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DrTx(1–42), a C-terminally truncated analogue of drosotoxin, is a candidate of analgesic drugs

Shunyi Zhu *, Lan Luo, Ping Li, Bin Gao, Limei Zhu, Yuzhe Yuan [1]

Group of Animal Innate Immunity, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China

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ABSTRACT

Drosotoxin is an engineered tetrodotoxin-resistant (TTX-R) sodium channel-specific blocker with a non-toxic structural core (Zhu et al. Biochem Pharmacol 2010; 80:1296–302). Here, we report the discovery and functional characterization of a carboxyl-terminally truncated analogue of drosotoxin (named DrTx(1–42)) which selectively inhibited dorsal root ganglion (DRG) neuron TTX-R sodium current (I_{Na}) with an IC_{50} value of 1.74 ± 0.07 μM. Consistent with this effect, DrTx(1–42) significantly attenuates inflammatory hyperalgesia of mice in a formalin-induced pain model with stronger potency than indomethacin, a nonsteroidal anti-inflammatory and analgesic drug. Our mutational experiments indicate that the N-turn insertion is an essential functional determinant for the emergence of neurotoxicity from a non-toxic antifungal scaffold.

1. Introduction

Voltage-gated sodium channels (VGSCs) are integral membrane proteins composed of a pore-forming α-subunit associated with one or more β-subunits. They conduct Na^+ through plasma membrane of excitable cells and are vital determinants of neuronal excitability and signalling [1]. Mammal has nine VGSC α-subunits (Nav1.1–Nav1.9) with different tissue distributions [2]. For example, Nav1.3 is expressed in fetal neural tissue and its expression is down-regulated in adults. Nav1.2 is expressed in brain whereas Nav1.4 and Nav1.5 are respectively expressed in skeletal and cardiac muscle cells. As a major neuronal cell type, dorsal root ganglia (DRG) express all the other channel isoforms, including Nav1.1 and Nav1.6–Nav1.9 [3]. Pharmacologically, VGSCs can be divided into two groups based on their sensitivity to the neurotoxin tetrodotoxin (TTX), most of which can be blocked by nanomolar concentrations of TTX and are thus termed "TTX-sensitive (TTX-S)" sodium channels, such as Nav1.1–Nav1.4, Nav1.6, and Nav1.7. TTX-R sodium channels include Nav1.5, Nav1.8 and Nav1.9 [4]. Recent studies have highlighted key roles of the latter two TTX-R sodium channels as therapeutic targets in neurological disorders and pains [5–7].

Animal venoms represent a rich resource of peptide toxins that function as modulators or blockers of VGSCs to change the gated properties or Na^+ permeability of the channels [8,9]. The majority of these toxins were found to affect TTX-S sodium channels. From an evolutionary viewpoint, this is not unexpected in that targeting such channels will disrupt the functions of brain, skeletal and cardiac muscles, which undoubtedly is a benefit for prey and defence of venomous animals. At present, only one naturally occurring animal toxin was identified as a selective blocker of mammalian TTX-R sodium channels (μ-SIIIA from *Conus striatus*) [10]. Two additional conotoxins (μO-MrVIA and μ-CnIIIB) were found to have preferential ability in inhibiting mammalian TTX-R over TTX-S channels [11]. In addition, several conotoxins with selective blocking effect on amphibian TTX-R sodium channels have also been reported [12].

Very recently, an engineered neurotoxin – drosotoxin, was found to inhibit mammalian TTX-R sodium currents in rat DRG neuron with high potency and selectivity [13]. This peptide of 63 amino acids was designed by grafting the N-terminal turn and C-tail of a weak scorpion neurotoxin onto a non-toxic, antifungal scaffold (drosomycin) [14]. In the present work, we describe the molecular dissection of drosotoxin which clearly indicates the importance of the N-terminal turn insertion in emerging toxic function from a non-toxic scaffold. The C-terminally truncated peptide DrTx(1–42) exhibits high potency and selectivity on TTX-R sodium current (I_{Na}) and stronger analgesic effect than the clinical drug indomethacin, which make it an intriguing candidate for the development of therapeutic drugs for pain.

* Corresponding author. Tel.: +86 010 64807112.
E-mail address: Zhusy@ioz.ac.cn (S. Zhu).
[1] Present address: Institute of Blood Transfusion, Chinese Academy of Medical Sciences, Chengdu, Sichuan Province, China.

