A novel slow-inactivation-specific ion channel modulator attenuates neuropathic pain

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ABSTRACT

Voltage-gated ion channels are implicated in pain sensation and transmission signaling mechanisms within both peripheral nociceptors and the spinal cord. Genetic knockdown and knockout experiments have shown that specific channel isoforms, including NaV1.7 and NaV1.8 sodium channels and CaV3.2 T-type calcium channels, play distinct pronociceptive roles. We have rationally designed and synthesized a novel small organic compound (Z123212) that modulates both recombinant and native sodium and calcium channel currents by selectively stabilizing channels in their slow-inactivated state. Slow inactivation of voltage-gated channels can function as a brake during periods of neuronal hyperexcitability, and Z123212 was found to reduce the excitability of both peripheral nociceptors and lamina I/II spinal cord neurons in a state-dependent manner. In vivo experiments demonstrate that oral administration of Z123212 is efficacious in reversing thermal hyperalgesia and tactile allodynia in the rat spinal nerve ligation model of neuropathic pain and also produces acute antinociception in the hot-plate test. At therapeutically relevant concentrations, Z123212 did not cause significant motor or cardiovascular adverse effects. Taken together, the state-dependent inhibition of sodium and calcium channels in both the peripheral and central pain signaling pathways may provide a synergistic mechanism toward the development of a novel class of pain therapeutics.

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1. Introduction

Voltage-gated sodium (NaV) and calcium (CaV) channels are crucially involved in nociceptive signaling pathways, in part by mediating ionic currents that contribute to the excitability of peripheral nociceptors in the dorsal root ganglia (DRG). A specific subtype of T-type CaV channel (CaV3.2) is highly expressed in DRG neurons and is involved in the initiation of action potential (AP) firing and the generation of burst firing [6,19,31,42]. Both tetrodotoxin (TTX)-sensitive NaV1.7 and TTX-resistant NaV1.8 channels are also robustly expressed in DRGs and are important for setting the threshold and upstroke of AP firing, respectively, and further act to influence the frequency and sustainability of firing [10].

Within the spinal cord dorsal horn, second-order neurons in superficial layers (lamina I/II) relay nociceptive-specific signals from peripheral nociceptors to pain-processing regions of the brain. Evidence suggests that a variety of NaV and CaV channel isoforms are expressed within lamina I/II neurons [11,14,20] and that both NaV and CaV channels may increase the excitability of dorsal horn neurons linked to neuropathic and inflammatory pain signaling [11,14,20]. Specific NaV and CaV isoforms have been shown to play pronociceptive roles; knockout of either NaV1.7 or NaV1.8 channels or knockdown of CaV3.2 T-type channels reduces hyperalgesia and allodynia in animal models of acute and neuropathic pain [6,25,30]. In humans, loss-of-function mutations in the NaV1.7 channel lead to complete abolition of pain sensation, while gain-of-function NaV1.7 mutations cause severe chronic pain syndromes [10].
Neuropathic pain results from damage to the peripheral or central nervous system and persists long after the nerve injury has resolved [46]. Pharmaceutical approaches to the management of neuropathic pain are limited, and the continued use of some therapies can lead to a variety of adverse events and/or desensitization of drug effects. It has been hypothesized that the increased AP firing and sustained depolarization of neurons associated with neuropathic pain may drive a greater subset of NaV and CaV channels into a protective slow-inactivated state in order to dampen neuronal excitability [4,5,17,44]. In this regard, blockers selectively targeting the slow-inactivated channel state would be predicted to mitigate off-target effects by preferentially attenuating aberrantly hyperexcitable neurons while largely sparing normally firing neurons and other nonhyperexcited targets.

In the current study, we have designed and characterized a low-molecular-weight, orally available organic compound (Z123212) that stabilizes the slow-inactivated state of NaV and CaV channels, including TTX-resistant NaV and T-type CaV channels in DRG neurons and TTX-sensitive NaV channels in lamina I/II spinal cord neurons. Z123212 potently reduces the excitability of DRGs and lamina I/II neurons and is found to reverse thermal and mechanical hypersensitivity in animal models of acute and neuropathic pain. The identification of compounds such as Z123212 that have potentially synergistic effects by targeting multiple ion channels in components of the peripheral and central nociceptive signaling pathways through a state-dependent mechanism may lead to the development of novel classes of safe and effective pain therapeutics.

