The voltage-gated sodium channel Nav1.8 is preferentially expressed in peripheral nociceptive neurons and contributes to inflammatory and neuropathic pain. Therefore, Nav1.8 has emerged as one of the most promising analgesic targets for pain relief. Using large-scale screening of various animal-derived toxins and venoms for Nav1.8 inhibitors, here we identified \( \mu \)-EPTX-Na1a, a 62-residue three-finger peptide from the venom of the Chinese cobra (\textit{Naja atra}), as a potent inhibitor of Nav1.8, exhibiting high selectivity over other voltage-gated sodium channel subtypes. Using whole-cell voltage-clamp recordings, we observed that purified \( \mu \)-EPTX-Na1a blocked the Nav1.8 current. This blockade was associated with a depolarizing shift of activation and repolarizing shift of inactivation, a mechanism distinct from that of any other gating modifier toxin identified to date. In rodent models of inflammatory and neuropathic pain, \( \mu \)-EPTX-Na1a alleviated nociceptive behaviors more potently than did morphine, indicating that \( \mu \)-EPTX-Na1a has a potent analgesic effect. \( \mu \)-EPTX-Na1a displayed no evident cytotoxicity and cardiotoxicity and produced no obvious adverse responses in mice even at a dose 30-fold higher than that producing a significant analgesic effect. Our study establishes \( \mu \)-EPTX-Na1a as a promising lead for the development of Nav1.8-targeting analgesics to manage pain.

Voltage-gated sodium channels (VGSCs) are transmembrane proteins that form pores, permitting Na\(^{+}\) through the cell membrane in response to depolarization (1, 2). VGSCs contribute to the rising phase of action potentials in neurons and play a key role in transmitting peripheral pain signals to the central nervous system (CNS) (3, 4). Among the nine subtypes of VGSCs (Nav1.1–1.9) in mammals, tetrodotoxin-resistant (TTX-R) Nav1.8 is predominantly expressed in small- and medium-diameter primary sensory neurons and is involved in nociception processing (5, 6). Recent studies have indicated that Nav1.8 is becoming one of the most promising analgesic targets for pain relief. In humans, gain-of-function mutations in Nav1.8 were identified in patients with idiopathic small-fiber neuropathy, leading to dorsal root ganglion (DRG) neuron hyperexcitability and peripheral pain hypersensitivity (7). In desert-dwelling grasshopper mice, bark scorpion venom inhibits the mutant form of Nav1.8 that is expressed in the DRG neurons, which protects these mice from the acute pain induced by bark scorpion toxin (8). In diabetic neuropathy, abnormal glucose metabolism leads to the accumulation of high concentrations of the metabolite methylglyoxal, which modifies Nav1.8 and facilitates nociceptive neuron firing, finally leading to diabetes-related metabolic hyperalgesia (9). These significant findings have established the Nav1.8 as a crucial target for the treatment of various excruciating pain.

In fact, the small compound A-803467 potently inhibited Nav1.8 with significant analgesic efficacy in neuropathic and inflammatory pain, and a recent study showed that A-803467 blocked the cardiac Na\(^{+}\) currents mediated by Nav1.5 at 0.3 \( \mu \)M (10, 11). Some analogs of A-803467 and new compounds have also been developed and found to exert analgesic activity via Nav1.8 blockade (12, 13). The \( \mu \)-O-conotoxin MrVIB is a peptide blocker of Nav1.8 isolated from \textit{Conus marmoreus} venom and substantially alleviates chronic pain symptoms (14). Although MrVIB exhibited similar inhibitory activity against Nav1.8 and Nav1.4, the 10-fold selectivity for these channels over other VGSC subtypes contributed to MrVIB-induced analgesia. Taken together, Nav1.8 inhibitors with high selectivity and analgesic activity are urgently needed.

Snake venoms are complex mixtures that contain a variety of chemical compounds, including salts, small-molecule compounds, peptides, and proteins (15). These various compounds interact with ion channels/receptors in a variety of vertebrates and invertebrates. Therefore, the characterization of snake venoms has led to the development of invaluable research probes, diagnostic techniques, and pharmaceutical drugs (16). Over the past few decades, neurotoxins from snake venoms have been confirmed to have potential applications in pain control. Mam-
Balins are a new class of three-finger peptides from black mamba venom that can block pain via suppression of acid-sensing ion channel currents (17). Crotoxin from the venom of the South American rattlesnake *Crotalus durissus terrificus* has a long-lasting antinociceptive effect on neuropathic pain, which is mediated by central muscarinic receptors and 5-lipoxygenase–derived mediators (18). Cobratoxin from *Naja naja kaouthia* is an effective analgesic in rats with neuropathic pain, and this effect involves $\alpha_7$ nicotinic acetylcholine receptor inhibition (19). However, snake neurotoxins targeting Nav1.8 have not been reported to date.