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2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) were purchased from Sijiqing Biotech Co. Ltd. (Hangzhou, China) and Beijing Chemical Reagent Co. (Beijing, China), respectively; Enterokinase (EK) and dithiothreitol (DTT) were obtained from Sinobio Biotech Co. Ltd. (Shanghai, China) and SBS Genetech (Beijing, China), respectively; 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), tetrodotoxin (TTX), trypsin (type III), collagenase (IA), trypsin inhibitor (type II-S), Dulbecco's modified Eagle's medium (DMEM), tetraethylammonium chloride (TEA-Cl), D-glucose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), CsF, CsOH, CsCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, and NaOH were purchased from Sigma–Aldrich (Shanghai, China). All these reagents are of analytical grade.

2.2. Recombinant production of DrTx(1–42) and DrTx(3DB)

The method for recombinant production of DrTx(1–42) and drosotoxin in *Escherichia coli* has been described previously [13]. However, in such conditions, the yield of DrTx(1–42) is rather low. To improve this, we modified the preparation procedure by direct digesting fusion proteins with enterokinase (EK) in the presence of a suitable amount of DTT at 4 °C for several days. The digested product was separated by reversed-phase high performance liquid chromatography (RP-HPLC).

In addition, to study the impact of the fourth disulfide bridge to the function of drosotoxin, we constructed a mutant of drosotoxin whose two cysteines forming the fourth disulfide bridge were substituted by two serines (called DrTx(3DB)). Methods for the expression, purification and identification of DrTx(3DB) are similar to those previously described for recombinant drosotoxin [13] except DTT omitted.

2.3. Chemical synthesis and oxidative refold of peptides

Reduced peptides (DroTx(N-turn)(R) and Bm(N-turn)(R)) with >95% purity were synthesized by Xi'an Huachen Bio-Technology Co., Ltd. (Xian, China). To form an intra-molecular disulfide bridge, we oxidized these two peptides in 0.1 M Tris–HCl (pH 8.0) by air. Refolded peptide was purified to homogeneity by RP-HPLC.

2.4. MALDI-TOF mass spectra and Edman degradation

Molecular weights (MWs) of recombinant or synthetic peptides were determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry 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 the N-terminus of DrTx(1–42) (Shanghai GeneCore BioTechnologies Co., Ltd., Shanghai, China).

2.5. Electrophysiological assays for DRG neurons

Sprague–Dawley (SD) rats (100–150 g) were used for isolating DRG neurons in this work [13,15]. A rat was killed by decapitation without anesthetization and its dorsal root ganglia were rapidly removed and placed into Dulbecco's modified Eagle's medium (DMEM). After cut to pieces, they were digested for 15–25 min at 34 °C in DMEM containing 1.56 mg collagenase I and 0.66 mg trypsin (1:250). And then 1.0 mg trypsin inhibitor II-s was added to terminate the reaction. DRG neurons were distributed on coverslip in a 35-mm dish and incubated at 37 °C (5% CO_2 and 95% air) for 30–60 min, and then 2 ml DMEM containing 10% fetal calf serum and 100 U/ml penicillin–streptomycin were added into every dish. Cells were cultured in an incubator for 2–3 h before patch clamp experiments

Sodium channels from DRG cells were recorded by the whole cell patch clamp recording technique. Micropipettes were pulled by P-97 (Sutter Instrument Co.) and the resistances of micropipettes were 3.0–6.0 MΩ after filled with the internal solution containing (in mM): CsF 135, NaCl 10, HEPES 5, adjusted to pH 7.0 with 1 M CsOH. The external bathing solution contains (in mM): NaCl 30, KCl 5, CsCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, TEA-Cl 90, D-glucose 25, adjusted to pH 7.4 with NaOH. Membrane currents recordings were made by an AxoClamp 700A amplifier (Axon Inc.) and DIGIDATA 1322A (Axon Inc.). Pulse stimulation and data acquisition were controlled by Clampex 9.0 software (Axon Inc.). 200 nM TTX was used to separate the TTX-R from the TTX-S sodium currents.

2.6. Formalin test

Healthy male ICR (imprinting control region) mice (25–30 g) were housed in a temperature controlled room (22–25 °C) with free access to food and water. The formalin test was performed according to the standard method [16]. All animal handling procedures were in accordance with the China Animal Protection Law and officially approved by the Ethics Committee of Institute of Zoology, Chinese Academy of Sciences. Animals were treated with sterile saline (0.1 ml, i.v.), indomethacin (7.5 mg/kg, i.v.), or DrTx(1–42) (0.1, 1 mg/kg, i.v.), 30 min prior to formalin injection. 20 μl of 2.5% formalin (v/v in distilled water) was injected into the ventral surface of each mouse's right hind paw. Immediately after formalin injection, animals were individually placed in a suitable chamber for observation. The summation of time (in seconds) that animals spent licking the injected paws was recorded from 0 to 5 min (neurogenic phase, corresponding to a direct chemical stimulation of nociceptors) and from 15 to 30 min (inflammatory phase, involving release of inflammatory mediators) after injection of formalin.

