM, adjusted to pH 7.4 with triethanolamine-OH. The internal solution contains (in mM): $CsCl_2$ 120, Mg-ATP 5, Na_2-GTP 0.4, EGTA 10, HEPES-CsOH 20 (pH 7.2). Low-voltage activated (LVA) or high-voltage activated (HVA) calcium channel currents were evoked at −50 mV or 0 mV from a holding potential of −90 mV or −40 mV. For potassium current recordings, the bath solution contain (in mM): NaCl 160, KCl 4.5, $CaCl_2$ 2, $MgCl_2$ 1 and HEPES 10, pH 7.4 (with NaOH); The internal pipette solution used for measuring voltage-gated potassium currents contains (in mM) KF 155, $MgCl_2$ 2, EGTA 10 and HEPES 10, pH 7.2 (with KOH). Potassium currents were elicited with depolarizing voltage from −80 mV to +30 mV. Capacitative and linear leak currents were digitally subtracted by P/4 procedures. Voltage steps and data acquisition were controlled using a PC computer with software Pulsefit + Pulse 8.0 (HEKA Electronics, German).

2.6. Expression of para/tipE in Xenopus oocytes and electrophysiological recordings

Methods for the expression of para/tipE genes in *Xenopus* oocytes and two-electrode voltage-clamp recordings of these channels have been described previously [18].

3. Results

3.1. Molecular design of drosotoxin

Depressant toxins represent a distinct pharmacological group of scorpion Na^+ channel neurotoxins and most of them show high

preference for insect Na^+ channels [24], however, some members also exhibit activity against mammalian counterparts [18,25,26]. Such functional diversification could be a consequence of positive selection in several functional sites of their structural core [27,28]. These scorpion depressant toxins were found to share about 50% sequence similarity with the *Drosophila* antifungal drosomycins in their structural cores corresponding to the CSαβ region, in which 19 sites with identical or similar residues were identified (Fig. 1A). In fact, the toxins, represented by LqhIT2 whose 3D structure was recently determined [28], can be clearly divided into two distinct structural domains including the CSαβ structural core containing all secondary structure elements and an NC-domain comprising the N-terminal turn (N-turn) and C-terminal tail (C-tail), and the structural core of LqhIT2 can accurately match the overall scaffold of drosomycin, both having 35 Cα atoms well superimposed with a root mean square deviation (RMSD) of 1.41 Å (Fig. 1B). Given sequence and structural similarity as well as functional relatedness (both involved in defense) are crucial evidence for common genetic origin, the scorpion toxins are probably orthologs of the fly drosomycin. A putative ancestral toxicity feature of drosomycin was recently uncovered by the finding that this peptide can bind and induce conformational alteration of the fly Na^+ channel (para/tipE) despite no effect on the gating properties of this channel [29].

Based on the above facts, we reconstructed an evolutionary history of the scorpion depressant toxins (Fig. 1C): the gain of an NC-domain on a non-toxic progenitor scaffold (e.g. drosomycin-like molecule) by a small N-terminal insertion and C-terminal extension resulted in the emergence of an ancestral toxin with a single target, followed by positive selection to yield modern toxins targeting different channels from diverse origins. To reproduce the early event of the history, we transferred the NC-domain of BmKITc (a closest homologue to drosomycin) to the drosomycin scaffold