2. Methods

2.1. Chemistry

The synthesis of Z123212 is illustrated in Suppl. Fig. 1. Briefly, ethylenediamine-N,N-diacetic acid was cyclized under acidic conditions followed by N-tert-butoxycarbonyl (Boc) protection of piperazine nitrogen. Subsequent coupling with bis-CF3 aniline mediated by O-benzotriazol-1-y()-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) in N,N-dimethylformamide (DMF) provided the desired intermediate and was followed by deprotection of the Boc group to generate Z123212.

2.2. HEK 293 cell culture, transfection, and electrophysiology

Human embryonic kidney cells (HEK 293) were cultured and either stably or transiently transfected with recombinant NaV and CaV channel genes as previously described [18]. For NaV channel recordings, the external recording solution contained (in mM): 137 NaCl, 4 KCl, 1.8 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, adjusted to pH 7.4 with NaOH, while for CaV1.2, CaV2.1, and CaV2.2, the external solution contained (in mM): 139 CsCl, 5 BaCl2, 1 MgCl2, 10 HEPES, 10 glucose, adjusted to pH 7.4 with CsOH. For all recordings, the internal patch pipette solution contained (in mM): 126.5 CsMeSO4, 2 MgCl2, 11 EGTA, 10 HEPES, 2 Na3-ATP, adjusted to pH 7.3 with CsOH. For all voltage-clamp protocols liquid junction potentials were left uncorrected. Recordings were digitized at 5 kHz and low-pass filtered at 1 kHz.

2.3. Animals

All electrophysiological experiments involving animals and their care were performed in accordance with the recommendations of the Canadian Council on Animal Care and were according to the animal care regulations and policies of the University of British Columbia. For in vivo pain testing experiments, male Sprague–Dawley rats (225–300 g; Harlan; Indianapolis, IN) were maintained on a 12/12 h light/dark cycle and provided food and water ad libitum. All pain testing experiments were performed under protocols approved by the Institutional Animal Care and Use Committee in compliance with policies set forth by the National Institutes of Health of the United States.

2.4. Voltage-clamp recordings on DRG neurons

Male Wistar rats (P25 to P30) were anesthetized with isoflurane and decapitated. DRG were removed, cut into pieces, and placed in Ca2+- and Mg2+-free Hank balanced salt solution containing (in mM): 138 NaCl, 5.3 KCl, 0.4 KH2PO4, 0.3 Na2HPO4, 6 n-glucose, 10 HEPES, and 2 mg/ml collagenase (Type I, Worthington, Lakewood, New Jersey), and 200 units of DNaseI (Worthington, Lakewood, NJ). Ganglia were incubated for 45 min at 37 °C and subsequently placed in L-15 media supplemented with 10% fetal bovine serum, 100 units of penicillin, 100 μg streptomycin, 5 mHEPES, and 250 ng/ml nerve growth factor (all from Invitrogen, Carlsbad, CA). Cells were dispersed with fire-polished Pasteur pipettes and plated on glass coverslips coated with 1 mg/ml poly-γ-L-lysine and 5 μg/ml laminin (Sigma, St. Louis, MO). Coverslips were incubated at 37 °C for 1–2 h and transferred to 4 °C for storage. Within 72 h, neurons were subjected to voltage-clamp analyses with borosilicate glass patch pipettes with resistances of 2.5–5 MΩ. The external recording solution contained (in mM): 137 NaCl, 5 TEACl, 10 d-glucose, 1.8 CaCl2, 1 MgCl2, 10 HEPES, 0.0005 TTX, 0.001 LaCl3, adjusted to pH 7.4 with NaOH and 300 mMOSm with sucrose. The internal patch pipette solution contained (in mM): 120 CsCl, 2 MgCl2, 10 EGTA, 10 HEPES, 3 MgATP, 6 Tris2-phosphocreatine, and 0.4 Tris-GTP adjusted to pH 7.2 with CsOH and 290 mMOSm. Only neurons with stable leak currents less than 100 pA at –70 mV were used. Recordings were digitized at 20 kHz and low-pass filtered at 1 kHz.