2.7. MTT assay

The human GC-2 cell line was used in the MTT assay [17] for the evaluation of the cytotoxic effect of DrTx(1–42). GC-2 cells were cultured at 37 °C in a 5% CO_2: 95% air incubator in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). Cells distributed in 96-well plates at a density of 3×10^4 cells/well were incubated overnight. After removing the overnight medium, the cells were incubated with fresh medium alone (control) or various concentrations of peptides for 24 h at 37 °C. To determine cell viability, the cells were treated for 4 h with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide). Then the medium was removed and 200 μl dimethyl sulfoxide (DMSO) was added. Cell viability, as indicated by the absorbance at 570 nm of the purple formazan product formed by reduction of the MTT dye by live cells, was measured by the VersaMax reader (Molecular Devices).

3. Results and discussion

3.1. Purification and identification of recombinant DrTx(1–42)

In our previous studies, we isolated and identified a recombinant fragment of drosotoxin, which is a C-terminally truncated analogue composed of the N-terminal first 42 amino acids (called drosotoxin(1–42), hereafter abbreviated as DrTx(1–42)) [13] (Fig. 1). This truncated product was derived from non-specific cleavage between ^{42}K and ^{43}C of drosotoxin during recombinant

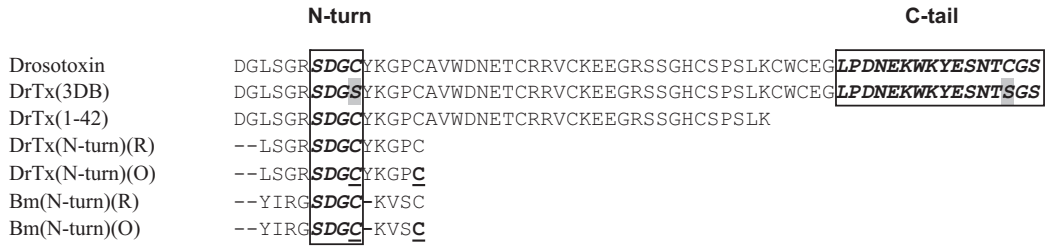

Fig. 1. Sequence alignment of drosotoxin and related peptides. The N-turn and C-tail derived from BmKITc are boxed and italicized. Two mutated sites (C10S and C61S) which remove the fourth disulfide bridge in drosotoxin are shadowed in grey. Cysteines of two reduced peptides that are involved in the formation of a disulfide bridge are boldfaced and underlined once.