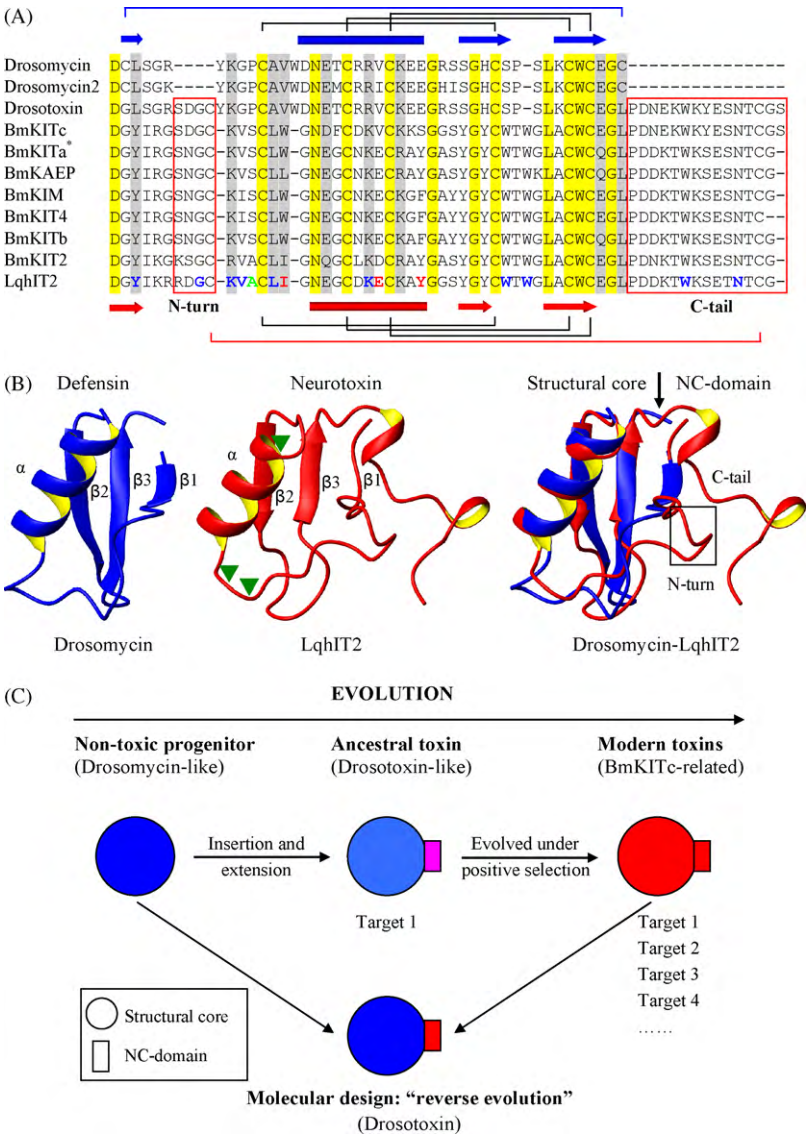

Fig. 1. Evolution-guided molecular design of drosotoxin. (A) Multiple sequence alignment of functionally known drosomycins [43] and toxins [18,27] highlighting the N-turn and C-tail unique to scorpion depressant toxins. *BmKITa was also named BmK dITAP3. All the toxin sequences are derived from the Chinese scorpion *Buthus martensii* Karsch except LqhIT2 that is from *Leiurus quinquestriatus hebraeus*. Arrow and cylinder respectively represent β-strand and α-helix extracted from their structures. Three identical disulfide bridges are indicated in black lines and the fourth disulfide bridge is shown in different colors. Functional residues of LqhIT2 important in interacting with para/tipE channels are colored: the pharmacophore (red); important for "voltage-sensor trapping" (green); conferring toxin specificity (blue). N-turn and C-tail are boxed in red; (B) structural comparison between drosomycin (pdb entry 1MYN) and LqhIT2 (pdb entry 2I61). The N-turn of LqhIT2 is boxed and the start point of its C-tail relative to drosomycin is indicated by an arrow. Three positively selected residues in LqhIT2 (Leu^{15}, Ile^{16} and Ala^{27}) [27] are indicated by green triangles; *C*. Evolutionary history of scorpion depressant toxins guides 'reverse evolution' design for improving target selectivity of modern toxins, in which substitution of structural core of toxins by evolutionarily related non-toxic scaffolds is proposed as a novel strategy in protein engineering.

and recombinantly produced the chimeric toxin-like peptide (named drosotoxin) for functional evaluation. Because the positively selected structural core of the toxin [27] was substituted by a non-toxic progenitor-like scaffold, we call this type of molecular design "reverse evolution" (Fig. 1C) which could aid in improving target selectivity of the toxin.