In the present study, by screening new Nav1.8 blockers from snake venoms, we identified $\mu$-EPTX-Na1a (hereafter Na1a) from the venom of *Naja atra* as a potent and selective Nav1.8 inhibitor. Moreover, this peptide exhibited effective analgesic properties in rodent pain models.

**Results**

*Purification and characterization of $\mu$-EPTX-Na1a*

Aiming to identify potent and specific inhibitors of Nav1.8, we performed a large-scale screening by using tens of snake venoms and other animal venoms (from spiders, scorpions, and centipedes) and their purified peptide toxins. As a result, a toxin from *N. atra* venom exhibited potent and specific inhibition. This unique peptide was purified from *N. atra* venom via a combination of RP-HPLC and anion-exchange RP-HPLC (Fig. 1, A, B, and C), and the molecular mass of the peptide was determined to be 7053.48 Da using electrospray ionization (ESI) MS (Fig. 1C, inset).

The amino acid sequence of this peptide was determined by N-terminal Edman degradation, cDNA cloning, and Edman degradation of enzyme (Glu-C)-digested fragments (Fig. 1D and Fig. S1). The peptide is composed of 62 amino acid residues with eight cysteine residues. This peptide belongs to the family of three-finger peptide toxins and shares high sequence identity with cardiotoxin A5, cardiotoxin-like basic polypeptides (CLBPs), and some other cardiotoxins (CTXs) (20, 21). These three-finger toxins are all $\beta$-sheet basic polypeptides that contain 60–62 amino acid residues with a three-fingered loop-folding topology. This peptide could also adopt the three-finger structure, as revealed by structure simulation based on the CTX A5 structure (Protein Data Bank (PDB) code 1KXI) (22).

The three-finger toxins from N-terminal Edman degradation, cDNA cloning, and Edman degradation of enzyme (Glu-C)-digested fragments (Fig. 1D and Fig. S1). The peptide is composed of 62 amino acid residues with eight cysteine residues. This peptide belongs to the family of three-finger peptide toxins and shares high sequence identity with cardiotoxin A5, cardiotoxin-like basic polypeptides (CLBPs), and some other cardiotoxins (CTXs) (20, 21). These three-finger toxins are all $\beta$-sheet basic polypeptides that contain 60–62 amino acid residues with a three-fingered loop-folding topology. This peptide could also adopt the three-finger structure, as revealed by structure simulation based on the CTX A5 structure (Protein Data Bank (PDB) code 1KXI) (22).

According to the peptide toxin nomenclature proposed by King *et al.* (23), we named this peptide toxin Na1a selectively blocks Nav1.8

![Figure 1. Purification and characterization of Na1a from the venom of *N. atra*. A, RP-HPLC separation of 3 mg of *N. atra* venom on a semipreparative C18 column (acetonitrile (ACN) gradient, 20 – 40% at an increasing rate of 1%/min and a flow rate of 3 ml/min). The fraction corresponding to the peak labeled by an asterisk potently inhibited Nav1.8 currents in DRG neurons. B, further purification of the target fraction by an anion-exchange RP-HPLC chromatographic column. The active fraction is labeled with an asterisk. C, desalting and further purification of the active fraction by RP-HPLC. The peptide in the fraction labeled by an arrow is Na1a. Inset, ESI mass spectral analysis of native Na1a revealed that this peptide has a molecular mass of 7053.48 Da. D, sequence comparison of Na1a with the toxins indicated from *N. atra* venom. The disulfide linkage is shown above the sequences. E, the structure of Na1a was modeled by SWISS-MODEL based on the CTX A5 structure (PDB code 1KXI).](http://swissmodel.expasy.org)
Na1a selectively blocks Nav1.8

μ-EPTX-Na1a (referred to as Na1a herein). The peptide identified in this study had no cytotoxicity, as revealed by the assays mentioned below. Notably, we discovered that Na1a is a potent inhibitor of Nav1.8.

Selectivity of Na1a for Nav1.8 in rat DRG neurons

Because Nav1.8 is primarily expressed in small-diameter DRG neurons, we first evaluated the ability and selectivity of Na1a to block Nav1.8 currents in rat DRG neurons. Generally, large neurons (>45 μm) mainly express TTX-S Na\(^+\) currents mediated by the Nav1.1, Nav1.6, and Nav1.7 subtypes, whereas small nociceptive neurons (<20 μm) contain both TTX-S and TTX-R Na\(^+\) currents mediated by Nav1.8 and Nav1.9 (24). TTX (1 μM) was added to the bathing solution to separate TTX-S Na\(^+\) currents from TTX-R Na\(^+\) currents. Additionally, to record Nav1.8 currents in small-diameter DRG neurons, the holding potential was clamped at −70 mV to inactivate the TTX-R Nav1.9 channel (25). As shown in Fig. 2, 1 μM Na1a potently blocked the slow inactivated TTX-R Nav1.8 current in small-diameter DRG neurons (Fig. 2B). In addition, the peptide did not alter the persistent Nav1.9 currents in small-diameter neurons evoked by depolarization to −60 mV from the holding potential of −100 mV (Fig. 2C) (26). Notably, only Nav1.9 currents were activated by this type of depolarization in rat DRG neurons. These data indicated that Nav1.8 was the specific target of Na1a in rat DRG neurons.