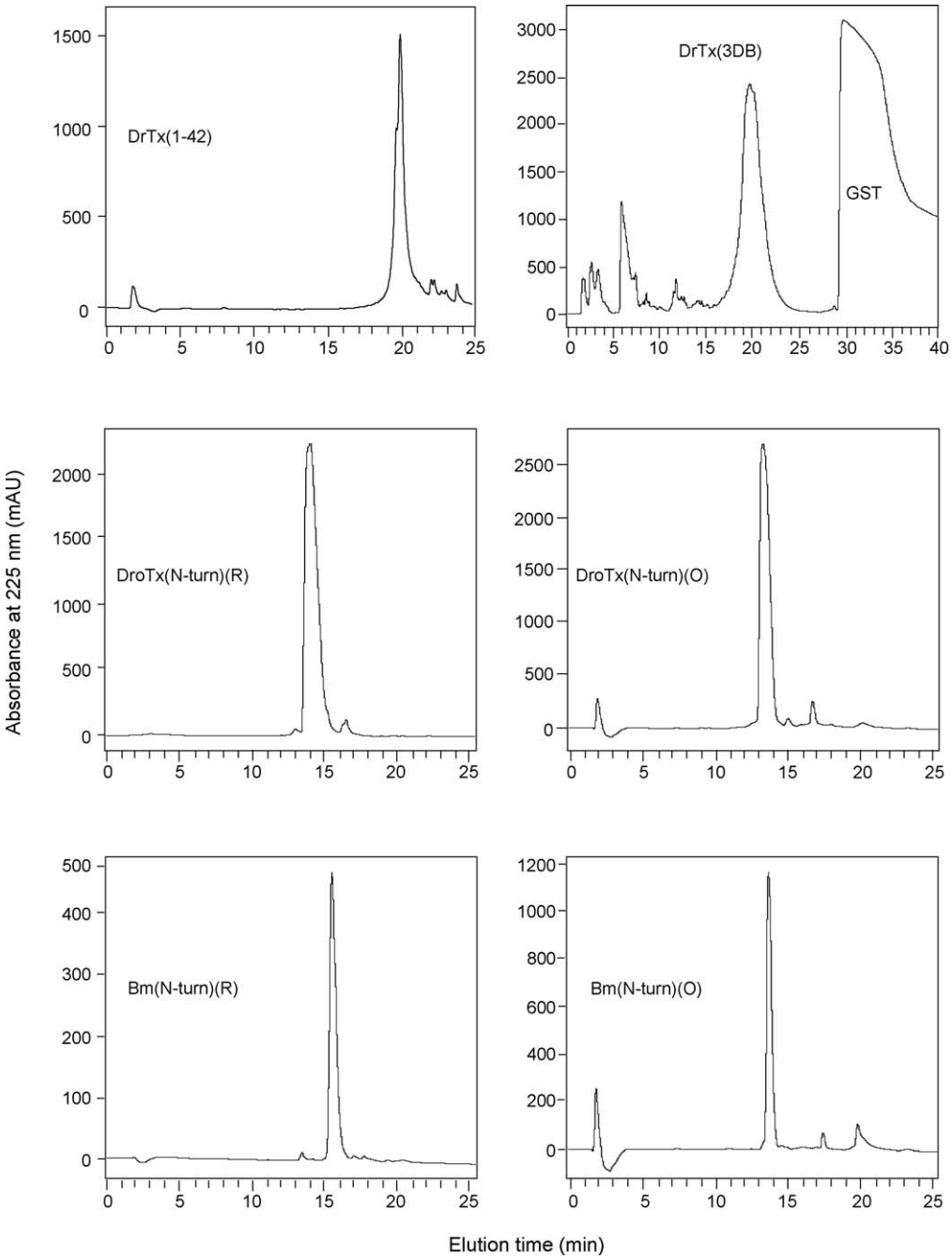

Fig. 2. RP-HPLC showing the purification of recombinant or synthetic peptides. All the elution was carried out using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (TFA) in water (v/v) within 40 min with a flow rate of 1 ml/min. For DrTx(3DB), the separation of EK-digested product is shown here.

Table 1
Molecular weights (MWs) of recombinant or chemically synthetic peptides related to drosotoxin.

Name	Sample	Theoretical	Experimental
DrTx(3DB)	Recombinant	6925.54 Da (−6H)	6926.23 Da
DrTx(1–42)	Recombinant	4528.01 Da (−4H)	4527.20 Da
DroTx(N-turn)(R)	Synthetic	1342.51 Da	1343.33 Da
DroTx(N-turn)(O)	Synthetic	1340.51 Da (−2H)	1341.25 Da
Bm(N-turn)(R)	Synthetic	1287.47 Da	1288.24 Da
Bm(N-turn)(O)	Synthetic	1285.47 Da (−2H)	1286.37 Da

Note: Experimental MWs were determined by MALDI-TOF.

production. The removed C-terminal 21 residues encompass the C-tail obtained from the neurotoxin BmKITc and the sequence "CWCEG" from the structural core of drosomycin.

Our initial functional assays showed that DrTx(1–42) was able to selectively block rat DRG neuron TTX-R I_{Na} and thus represents a functional unit. To prepare plenty of peptides for functional evaluation, we slightly modified the production procedure (Section 2). Under these conditions, a major RP-HPLC peak appeared at the retention time of about 20 min (Fig. 2), which was collected for MALDI-TOF analysis and Edman degradation. The first five amino acids obtained by Edman degradation are DGLSG, identical to the sequence of drosotoxin. The MW measured by MALDI-TOF is 4527.20 Da, about 4 Da smaller than the theoretical MW (4532.01 Da) calculated from its primary sequence, suggesting that 4 hydrogens may be removed from the four cysteines to form two disulfide bridges (Table 1). In this method, the full-length drosotoxin was nearly completely digested into DrTx(1–42) due to non-specific cleavage after the residue ^{42}Lys and thus the yield is significantly increased.