3.2. Recombinant production of drosotoxin

The prokaryotic expression vector pGEX-6P-1-drosotoxin was constructed by using pGEX-6P-1-drosomycin [20] as an initial template to undertake PCR amplifications (Fig. S1A). Recombinant drosotoxin was produced according to the method previously described for drosomycin [22]. The fusion protein of GST-DDDDK-drosotoxin (about 33 kDa) was expressed in a soluble form (Fig. S1B) and thus directly purified by GST affinity chromatography. The purified fusion protein released a product of 6.9 kDa after EK digestion, as identified by SDS-PAGE, which was further isolated by RP-HPLC where drosotoxin displayed a heterogeneous state (Fig. S1B). To prepare homogeneous drosotoxin for functional evaluation, we collected the HPLC component corresponding to the retention time of 18–22 min for DTT treatment. By using this approach, we obtained three well-defined peaks in the HPLC chromatogram (Fig. 2), that all represent homogeneous components. MALDI-TOF combined with N-terminal sequencing identified that these peaks respectively correspond to two C-terminally truncated fragments and the full-length drosotoxin. All three peaks start with the sequence DGLSG and their MWs are 4528.1, 4399.8 and 6958.7 Da (Fig. S2), respectively, which are in agreement with the theoretical MWs of the fragment 1–42 (4528.01 Da) (named drosotoxin (1–42)), 1–41 (4399.8 Da) (named drosotoxin (1–41)) and drosotoxin (6955.7 Da). The final yield of the recombinant drosotoxin is approximately 2.5 mg/l *E. coli* culture.

3.3. Functional evaluation of drosotoxin

Both drosotoxin and BmKITc exhibited no activity against *Neurospora crassa* (Fig. 3), a most sensitive fungus to drosomycin [19,20,22], suggesting their antifungal surface is likely masked by the NC-domain. Next, we evaluated the activity of drosotoxin against the *Drosophila* para/tipE channels expressed in *Xenopus* oocytes and found that 2 μM drosotoxin neither shifts the steady-state activation of the channels to more hyperpolarizing potentials nor inhibits the Na^+ influx, nor affects the steady-state inactivation

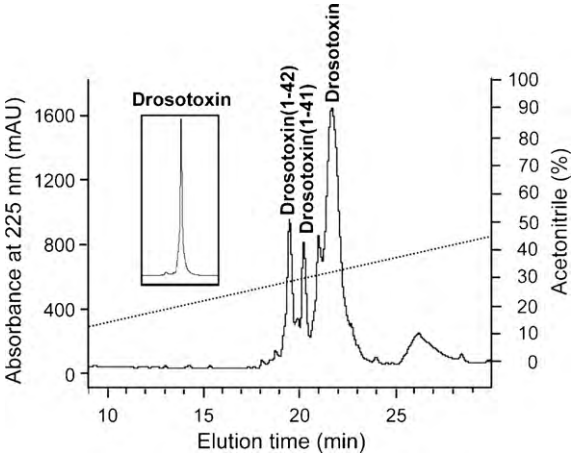

Fig. 2. RP-HPLC showing the purification of drosotoxin. Inset: the purified drosotoxin from peak 3. Elution was carried out using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (TFA) in water (v/v) within 40 min with a flow rate of 1 ml/min.