2.5. Current-clamp recordings on DRG neurons

Male Sprague–Dawley rats (P1 to P4) were anesthetized with CO2 and decapitated. DRG were removed and placed in Ham F-12 supplemented with 10% horse serum, 50 units of penicillin, and 50 μg streptomycin (all media components from Invitrogen). Ganglia were incubated for 15 min at 37 °C in F-12 media supplemented with 0.05% collagenase (Type XI, Sigma), rinsed, and incubated for 10 min at 37 °C in phosphate-buffered saline supplemented with 0.12% trypsin (Invitrogen). Cells were dispersed with fire-polished Pasteur pipettes and plated on glass coverslips coated with 15 μg/ml poly-γ-L-lysine (Sigma) in F-12 media supplemented with 40 ng/ml nerve growth factor (Invitrogen). Within 72 h of plating, neurons were current-clamped with borosilicate glass patch pipettes with resistances of 2.5–5 MΩ. The external recording solution contained (in mM): 137 NaCl, 5 KCl, 10 d-glucose, 2 CaCl2, 2 MgCl2, 10 HEPES adjusted to pH 7.4 with NaOH and 300 mMOSm with sucrose. The internal patch pipette solution contained (in mM): 130 KCl, 2 MgCl2, 10 EGTA, 10 HEPES, 3 MgATP, 6 Tris2-phosphocreatine, and 0.4 Tris-GTP adjusted to pH 7.2 with CsOH and 290 mMOSm. Only neurons with stable leak currents less than 100 pA at –70 mV were used. A calculated liquid junction potential of 12.2 mV was corrected in all DRG current-clamp recordings. Recordings were digitized at 5 kHz and low-pass filtered at 1 kHz.

2.6. Recordings on lamina I/II spinal cord neurons

Male Wistar rats (P6 to P9 for voltage-clamp and P15 to P18 for current-clamp recordings) were anesthetized through
intraperitoneal injection of Inactin (Sigma). The spinal cord was then rapidly dissected out and placed in an ice-cold protective sucrose solution containing (in mM): 50 sucrose, 92 NaCl, 15 d-glucose, 26 NaHCO3, 5 KCl, 1.25 Na2HPO4, 0.5 CaCl2, 7 MgSO4, and 1 kynurenic acid, and bubbled with 5% CO2/95% O2. The meninges, dura, and dorsal and ventral roots were then removed from the lumbar region of the spinal cord under a dissecting microscope. The “cleaned” lumbar region of the spinal cord was glued to the vibratome stage and immediately immersed in ice-cold bubbled sucrose solution. For current-clamp recordings, 300- to 350-μm parasagittal slices were cut to preserve the dendritic arbor of lamina I neurons, while 350- to 400-μm transverse slices were prepared for voltage-clamp recordings. Slices were allowed to recover for 1 h at 35 °C in Ringer solution containing (in mM): 125 NaCl, 20 d-glucose, 26 NaHCO3, 3 KCl, 1.25 Na2HPO4, 2 CaCl2, 1 MgCl2, 1 kynurenic acid, and 0.1 picrotoxin, bubbled with 5% CO2/95% O2. The slice recovery chamber was then returned to room temperature (20–22 °C), and all recordings were performed at this temperature.

Neurons were visualized with IR–DIC optics (Zeiss Axioskop 2 FS plus, Gottingen, Germany), and neurons from lamina I and the outer layer of lamina II were selected on the basis of their location relative to the substantia gelatinosa layer. Neurons were subjected to patch-clamp analyses with borosilicate glass patch pipettes with resistances of 3–6 MΩ. Voltage-clamp recordings of NaV currents in lamina I/II neurons were performed after slowly (2–5 min) pulling the neurons off the slice to enable adequate space clamp (entire soma isolation [ESI], technique as in Safronov et al. [38]; see Suppl. Fig. 4). For current-clamp recordings of lamina I/II neurons in the intact slice, the external recording solution was the above Ringer solution, while the internal patch pipette solution contained (in mM): 140 KGlucose, 4 NaCl, 10 HEPES, 1 EGTA, 0.5 MgCl2, 4 MgATP, 0.5 Na2GTP, adjusted to pH 7.2 with 5 M KOH and to 290 mOsm with d-mannitol (if necessary). Only tonic firing neurons were selected for current-clamp experiments, while phasic, delayed-onset, and single-spike neurons were discarded [34]. For voltage-clamp recordings of pharmacologically isolated NaV currents [38] in ESI lamina I/II neurons, the external recording solution was a modified TEA–Ringer solution containing (in mM): 95 NaCl, 20 TEACL, 11 d-glucose, 25 NaHCO3, 5.6 KCl, 1 NaH2PO4, 0.1 CaCl2, 5 MgCl2, 1 kynurenic acid, 0.1 picrotoxin, while the internal patch pipette solution contained (in mM): 140 CsCl, 5.8 NaCl, 1 MgCl2, 3 EGTA, 10 HEPES, 4 MgATP, 0.5 Na2GTP, adjusted to pH 7.3 with NaOH and 290 mOsm with d-mannitol (if necessary). Only neurons with stable leak currents less than 100 pA (at −100 mV) for voltage-clamp and with resting membrane potentials (\(V_{\text{rest}}\)) more negative than −50 mV for current-clamp were used for subsequent experiments. A calculated liquid junction potential of 14.6 mV was corrected for current-clamp recordings. Recordings were digitized at 50 KHz and low-pass filtered at 2.4 or 10 kHz for voltage-clamp and current-clamp recordings, respectively.