3.2. Functional features of DrTx(1–42)

Under voltage-clamp conditions, the inward I_{Na} was elicited by 50 ms depolarization from the holding potential of −80 mV to −10 mV. At 1.5 μM concentration, DrTx(1–42) significantly inhibited TTX-R I_{Na} but did not affect TTX-S I_{Na} even at 15 μM concentration (Fig. 3A), consistent with the functional feature of the intact molecule of drosotoxin. The time course of inhibition of DrTx(1–42) on TTX-R I_{Na} is shown in Fig. 3B, from which we can see a rapid blockade of the current occurring within 50 s, similar to drosotoxin but distinct from μ-SIIIA, a selective TTX-R I_{Na} blocker isolated from *C. striatus*. It was found that μ-SIIIA completely inhibited TTX-R I_{Na} only after 30 min. The effect of DrTx(1–42) on the current–voltage (*I–V*) curves of TTX-R I_{Na} is illustrated in

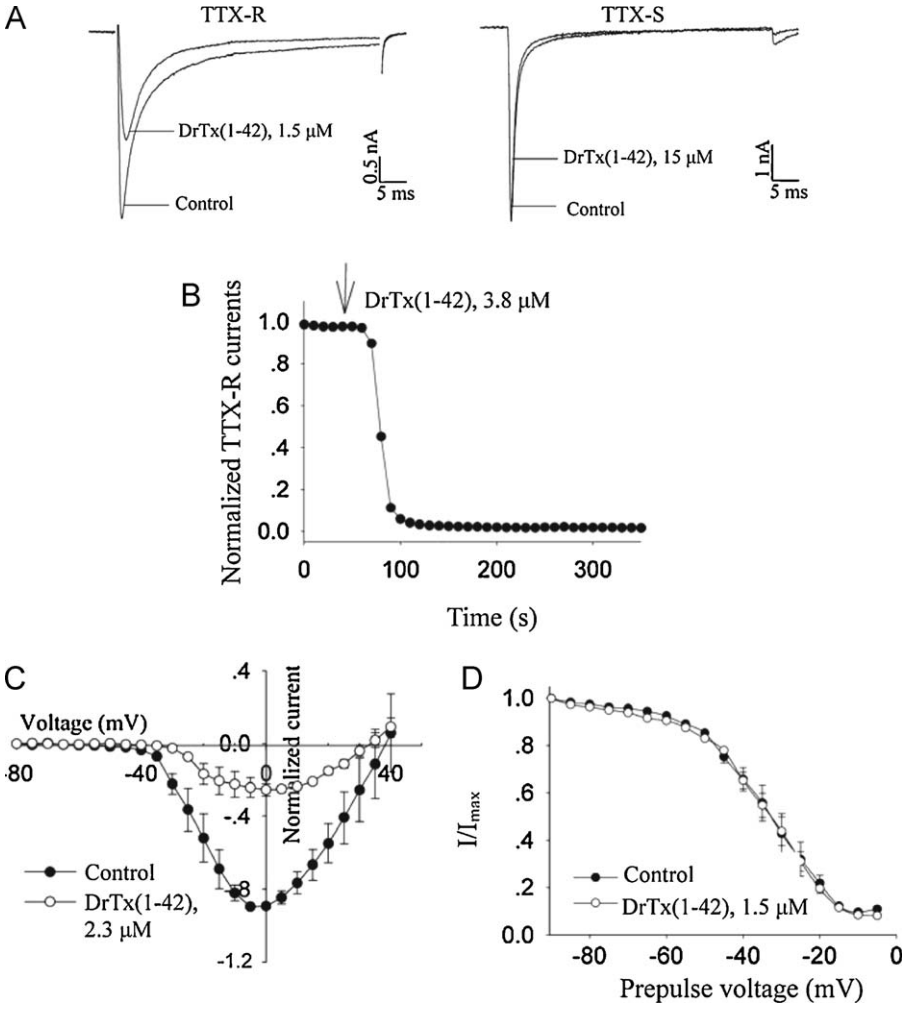

Fig. 3. Functional features of DrTx(1–42). (A) Representative traces of TTX-R and TTX-S sodium currents in DRG neurons in the absence or presence of DrTx(1–42); (B) time–effect curves of 3.8 μM peptide on TTX-R sodium peak currents. The channels were depolarized to −10 mV from a holding potential of −80 mV; (C) the *I–V* relationship of TTX-R sodium currents in control solution (●) and in 2.3 μM DrTx(1–42) solution (○), cells were held at −80 mV, and a series of sodium currents were elicited by 50-ms depolarizing steps to various potentials ranging from −80 mV to +40 mV in 5-mV increments (n = 6); (D) steady-state inactivation of TTX-R sodium channels in control solution (●) and in 1.5 μM DrTx(1–42) solution (○), the currents were induced by a 50-ms depolarizing potential of −10 mV from various prepulse potentials for 25 ms which ranged from −90 to 0 mV in 5-mV increments (n = 6). The data represent the mean ± S.E.