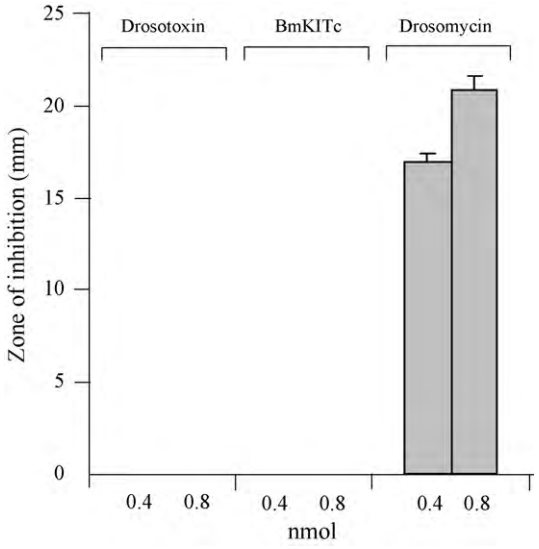

Fig. 3. Drosotoxin and BmKITc being inactive against *N. crassa*. Each peptide (0.4 nmol/well and 0.8 nmol/well) was aliquoted into wells and zones of inhibition were measured after 24 h of incubation at 30 °C. Data are shown as mean ± SE ($n = 3$).

of the channels (Fig. S3). The lack of the activity is not unexpected because (i) among 14 residues of the scorpion depressant toxin LqhIT2 identified as crucial for interacting with the para/tipE channels, 11 are located in its structural core, and only three in the NC-domain [28] (Fig. 1A); (ii) Even 10 μM drosomycin has no effect on the conductance and gating properties of the insect channels [29].

Subsequently, we assayed the activity of drosotoxin and drosomycin against TTX-S and TTX-R channels on rat DRG neurons for comparison with BmKITc. TTX-S and TTX-R channels display differential sensitivity for TTX, and the former activates and inactivates more quickly than the latter under whole-cell configuration [30,31]. In our study, 200 nM TTX was used to discriminate between these two types of channels because TTX at this dose is sufficient to eliminate TTX-S currents but does not affect TTX-R currents. Under voltage-clamp conditions, the inward Na^+ currents were elicited by 50 ms depolarization from the holding potential of −80 mV to −10 mV. As a control, BmKITc weakly inhibited both TTX-S and TTX-R currents at 5–10 μM concentrations (Fig. 4A). On the contrary, drosomycin exhibited no effect on these two types of Na^+ channels at 10 μM concentration ($n = 3$) (Fig. 4B). At this concentration, drosotoxin was also ineffective on TTX-S currents (Fig. 4C). However, 1 μM drosotoxin significantly inhibited TTX-R currents (Fig. 4C, $n = 5$) whereas no detectable effects were observed on K^+ and Ca^{2+} currents in rat DRG neurons ($n = 3$) at this concentration (Fig. S4). The dose-dependent effects of drosotoxin on TTX-R currents was also verified by the experiments with seven different peptide concentrations (from 0.001 μM to 100 μM with a tenfold increment) and the 50% inhibitory concentration (IC_{50}) calculated from these data is 2.6 ± 0.5 μM (Fig. 5A). The time course of inhibition of this peptide on TTX-R channels is shown in Fig. 5B. Rapid blockade of the currents may indicate a direct interaction between drosotoxin and the channels.

Effects of 1 μM drosotoxin on the current–voltage (I–V) curves of TTX-S and TTX-R channels are respectively illustrated in Fig. 5C and D ($n = 5$). From the curves, it can be seen that no effect was observed for TTX-S channels. For TTX-R channels, the inhibition of currents was observed at all tested potentials after the depression of current amplitude by 1 μM drosotoxin, and the current inhibition was not associated with a change of the shape of the