2.7. In vivo pain testing

Spinal nerve ligation (SNL) injury was performed by tight ligation of the L5 and L6 spinal nerves according to the procedure of Kim and Chung [24] in Harlan Sprague–Dawley rats. Rats that exhibited motor deficiency (such as paw dragging or dropping) or showed no tactile or thermal hypersensitivity were excluded from further testing. The experimenter was blinded to the drug pretreatment. Fourteen days after SNL injury, tactile paw withdrawal threshold and thermal paw withdrawal latency were measured. Response thresholds to innocuous mechanical stimuli were evaluated by determining paw withdrawal threshold after probing the paw with a series of calibrated Von Frey filaments [7]. The withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength (up-and-down method) and analyzed by a Dixon nonparametric test. Data are expressed as the mean withdrawal threshold. Response thresholds to noxious thermal stimuli were determined by measuring the latency of paw withdrawal from a focused beam of radiant heat on the surface of the hind paw using a plantar analgesia meter (Ugo Basile, Italy) by the Hargreaves method [16]. A maximum cutoff of 33 s was used to prevent tissue damage. For the hot-plate test, naive rats were placed on a 52 °C metal hot plate to measure the latency of paw flinching or licking before or 1 h after drug administration. A cutoff of 15 s was used.

2.8. Cardiovascular liability studies

Isolated New Zealand White rabbit (2.5–3.5 kg) hearts were AV ablated, perfused in a retrograde manner, and paced at a stimulation rate of 1 Hz (basic cycle length = 1 s). The stabilization period was at least 15 min long before obtaining control responses. Experiments were performed at 37 ± 3 °C. Each heart acted as its own vehicle control before application of Z123212. Concentrations of 3, 10, and 30 μM Z123212 were applied sequentially, in ascending order, for exposure periods of at least 15 min/concentration to allow for equilibration within the heart tissue. The QT interval and QRS duration were calculated by ECG Auto software (EMKA Technologies, Falls Church, VA).

2.9. Pharmacokinetic studies

Z123212 was provided as the HCl salt for pharmacokinetic analysis. All dosing was based on the free base weight of the compound. Harlan male Sprague–Dawley rats were fasted overnight before dose administration of Z123212 in 0.5% carboxy methyl cellulose. Plasma samples were collected via jugular cannulae from 3 animals per time point at 0.25, 0.5, 0.75, 1, 2, and 4 h. Brains were collected from 3 animals per time point at 1 and 4 h. Plasma and brain samples were stored below −70 °C until analysis could be performed by a research-grade liquid chromatography/tandem mass spectrometry assay. Mean Z123212 concentrations in the plasma and brain and noncompartmental pharmacokinetic analysis of the plasma data were performed by WinNonlin software, version 5.0.1 (Pharsight, Mountain View, CA).

2.10. Compounds and perfusion

Unless otherwise indicated, all compounds were obtained from Sigma. For in vitro studies, Z123212 was prepared as 30 or 100 mM stock solutions in dimethyl sulfoxide and stored at −80 °C. Stock aliquots were thawed and used for a maximum of 2 weeks. The highest concentration of dimethyl sulfoxide in the extracellular solutions did not exceed 0.1%, a concentration that did not detectably affect current-clamp or voltage-clamp recording properties. A closed perfusion system (10 mL) was used for spinal cord slice recordings, with a flow rate of between 2 and 4 mL/min. For in vivo studies, Z123212 was dissolved in 0.5% carboxy methyl cellulose at a concentration of 6 mg/mL.