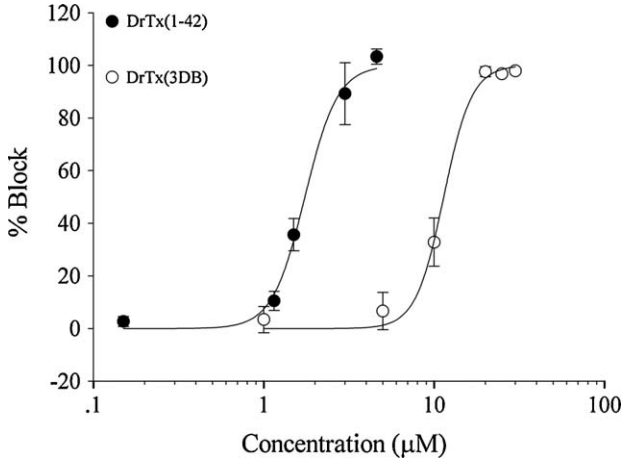

Fig. 4. The concentration-dependent inhibition of TTX-R peak currents by DrTx(1–42) and DrTx(3DB). The data points were fitted according to the Hill equation ($n \geq 5$ for DrTx(1–42); $n \geq 3$ for DrTx(3DB)) and expressed as mean ± S.E. The channels were depolarized to −10 mV from a holding potential of −80 mV.

Fig. 3C. From the curves, it can be seen that the inhibition of currents was observed at all tested potentials after the depression of current amplitude by 2.3 μM DrTx(1–42), and the current inhibition was not associated with a change of the shape of the *I–V* relationship. No shift in the membrane reversal potential of the TTX-R channels was observed (Fig. 3C), implying that DrTx(1–42) does not change the ion selectivity of the channels. DrTx(1–42) also displayed no effect on steady-state inactivation of TTX-R I_{Na} (Fig. 3D). The dose-dependent effects of DrTx(1–42) on TTX-R I_{Na} were verified by the experiments with different peptide concentrations and the 50% inhibitory concentration (IC_{50}) calculated from these data is 1.74 ± 0.07 μM (Fig. 4), slightly lower than that of drosotoxin (2.60 ± 0.50 μM). Overall, this truncated peptide retains the activity of the full-length molecule and our results thus indicate the insertion of the N-turn rather than the extension of the C-tail as a crucial event for the emergence of toxic function on the non-toxic drosomycin scaffold.

3.3. The N-turn-associated fragments lack TTX-R I_{Na} blocking activity

To assay whether smaller N-turn-associated fragments exhibit similar TTX-R I_{Na} blocking activity, we chemically synthesized two such peptides: (1) DrTx(N-turn)(R) (13-mer) that is composed of the N-turn and its flanking amino acids in drosotoxin; (2) Bm(N-turn)(R) (12-mer), including the N-turn and its flanking sequence in BmKITc. DrTx(N-turn)(R) or Bm(N-turn)(R) contains two cysteines that could form an intra-molecular disulfide bridge. To evaluate possible importance of the structural factor, we also prepared the oxidized products of these two reduced peptides, named DrTx(N-turn)(O) and Bm(N-turn)(O), respectively. Obviously, the oxidized peptides altered their retention times in RP-HPLC from 14.2 min to 13.5 min for DrTx(N-turn) and from 15.2 min to 13.8 min for Bm(N-turn) (Fig. 2). The MWs of the two oxidized products are 2 Da less than their respective reduced peptides, indicating one intra-molecular disulfide bridge has been formed (Table 1). All these peptides showed no activity on TTX-R I_{Na} even at 20 μM concentration (Fig. 5), emphasizing the importance of the N-turn combined with the sequence of drosomycin.