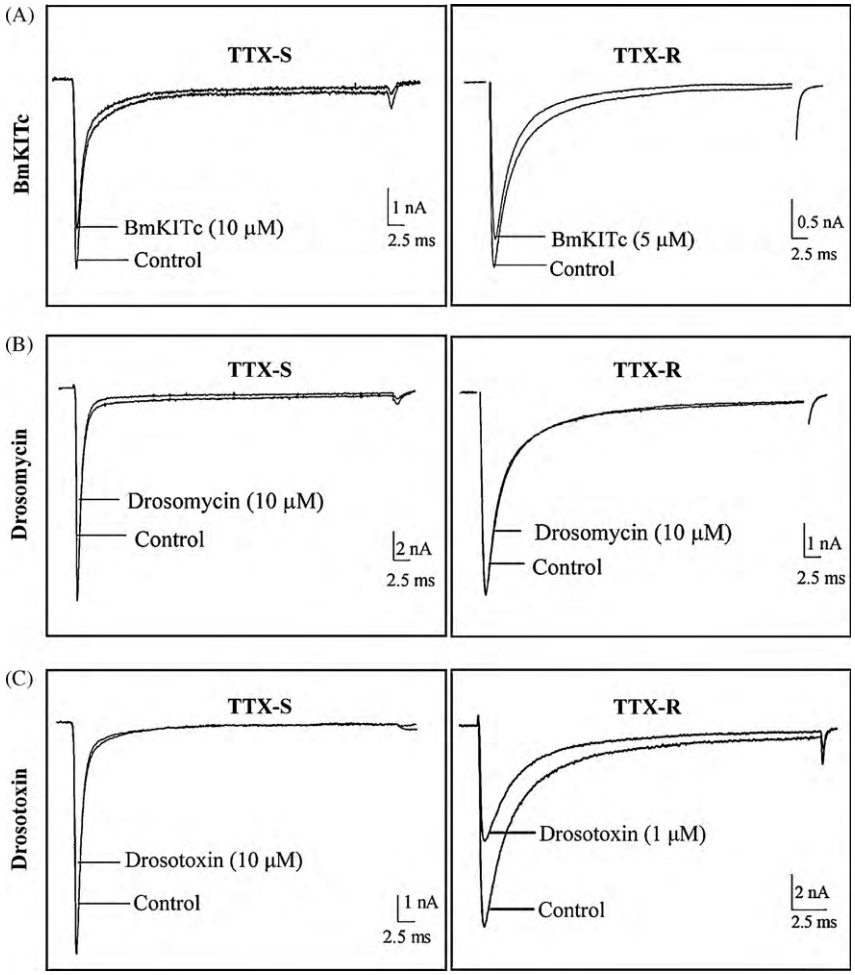

Fig. 4. Drosotoxin selectively inhibiting TTX-R Na^+ currents in rat DRG neurons. (A) High concentration (5–10 μM) BmKITc weakly inhibits both TTX-S and TTX-R currents; (B) 10 μM drosomycin is without effects on both TTX-S and TTX-R currents; (C) 1 μM drosotoxin reduces TTX-R sodium currents amplitude.

I–V relationship. Furthermore and as shown in Fig. 5D, no shift in the membrane reversal potential of the TTX-R channels was observed, implying that drosotoxin does not change the ion selectivity of the channels. Drosotoxin also displayed no effect on steady-state inactivation of DRG Na^+ channels (Fig. 5E and F).

4. Discussion

Evolutionary emergence of toxins represents a successful paradigm for ecological adaptation of venomous animals, however, little is known regarding the molecular mechanism of their origin. Elucidation of such mechanism is of general significance in both evolutionary biology and protein engineering. Phylogenetic evidence supports that snake venom-derived toxins likely originated from some related body proteins [32,33]. Computational analyses highlight convergent evolution of protein toxicity in shrew and lizard venom via acquisition of small insertions and subsequent accelerated sequence substitution to introduce a novel chemical environment into the catalytic cleft of a toxin [34]. In addition, at least three different folds were found to be shared by antimicrobial defensins and ion channel-targeted neurotoxins, which are CSαβ (16, 17), inhibitor cystine knot (ICK) [35] and β-defensin [36]. A very recent study combining evolutionary, structural, and mechanistic investigations illustrates that antimicrobial and cytotoxic peptides have ancestral structure-function homology [37]. All these observations provide new clues for studying how proteins evolve novel functions.