The inhibition by Na1a of the Nav1.8 current was dose-dependent, and the IC\(_{50}\) was calculated as 141 nM (Fig. 2D). The time course for Na1a-mediated inhibition was rapid (τ\(_{on}\) = 16.9 ± 0.5 s in the presence of 1 μM Na1a), and relatively slow recovery (τ\(_{off}\) = 69.4 ± 0.9 s) by washing was observed (Fig. 2E). With treatment with 200 nM Na1a, the current–membrane voltage relationship for the Nav1.8 was positively shifted by 10 mV (Fig. 2F); in addition, an approximately −11 mV shift in steady-state inactivation was observed (Fig. 2H). Herein, Na1a was found to be a gating modifier toxin targeting Nav1.8.

Selectivity of Na1a for VGSC subtypes

We next evaluated whether Na1a was selective for individual VGSC subtypes expressed in HEK-293 or ND7/23 cells. As shown in Fig. 3, 1 μM Na1a potently blocked hNav1.8 current...
Na1a selectively blocks Nav1.8

Figure 3. Effects of Na1a on VGSC subtypes. A–H, effects of Na1a on TTX-S VGSC currents in hippocampal neurons (A), hNav1.2 (B), hNav1.3 (C), hNav1.4 (D), hNav1.5 (E), hNav1.6 (F), hNav1.7 (G), and hNav1.8 (H). The inward Na+ currents were triggered by a 50-ms depolarizing potential of −10 mV from a holding potential of −80 mV. hNav1.2–1.7 were expressed in HEK-293 cells, and hNav1.8 was expressed in ND7/23 cells. I, concentration-response relationship for the inhibition of hNav1.8, hNav1.5, and hNav1.4 by Na1a. The curves were fitted to the Hill equation. Data points are expressed as the mean ± S.E. (n = 4–7), and error bars are S.E.

expressed in ND7/23 cells (Fig. 3H), whereas 10 μM toxin hardly affected the currents of hNav1.2, hNav1.3, hNav1.6, and hNav1.7 (Fig. 3) and only partially decreased the peak currents of hNav1.4 (Fig. 3D) and hNav1.5 (Fig. 3E). The apparent IC50 values for Nav1.8 and hNav1.5 were ~0.38 and 8.51 μM, respectively, whereas for hNav1.4, the value might be greater than 10 μM because this concentration of toxin reduced the hNav1.4 current by only ~40% (Fig. 3D). For the subtype Nav1.1, which was not examined in HEK-293 cells, we further tested the sensitivity to Na1a in rat hippocampal neurons, which express the Nav1.1–1.2 and Nav1.6 subtypes at high levels (27). We observed that 10 μM Na1a exhibited very weak inhibition of these composite currents (Fig. 3A). Therefore, these data established Na1a as a potent inhibitor of Nav1.8 with more than 22-fold selectivity for Nav1.8 against other VGSC subtypes. To the best of our knowledge, Na1a is the most selective peptide inhibitor of Nav1.8 identified to date.

To determine whether Na1a has effects on other pain-related targets, we then evaluated the activity of Na1a on TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, TRPM8, and TRPC3–6. No obvious effect of 10 μM Na1a on these pain targets was observed (Table S1).

We then determined the influence of Na1a on the steady-state activation and inactivation of individual VGSCs subtypes. As shown in Fig. 4, Na1a had no apparent modulatory effects on the steady-state activation and inactivation of Nav1.2–1.7 or on the Na+ current in rat hippocampal neurons. Similarly, Na1a caused a depolarizing shift of the half-maximal activation of steady-state activation of hNav1.8 and a hyperpolarizing shift of half-maximal inactivation of steady-state inactivation by ~−9 mV.

Analgesic effect of Na1a on acute inflammatory pain

The analgesic effect of Na1a was determined in rodent pain models. First, the ability of Na1a to attenuate acute inflammatory pain was examined. Na1a was intraperitoneally (i.p.) injected to attenuate acute inflammatory pain in mice, and the effects were examined. For the abdominal constriction test, acetic acid was i.p. injected into mice to generate an abdominal constriction response consisting of a wave of constriction and elongation passing caudally along the abdominal wall. Na1a (i.p. injected) decreased the number of writhing movements by 9.7% (i.p. injected) at equivalent doses. Na1a caused significantly greater reduction than morphine when the doses were 7 and 70 nmol/kg (n = 10, p < 0.01) (Fig. 5A).