3.4. The role of the fourth disulfide bridge

As shown previously, the antifungal drosomycin and the neurotoxic BmKITc share three conserved disulfide bridges, but differ by the fourth disulfide bridge (also called the wrapper disulfide bridge) [13,18]. In our molecular design, we rebuilt this unique disulfide bridge in drosotoxin. To investigate the contribution of this disulfide bridge to the function, we designed and recombinantly produced a mutant (DrTx(3DB)) with the two cysteines (C1 and C8) substituted. DrTx(3DB) was expressed as a soluble fusion protein with an N-terminal GST tag and thus directly subjected to EK digestion. Pure DrTx(3DB) obtained by RP-HPLC separation of the digested product was eluted at the retention time of about 20 min (Fig. 2). Recombinant DrTx(3DB) has an experimental MW of 6926.23 Da measured by MALDI-TOF, which perfectly matches its theoretical MW calculated from its oxidized form (Table 1), suggesting that six hydrogen atoms in the cysteines may be removed to form three disulfide bridges. The final yield of the recombinant peptide is approximately 5 mg/L *E. coli* culture. Functional assays indicate that the mutation of this disulfide bridge cannot completely abolish the blocking activity of drosotoxin on TTX-R I_{Na}. The IC_{50} value of DrTx(3DB) is 11.38 ± 0.40 μM (Fig. 4), showing about 4.5-fold lower potency than drosotoxin. The decrease in the activity appears not to be associated with the removal of the disulfide bridge because DrTx(1–42) also lacks this disulfide bridge due to the C-terminal deletion but shows even stronger potency than the intact molecule. Therefore, the mutation of the first cysteine in the N-turn could be a direct reason.

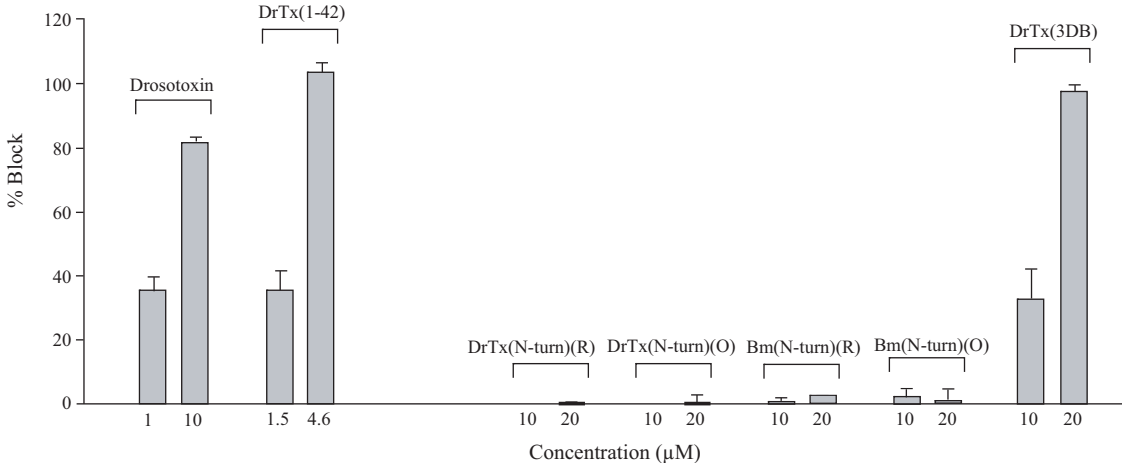

Fig. 5. Comparison of blocking effects of drosotoxin and related peptides on rat DRG neuron TTX-R I_{Na}. The channels were depolarized to −10 mV from a holding potential of −80 mV. Data are treated as mean ± S.E. for $n \geq 3$.