Based on sequence similarity and structural conservation between scorpion depressant toxins and antimicrobial defensins, we reconstructed the early evolutionary history of these toxins from the defensins, in which we believe two major genetic modification events in the evolution of proteins have taken place: (i) an N-turn was presumably developed by a small insertion, and (ii) a C-tail was added. The gain of functional data, as reported in our work here, supports our hypothesis and can be seen as an achievement of the purpose of this study. Our work presented here indeed confirms that an antimicrobial defensin with the CSαβ scaffold can switch its target from fungi to animals, by mimicking the two natural evolutionary processes (i.e. insertion and terminal extension) in *ex vivo* laboratory experiments. This work provides new insights into the early origin of scorpion neurotoxins because the gain of an NC-domain alone is sufficient to make a non-toxic scaffold into a toxin. Remarkably, drosotoxin only selectively inhibits TTX-R Na^+ channels, and not TTX-S channels or para/tipE. In comparison with drosotoxin, BmKITc can not only inhibit rat DRG TTX-R Na^+ channels, but also inhibit TTX-S channels. In addition, a maintained current component during the last phase of the inactivation of the insect sodium channels (para/tipE) is seen [18]. This suggests that the structural core is a key determinant of channel specificity, consistent with the feature of accelerated evolution in this structural core [27]. Because low channel selectivity (*i.e.* multiple targets) of modern toxins represents an evolutionary advantage for prey and defense of venomous animals and because it was likely evolved from an ancestor toxin targeting