Intraplantar injection of formalin caused a biphasic pain response in mice: an early nociceptive response (phase I, 0–5 min) followed by a quiescent period that precedes a second phase of nociceptive behavior (phase II, 15–30 min) (28). Pretreatment with Na1a significantly reduced both the phase I and phase II responses (Fig. 5B). Compared with the control, Na1a was effective at attenuating paw licking time by 9.7 ± 0.8 (n = 12), 25.6 ± 2.3 (n = 12, p < 0.05), and 40.3 ± 1.8% (n = 12, p < 0.01) during phase I (Fig. 5C) and 8.9 ± 0.4 (n = 12), 27.4 ± 1.6 (n = 12, p < 0.05), and 67.7 ± 2.3% (n = 12, p < 0.01) during phase II (Fig. 5D) at concentrations of 1, 7, and 70 nmol/kg, respectively. As a control, pretreatment with morphine also
decreased paw licking time at equivalent doses. In general, Na1a appeared to be more effective than morphine in the two phases. The difference was especially significant at a dose of 70 nmol/kg (\(n = 11005\), \(p < 0.01\)).

### Analgesic effect of Na1a on chronic pain

We further determined the ability of i.p. injected Na1a to abolish inflammatory and neuropathic chronic pain in rats. The complete Freund’s adjuvant (CFA)-induced rat pain model was used to evaluate the antinociceptive effect of Na1a on chronic inflammatory pain. Two days after CFA injection into the plantar area, inflammatory hyperalgesia developed on the injured hind paw, as revealed by the mechanical paw withdrawal threshold (PWT) in response to von Frey hair stimulation. The PWT values were 1.3 ± 0.5 g for the CFA injection group and 15.6 ± 1.3 g for the sham group (\(n = 10–12\)) (two-way ANOVA; *, \(p < 0.05\) and **, \(p < 0.01\), Na1a or morphine versus saline; ##, \(p < 0.01\), Na1a versus morphine).

![Figure 4. Effects of Na1a on steady-state activation and inactivation of VGSC subtypes.](image)

**Figure 4.** Effects of Na1a on steady-state activation and inactivation of VGSC subtypes. Na1a (10 \(\mu\)M) induced no apparent shift in either the steady-state activation or inactivation of TTX-S Na\(^+\) current in hippocampal neurons (A), hNav1.2 (B), hNav1.3 (C), hNav1.4 (D), hNav1.5 (E), hNav1.6 (F), and hNav1.7 (G) but induced a depolarizing shift in the steady-state activation of hNav1.8 (H) by −7 mV and a hyperpolarizing shift in steady-state inactivation by −9 mV. hNav1.2–1.7 were expressed in HEK-293 cells, and hNav1.8 was expressed in ND7/23 cells. Data points are expressed as the mean ± S.E. (\(n = 4–7\)), and error bars are S.E., and the curves of steady-state activation and inactivation were fitted to the Hill equation and Boltzmann equation, respectively.

![Figure 5. Analgesic effects of Na1a on acute pain in mice.](image)

**Figure 5.** Analgesic effects of Na1a on acute pain in mice. A, Na1a reduced the abdominal writhing induced by i.p. injected acetic acid. B, Na1a had analgesic effects on a formalin-induced mouse pain model, as revealed by the recorded licking times. Phase I (0–5 min postinjection) (C) and phase II (15–30 min postinjection) (D) followed intraplantar injection of formalin. Data points are the mean ± S.E., and error bars are S.E. (\(n = 10–12\)) (two-way ANOVA; *, \(p < 0.05\) and **, \(p < 0.01\), Na1a or morphine versus saline; ##, \(p < 0.01\), Na1a versus morphine).
Na1a was long-lasting. Even at 300 min postinjection, the PWT remained 5.8 ± 0.6 g (n = 11). Treatment with morphine at a dose of 70 nmol/kg also increased the PWTs within the recording time. Under the same conditions, Na1a had no effect on the PWT of the contralateral noninflamed paw, which was indicative of a specific anti-hyperalgesic effect in this model.

The antinociceptive effect of Na1a on chronic neuropathic pain was assessed by a rat model of partial nerve ligation (PNL)-induced allodynia. Two weeks after surgery, significant mechanical allodynia developed on the injured hind paw, and the PWT was 1.6 ± 0.4 g, whereas that of the sham group was 16.8 ± 1.5 g (n = 10, p < 0.01). Similar to that in the CFA model, i.p. injected Na1a or morphine at a dose of 70 nmol/kg alleviated the mechanical allodynia induced by PNL (Fig. 6B). The PWT values were 10.3 ± 0.8 g (n = 10, p < 0.05) for Na1a and 7.8 ± 0.8 g (n = 10, p < 0.05) for morphine at 60 min postinjection. The effects were maintained for a long duration, although no significant difference was observed beyond 60 min. In both the CFA and PNL models, Na1a was more effective than morphine. At 60 min postinjection, the augmentation of PWT by Na1a was significantly higher than that by morphine at the same dose of 70 nmol/kg.