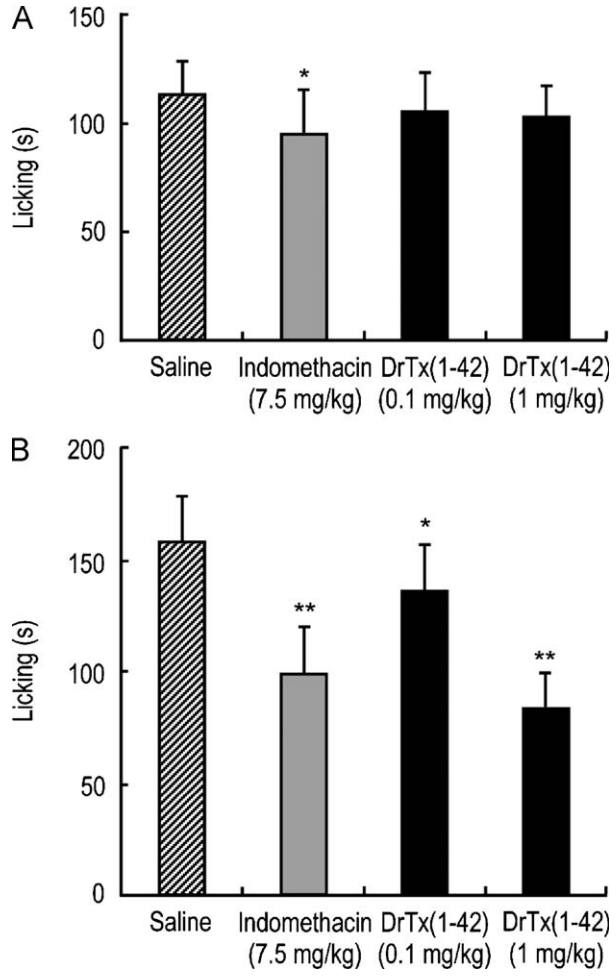

Fig. 6. Effects of DrTx(1–42) on the time spent licking during the neurogenic (A) and inflammatory (B) phases of the formalin test in mice. Data are treated as mean ± S.D. for $n = 6–8$ per group. Statistical analysis was performed using one-way ANOVA followed by Student–Newman–Keuls post hoc test to compare control (saline, 0.9% NaCl in ddH_2O) and the treatment group with SPSS (SPSS Inc.). $^{*}P < 0.05$; $^{**}P < 0.01$.

3.5. The analgesic effect of DrTx(1–42)

DRG neuron TTX-R sodium channels have been demonstrated as the next target for analgesic drugs and low doses of channel blockers have been used for many years as analgesics [5–7,19]. We thus evaluated the analgesic effect of DrTx(1–42) on mice by using a formalin-induced pain model. In this assay, the nociceptive response produced by formalin can be distinguished pharmacologically into two phases: phase 1 (neurogenic) begins immediately after the formalin injection and lasts about 5–10 min and phase 2 (inflammatory) typically begins 15–20 min after the formalin injection and continues for ≥60 min [16]. Indomethacin, a nonsteroidal antiinflammatory agent (NSAIDs) which is ineffective in attenuating the phase 1 response, but is effective in attenuating phase 2, was used as a control in this assay. As shown in Fig. 6, DrTx(1–42) did not affect the neurogenic phase of mice but significantly attenuated the inflammatory response in a dose-dependent manner. At a high concentration (7.5 mg/kg), indomethacin only slightly attenuated the neurogenic response but significantly inhibited inflammatory pain ($P < 0.01$), as identified by the decrease of 37% licking time. Compared with this drug, 1 mg/kg of DrTx(1–42) resulted in the decrease of 50% licking time, demonstrating that this peptide has stronger analgesic effect than indomethacin.

The MTT assay confirmed the DrTx(1–42) lacks detectable cytotoxicity on the human GC-2 cell line (Fig. 7). At 10 mg/kg dose, this peptide was also non-toxic on mice (data not shown).

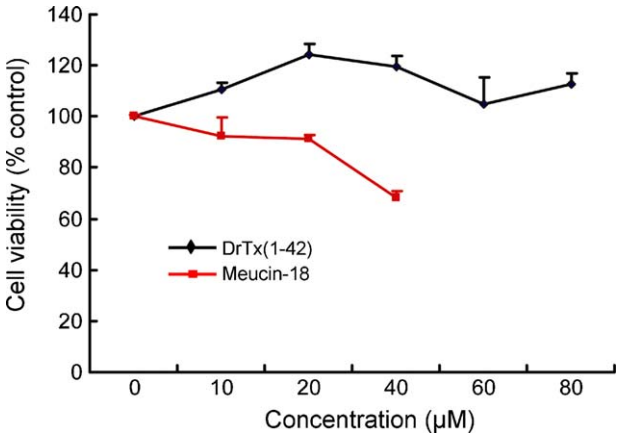

Fig. 7. MTT assay for the evaluation of cell viability of GC-2 treated by various concentrations of DrTx(1–42) for 24 h. Data are shown as mean ± S.D. ($n = 3$). Meucin-18, a known scorpion cytolytic peptide active on bacteria, yeasts, fungi and mammalian erythrocytes was used as a positive control.

4. Conclusion

Drosotoxin is the only one engineered toxin with a non-toxic structural core and exhibits selective activity on TTX-R $I_{Na.}$ The work described here reports the importance of the N-turn insertion in the emergence of toxic function from a non-toxic scaffold. The unique functional feature of DrTx(1–42), including high potency and selectivity on TTX-R I_{Na}, the lack of toxicity on mice, and stronger analgesic effect than the clinical drug indomethacin, make it an intriguing candidate for the development of therapeutic drugs for pain. The elucidation of bioactive sites and precise mechanism for this peptide action on TTX-R I_{Na} is under way.

Acknowledgements

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