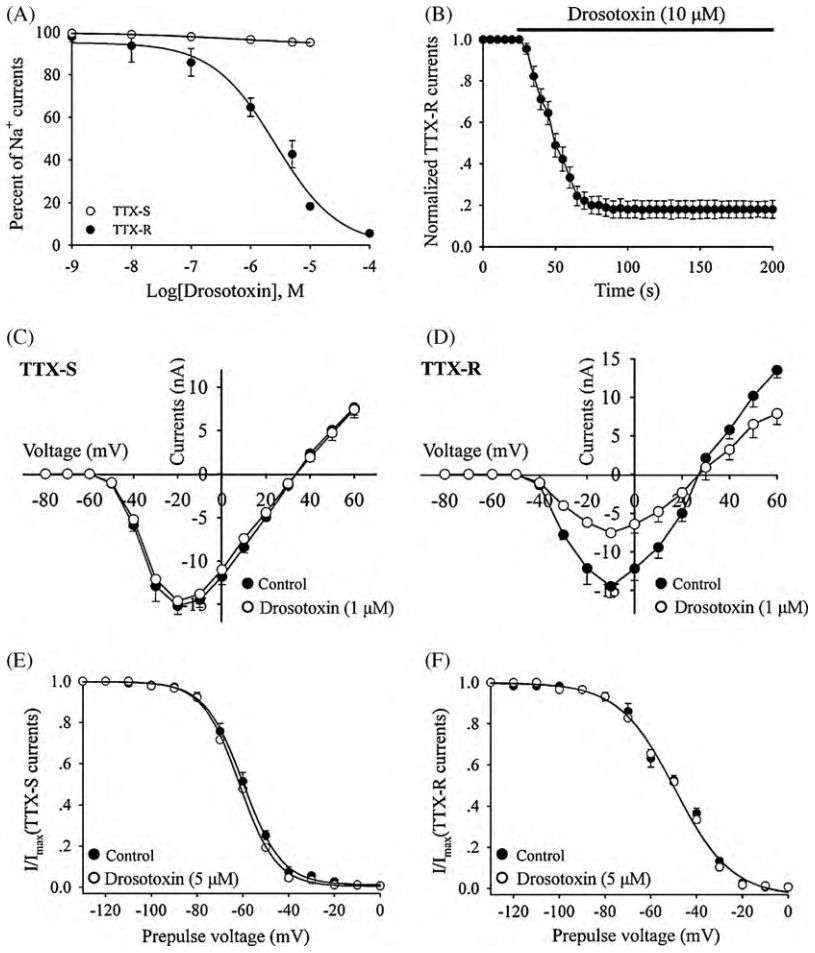

Fig. 5. Functional features of drosotoxin on TTX-R currents. *A.* The concentration-dependent inhibition of Na^+ channels by drosotoxin. Every point (mean ± S.E.) originates from 3 to 5 separated experimental cells. These data points were fitted according to the Hill equation, the yielded IC_{50} value was 2.6 ± 0.5 μM; *B.* Time–effect curves of 10 μM drosotoxin on TTX-R sodium currents; (C and D) The current–voltage (*I–V*) relationships of TTX-S and TTX-R currents before (filled circles) and after (open circles) the treatment of 1 μM drosotoxin, respectively. Cells were held at −80 mV, and families of sodium currents were elicited by 50-ms depolarizing steps to various potentials ranging from −80 mV and +60 mV at increments of +10 mV. (E and F) Drosotoxin has no effect on steady-state inactivation of TTX-S (E) and TTX-R (F) channels. The TTX-S and TTX-R currents were induced by a 50-ms depolarizing potential of −10 mV from various prepulse potentials for 1 s which ranged from −130 mV to 0 mV in 10-mV increments.

only a single channel, assembly of such ancestors in a laboratory environment (*ex vivo* experiments) provides promising clues for improving target selectivity. A possibility, as described here, is to establish an evolutionary link between toxins and non-toxic peptides. Once such relationship is determined, we can use a non-toxic scaffold to substitute the structural core of a toxin with multiple targets presumably evolved by natural selection, and thus obtain novel peptides with higher target selectivity. It is also worth mentioning that although the strategy used here is successful, it appears there was no way to predict target selectivity of a constructed toxin-like molecule. Despite this, our strategy might still be useful in engineering snake toxins from the related body proteins [32,33].

Although no mutagenesis data are available to define the functional surface of drosotoxin, its stronger potency towards TTX-R channels than BmKITc suggests that the drosomycin scaffold could provide critical residues to interact with the TTX-R channels. Divergent residues flanking the N-turn between drosotoxin and BmKITc constitute a candidate for mutagenesis analysis to speculate their functional difference. Therefore, it appears that ancestral antifungal defensins (e.g. drosomycin-like peptides) could have developed a partially toxic surface and that the lack of toxicity is likely due to the absence of the NC-domain responsible for a correct location of ancestral functional sites. In this case, grafting the NC-domain on the antifungal scaffold may activate its potential functional sites and lead to the origin of an ancestral toxin with single channel targets (i.e. TTX-R channels) which finally evolved into modern toxins with multiple targets by positive selection-driven accelerated amino acid changes in specific functional regions [27,28].

Two μO-conotoxins (MrVIA and MrVIB) showing preferential ability in inhibiting mammalian TTX-R over TTX-S current have been reported previously [38,39]. MrVIA and MrVIB inhibited the TTX-R current with a 10-fold higher potency than the TTX-S current in rat DRG neurons. Our results showed that at all the concentrations tested here, drosotoxin was not able to inhibit TTX-S current, suggesting that this peptide exhibits high selectivity for TTX-R over TTX-S channels. In addition, several conotoxins were found to selectively block amphibian TTX-R Na^+ channels, such as μ-SmIIIA, μ-KIIIA, μ-CnIIIA, μ-CnIIIB, μ-CIIIA, μ-MIIIA. However, μ-KIIIA is also active on mammalian TTX-S Na^+ channels [40]. Drosotoxin represents the first chimeric peptide with the CSαβ scaffold that selectively acts on mammalian TTX-R Na^+ channels. Although the molecular and structural basis for the emergence of new function in the drosomycin scaffold and the Na^+ channel subtypes and binding sites of drosotoxin await to be determined, the high TTX-R channel selectivity and rapid blockade of the currents make it a candidate for development of a pharmacological tool to discriminate between Na^+ channel types in DRG neurons and of an analgesic drug [41,42]. Finally, our data also provide

functional evidence strengthening evolutionary link between arthropod-derived antimicrobial defensins and neurotoxins.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.07.008.

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