Adverse effect profile of Na1a

Our data indicate that Na1a had an antinociceptive effect on rodent pain models. Next, the potential toxicity of Na1a was evaluated by the following assays. Na1a did not cause death, and there were no evident changes in the motor functions of the mice, as revealed by the forced swimming test (Fig. 7A) at a dose of 2.1 μmol/kg (30-fold higher than the highest dose used in the antinociceptive test). Na1a did not significantly affect cell viability (HEK-293, CHO, Mat-Ly-Lu, and HeLa) at a concentration of 56 μM (Fig. 7B) and showed no detectable effect on endothelial cell (human umbilical vein endothelial cells (HUVECs) and calf pulmonary artery endothelial (C-PAE) cells) proliferation at a concentration of 10 μM (Fig. 7, F and G), as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, the hemolytic activity test showed no evidence of lytic effects on rat red blood cells upon treatment with 36 μM Na1a (Fig. 7C). Moreover, the effect of Na1a on the cardiac safety–related hERG K+ channel was examined. 10 μM Na1a could inhibit only ~13.4% of the hERG currents expressed in HEK-293 cells, whereas 1 μM toxin had no inhibitory effect; as a control, the antipsychotic drug haloperidol completely blocked the current at a concentration of 1 μM (Fig. 7, D and E). Taken together with the low inhibition of Nav1.5 by the toxin, the results indicate the low toxicity of Na1a.

Discussion

Recently, Nav1.8 was proven to be a crucial target for pain management, and therefore, identification of Nav1.8-selective inhibitors is urgently needed. Based on natural evolution and structural diversity, animal venom peptides afford elaborate templates and leads for the development of selective inhibitors targeting Nav1.8.

A large-scale screening was performed for the identification of a Nav1.8-selective inhibitor from tens of animal venoms. The novel three-finger peptide Na1a was finally identified. The Nav1.8 inhibition activity was first evaluated in rat DRG neurons. After the TTX-R Nav1.9 channel was inactivated by a voltage clamp at a holding potential of ~70 mV, Na1a robustly inhibited the TTX-R Nav1.8 current in small-diameter DRG neurons. Na1a had no detectable effect on either TTX-S current in large DRG neurons or the persistent Nav1.9 current in small-diameter DRG neurons. Among the different VGSC sub-
Na1a selectively blocks Nav1.8

Figure 7. Adverse effect profile of Na1a. A, i.p. injected Na1a had no effect on motor function as judged by a swim test (n = 10). B, Na1a did not affect cell viability. C, the hemolytic activity tests showed low effects of Na1a on rat red blood cells. D, Na1a had negligible effects on the hERG channel expressed in HEK-293 cells. Shown are representative K$^+$ current traces before (black) and after (blue and dark red) the addition of Na1a and the control drug haloperidol (dark green). E, Na1a inhibited only ~13.4% of the hERG channel currents at a concentration of 10 μM (n = 4). F and G, Na1a did not affect the proliferation of HUVECs and C-PAE cells. For B, C, F, and G, the experiments were performed thrice with each in triplicate. Data points are presented as mean ± S.E., and error bars are S.E.

types, Na1a possesses almost 22-fold selectivity for Nav1.8 over other subtypes. Some selective inhibitors have been discovered via a great deal of effort by different groups, including the small compound A-803467, its analog PF-01247324, and the μO-conotoxin MrVIB (11, 13, 14). The selectivity of Na1a is comparable with that of A-803467 (blocked Nav1.5 at 0.3 μM) and MrVIB (10-fold selectivity). Although data for Nav1.3 are lacking, the compound PF-01247324 exhibited higher selectivity than Na1a. Therefore, Na1a is a peptide-derived Nav1.8 inhibitor with high selectivity for VGSC subtypes. Moreover, evaluation of the effect of Na1a on other pain targets showed that Na1a has comparable selectivity for other targets.

Characterization of the properties of Na1a modification of Nav1.8 in DRG neurons indicates that Na1a is a gating modifier toxin with properties distinct from other VGSC gating modifiers. Macroscopically, Na1a only blocked the peak currents of Nav1.8-like pore-occluding toxins, whereas kinetically, this peptide not only shifted the activation to a more depolarizing potential but also caused a repolarizing shift in inactivation, a phenomenon that has not been observed for other gating modifier toxins. Therefore, Na1a must possess a distinct mechanism of action, elucidation of which via determination of the binding site on Nav1.8 will expand our understanding of toxin–VGSC interactions and be helpful for the design of therapeutic drugs targeting Nav1.8.

Nav1.8 is highly expressed in small nociceptive neurons and contributes to the rising phase of action potential (29). Inflammatory mediators enhance Nav1.8 activity by up-regulation and modulation of Nav1.8 (30). Sciatic nerve injury can cause the up-regulation of both Nav1.8 mRNA and protein (31, 32). A-803467 and its analog PF-01247324 relieve both inflammatory and neuropathic pain. Molecular biological evidence and pharmacological evidence support the curative role of Nav1.8 in inflammatory and neuropathic pain. Here, we comprehensively evaluated the analgesic activity of Na1a in different rodent pain models. Na1a exhibited significant efficacy in the treatment of acetic acid–, CFA–, and formalin–induced inflammatory pain and relieved tactile allodynia in PNL-induced chronic neuropathic pain. Moreover, the efficacy of Na1a was comparable with that of morphine at doses up to 70 nmol/kg in the treatment of both acute and chronic pain. Hence, Na1a is a useful probe for further investigation of Nav1.8 in pain management.

Na1a, as a three-finger peptide, shares high sequence identity with some snake venom peptides from N. atra, including CTX A5, CLBP, and CTX A3. Cobra CTXs constitute major components of cobra venom and play critical roles in cobra venom toxicity. Some CTXs, e.g. CTX A3 from Taiwan cobra, exhibit potent cytotoxicity, which is responsible for the severe tissue necrosis that is observed after cobra snake bites (33). However, unlike CTX A3, CTX A5 and CLBP from Taiwan cobra are noncytotoxic (20, 21). In fact, the activity and molecular targets of these CTXs had not been determined until 2006. That year, Wu et al. found that CTX A5 is able to specifically bind to αβ3 integrin and inhibit bone resorption activity (34). αβ3 integrin is essential for endothelial cell (HUVECs and C-PAE cells) proliferation and survival. Inhibition of αβ3 integrin leads to HUVEC and C-PAE cell apoptosis, whereas activation of αβ3 integrin can promote HUVEC proliferation (35–37). We found that Na1a exhibited no detectable promotion or inhibition of the proliferation of HUVECs and C-PAE cells, suggesting that αβ3 integrin is not the target of Na1a. Based on this toxicity-related concern, we performed a toxicity test for comprehensive assessment. The results confirmed that Na1a has no obvious cytotoxicity at high concentrations in both normal and cancer cell lines. Moreover, there was no detectable hemolytic effect on rat red blood cells. These data indicate that Na1a is divorced from cytotoxicity, which is consistent with previous studies (20, 21). The hERG K$^+$ channel is commonly recommended for evaluation of cardiac safety during preclinical drug development because many drugs associated with acquired QT prolongation are known to act on the hERG channel (38). Na1a
(10 μM) caused only weak inhibition of the hERG current, whereas the positive control haloperidol (1 μM) completely blocked the current. The cardiac sodium channel Nav1.5 initiates the up-regulation of the action potentials (phase 0) of atrial and ventricular myocytes. Nav1.5 inhibition reduces the rate of cardiac depolarization, which then leads to decreased conduction velocity, leading to potential risks of arrhythmia and sudden cardiac death (39). Here, Na1a caused weak inhibition of the hNav1.5 current. Therefore, based on our data, Na1a has relatively high safety for the two key safety-related channels. Based on in vivo observations, there were no deaths or motor function deficits observed at a dose that was 30-fold higher than the highest dose used in the antinociceptive test. Therefore, the low toxicity of Na1a indicates the potential for further research.

In conclusion, Nav1.8 has been considered to be an important and challenging target for the discovery and development of human pain therapeutics. In the present study, we identified a three-finger neurotoxin, Na1a, from N. atra venom. Na1a potently inhibited Nav1.8 currents in rat DRG cells with high selectivity over other VGSC subtypes. This peptide functioned as a gating modulator by shifting activation in the positive direction and inactivation in the negative direction. Interestingly, Na1a led to significantly decreased nociceptive sensitivity in rodent pain models of inflammatory and neuropathic pathways. Evaluation of the adverse effects of Na1a demonstrated that selective inhibition of Nav1.8 by Na1a was not accompanied by considerable side effects in cells and animals. Taken together, these findings suggest that the snake venom peptide Na1a might be a promising lead or prototype drug for the development of Nav1.8-selective analgesic drugs.

Experimental procedures

**Purification of μ-EPTX-Na1a**

Lyophilized crude venom of *N. atra* was purchased from a company that sells domesticated snakes in Jiang Xi Province, China. The *N. atra* venom was dissolved in double distilled H2O to a final concentration of 6 mg/ml and subjected to semi-preparative C18 RP-HPLC purification (acetoniitrite gradient, 20–40% at an increasing rate of 1%/min). The fraction containing Na1a was then collected, lyophilized, and subjected to anion-exchange RP-HPLC (1 mM sodium perchlorate, 10 mM phosphate buffer, pH 6.0; acetoniitrite gradient, 20–50% at 0.66%/min). An analytical C18 RP-HPLC column was used for further purification with a slow acetoniitrite gradient (increasing rate of 0.5%/min). The molecular weight of Na1a was determined by ESI MS.

**Culture and transfection of HEK-293 and ND7/23 cells**

HEK-293 and ND7/23 cells were grown under standard culture conditions (5% CO2, 95% humidity at 37 °C) in DMEM supplemented with 10% fetal bovine serum. For VGSC subtypes, an hNav1.2–1.7 channel plasmid and a plasmid encoding GFP were transiently transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and an hNav1.8 channel plasmid and a plasmid encoding GFP were transiently transfected into ND7/23 cells. Cells emitting green fluorescence were selected for patch-clamp recording 24–48 h after transfection.

**Isolation and culture of primary neurons**

Animal experimentation protocols were approved by the Animal Experimentation Ethics Committee of Hunan Normal University. All experiments conformed to the general rule of minimizing animal suffering and numbers. Rat DRG neurons were acutely dissociated from Sprague-Dawley rats (30 days old of either gender) and maintained in short-term primary culture according to the method described previously (40). DRG neurons were treated with DMEM (serum-free; 5 mg/ml protease and 2 mg/ml collagenase) for 35 min. The dissociated neurons were plated into poly-L-lysine–coated (50 μg/ml) 35-mm diameter dishes (Corning) and maintained in DMEM supplemented with 10% fetal bovine serum. Large-diameter DRG neurons (>45 μm) were selected for measurement of TTX-S Na+ currents, and small-diameter neurons (<20 μm) were selected for measurement of TTX-R Na+ currents. TTX (final concentration of 1 μM) was used to separate TTX-R Na+ currents from TTX-S Na+ currents.

Hippocampal tissues of neonatal rats were dissected and treated with 0.25% trypsin in Ca2+- and Mg2+-free Hanks’ buffered salt solution at 37 °C for 15 min, and then the neurons were dissociated by trituration with glass Pasteur pipettes. The dissociated hippocampal neurons were plated into poly-L-lysine–coated (50 μg/ml) 35-mm diameter dishes at a density of 3 × 104 cells/dish. After 24 h of culture, the culture medium was replaced with serum-free Neurobasal medium (supplemented with 2% B27), and 500 μM glutamine was added to prevent the growth of glial cells. The hippocampal neurons were maintained at standard conditions (5% CO₂, 95% humidity at 37 °C), and half the culture medium was replaced with fresh medium every other day. The neurons were used at days in vitro 14–17 (41).

**Whole-cell voltage-clamp recording**

Whole-cell patch-clamp was used to record the Na+ currents in rat DRG neurons, hippocampal neurons, and HEK-293 and ND7/23 cells expressing VGSC subtypes using an EPC-10 amplifier (HEKA Electronics, Ludwigshafen, Germany) at room temperature. Fire-polished electrodes (1.5–2.5 megohms) were fabricated from a 1.5-mm glass capillary by two-step pulling on a vertical micropipette puller. For recording the Na+ current in rat DRG and hippocampal neurons, the pipette solution was 140 mM CsF, 10 mM NaCl, 10 mM HEPES, and 1.1 mM EGTA (pH 7.3, adjusted with CsOH). The external solution was 140 mM NaCl, 3 mM KCl, 1 mM CaCl2, 1 mM MgCl2-6H2O, and 10 mM HEPES (pH 7.3, adjusted with NaOH). For recording the Na+ current in HEK-293 or ND7/23 cells, the pipette solution was 145 mM CsCl, 4 mM MgCl2, 6H2O, 10 mM HEPES, 10 mM EGTA, 10 mM glucose, and 2 mM ATP (pH 7.3 adjusted with CsOH). The external solution contained 145 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl2, 1.2 mM MgCl2-6H2O, 10 mM HEPES, and 10 mM glucose (pH 7.4, adjusted with NaOH). Experimental data were collected and analyzed with PatchMaster software (HEKA Electronics) and SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA).

Concentration-response curves of Na1a on the channels were fitted to the Hill equation as follows: $I_{\text{norm}} = C + A/(1 + I_{\text{norm}})$.
**Complete Freund’s adjuvant–induced pain**

The steady-state inactivation curve was fitted to the Boltzmann equation as follows: \( y = 1/(1 + \exp((V_{1/2} - V)/k)) \), where \( V_{1/2} \), \( V \), and \( k \) are the midpoint voltage of kinetics, test potential, and slope factor, respectively. \( \tau_{on} \) and \( \tau_{off} \) values were fitted using following the single-exponential fit equations: \( I(t) = a_0 + a_1[1 - \exp(-t/\tau_{on})] \) and \( I(t) = a_0 + a_1\exp(-t/\tau_{off}) \), respectively.

**Acetic acid–induced abdominal writhing**

The formalin-induced mouse pain model features stability and repetition. Therefore, mice were used for the Na1a analgesic activity test in the acetic acid–induced pain model (42). Abdominal writhing action induced by acetic acid leads to abdominal constriction and hind limb extension (42). Mice were injected i.p. with 0.1 ml of saline containing Na1a or morphine 30 min before being i.p. injected with acetic acid (1% solution, 0.1 ml/20 g). Mice were placed into individual, open polyvinyl cages (20 x 40 x 15 cm) immediately after acid administration, and the number of abdominal contractions was counted cumulatively in 30 min.

**Formalin-induced paw licking**

The formalin-induced mouse pain model is stable and repeatable, so mice were used for the Na1a analgesic activity test in the formalin-induced pain model as described previously (43). In the formalin-induced mouse pain model, pain was induced by injection of formalin (1%, 20 μl) into the plantar region of the right hind paw. Either Na1a or morphine was i.p. injected 30 min before formalin administration. Following formalin injection, the mice were placed in individual, open polyvinyl cages (20 x 40 x 15 cm) that permitted unrestricted observation. The time that the mice spent licking the injected paw was recorded to represent nociception. The first phase (neurogenic) of the nociceptive response normally peaks at 0–5 min, and the second phase (inflammatory) normally peaks at 15–30 min after formalin injection (43).

**Complete Freund’s adjuvant–induced pain**

Experiments were performed on Sprague-Dawley rats of both genders that weighed 160–210 g. Rats were injected with 0.1 ml of undiluted CFA (Sigma) at the plantar surface of the left hind paw, which induced inflammation-related pain after 24 h. To assess mechanical allodynia, mechanical PWTs were measured using a set of von Frey hairs by the up-down paradigm (44). On day 2 postinjection, the rats were i.p. injected with 0.1 ml of saline containing Na1a or morphine, and PWTs were determined at 30, 60, 120, 180, 240, and 300 min postinjection.

**Partial nerve ligation–induced allodynia**

Rats were anesthetized by i.p. injection with sodium pentobarbital (60 mg/kg). PNL surgery was performed under sterile conditions using the method described by Seltzer et al. (45). The rat’s left sciatic nerve was exposed, and one-third of the nerve diameter was ligated using a nonabsorbable monofilament polypropylene suture. Another group of rats was subjected to sham surgery in which the sciatic nerve was exposed but not ligated. Animals were inspected every day and tested on day 14 after surgery. Na1a or morphine was administered, and PWTs were determined in a manner similar to those in the CFA-induced pain model as described above.

**Adverse effect test**

A forced swimming test was performed as reported previously to evaluate the influence of Na1a on motor function (46). The mice were i.p. injected with Na1a or an equal volume of saline and then subjected to an exhaustive swimming test after 30 min. The cytotoxicity and inhibition of proliferation by Na1a were evaluated by the MTT assay as described previously (47). The MTT assay is widely used to measure cell viability, proliferation, and cytotoxicity. The effects of Na1a on cell viability (HEK-293, CHO, Mat-Ly-Lu, and HeLa) and endothelial cell (HUVECs and C-PAE cells) proliferation were tested after incubation of Na1a for 24 and 48 h, respectively. The hemolytic activity of Na1a was assayed as described previously (48). The lytic effect of Na1a on rat red blood cells was detected after pretreatment with Na1a or an equal volume of Triton X-100 (1%).

The effect of Na1a on the hERG channel stably expressed in HEK-293 cells was tested. The hERG channel currents were recorded with an EPC-10 amplifier using the whole-cell patch-clamp technique as described previously (49). Briefly, the suction pipettes (1.5–2.5 μm) contained 130 mM KCl, 1 mM MgCl2, 5 mM MgATP, 5 mM EGTA, and 10 mM HEPES (pH 7.2, adjusted with KOH). The bathing solution was 140 mM NaCl, 2.5 mM CaCl2, 1 mM MgCl2, 4 mM KCl, 10 mM glucose, and 5 mM HEPES (pH 7.4, adjusted with NaOH). The peak \( I_{hERG} \) tail current was triggered at −50 mV after a test potential of 20 mV from a holding potential of −80 mV. The positive control was the antipsychotic drug haloperidol, which is a potent inhibitor of the hERG channel.

**Statistics**

Data were analyzed by PatchMaster, SigmaPlot 10.0, and GraphPad Prism 5 (GraphPad Software, La Jolla, CA) software. All data are shown as means ± S.E. \( n \) is the number of the separate experimental cells, rats, and mice. Statistical significance was examined by a two-way ANOVA. \( p < 0.05 \) was considered statistically significant.


**References**