2.11. Data analysis

Figures and fittings utilized Microcal Origin 7.5 (Northampton, MA). Current–voltage relationships were fitted with the modified Boltzmann equation: 

\[ I = \frac{G_{\text{max}} \times (V_m - E_{\text{rev}})}{1 + \exp \left( \frac{V_m - V_{\text{rev}}}{k_v} \right)} \]

where \(V_m\) is the test potential, \(V_{\text{rev}}\) is the half-activation potential, \(E_{\text{rev}}\) is the extrapolated reversal potential, \(G_{\text{max}}\) is the maximum slope conductance, and \(k_v\) reflects the slope of the activation curve. Data from concentration dependence studies were fitted with the equation 

\[ y = [A_1 - A_2] \left(1 + \left(\frac{X}{C^*}\right)^{k_v}\right) + A_2 \]

where \(A_1\) is initial amplitude.
potential \(V_{50}\); \(-110\) mV) that would place the channels largely in the closed state. Somewhat surprisingly, even at relatively high concentrations, Z123212 caused minimal inhibition of recombinant CaV3.2 channels when activated from the closed state (IC\(_{50} > 10 \) \(\mu\)M, Fig. 2A and B). We assessed the effects of Z123212 on other CaV3.2 channel properties and found that 10 \(\mu\)M Z123212 also had no significant (\(P > .05\)) effect on the voltage dependence of fast channel inactivation (control, \(V_{1/2FastInact} = -61 \pm 1\) mV, \(n = 5\); 10 \(\mu\)M; Z123212, \(V_{1/2FastInact} = -66 \pm 3\) mV, \(n = 4\); data not shown). As previously observed for CaV3.1 channels [17], we next examined whether CaV3.2 channels undergo a slow inactivation process (Fig. 2C). Z123212 (10 \(\mu\)M) significantly (\(P < .05\)) increased the extent of CaV3.2 channel slow inactivation at specific membrane potentials and also caused a significant 6 mV hyperpolarizing shift in the voltage dependence of CaV3.2 channel slow inactivation (Fig. 2C; \(P < .05\)). The recovery from inactivation

3. Results

3.1. Design and synthesis of Z123212

Given the well-documented roles of CaV3.2 T-type channels and Na\(_V\) channels in modulating the excitability of DRG neurons, we set out to rationally design a mixed ion channel blocker that may have synergistic effects in attenuating pain signaling. As part of a rationale scaffold-based design and screening program to develop subtype-selective N-type and T-type Ca\(_V\) blockers, the small organic compound Z121912 (molecular weight = 521; Fig. 1) was initially designed on the basis of previous backbones identified as exhibiting promising Ca\(_V\) blocking and preclinical characteristics [32,47]. In particular, the bis-C\(_{2}\) aryl amide group of Z121912 was a preferred structural feature from the aspect of T-type Ca\(_V\) blocking potency (IC\(_{50} = 64\) nM). However, from a drug discovery perspective Z121912 possessed potential cardiovascular liability in that it also potently blocked the hERG potassium channel (IC\(_{50} \sim 100\) nM). In order to reduce the hERG liability, the benzhydro group of Z121912 was either removed (Z123875; molecular weight = 355) or replaced with an oxygenated piperazine (Z123212; molecular weight = 369; Fig. 1; Suppl. Fig. 1). Both derivatives exhibited significantly improved profiles against the hERG potassium channel (IC\(_{50} > 10\) \(\mu\)M) with Z123212 being selected for further preclinical assessment on the basis of its favorable pharmacokinetic properties.

3.2. Z123212 stabilizes the slow-inactivated state of CaV3.2 T-type channels

The blocking activity of Z123212 was initially tested against recombinant CaV3.2 channels expressed in HEK cells by using standard depolarizing test pulses from a hyperpolarized holding potential.

**Fig. 2.** Z123212 selectively alters the slow-inactivated state of CaV3.2 channels. (A) Representative traces of recombinant CaV3.2 channels during depolarizing steps from \(-110\) mV to \(-30\) mV demonstrating that perfusion of 10 \(\mu\)M Z123212 caused minimal inhibition of CaV3.2 channels when activated from hyperpolarized potentials. Scale bar: \(x = 20\) ms, \(y = 500\) pA. (B) Average time course of normalized CaV3.2 channel peak current values before and during perfusion of 10 \(\mu\)M Z123212 (\(n = 11\)). (C) Z123212 (10 \(\mu\)M) caused a significant (\(P < .05\)) hyperpolarizing shift in the voltage dependence of CaV3.2 channel slow inactivation (control, \(V_{1/2SlowInact} = -74 \pm 1\) mV, \(n = 4\); 10 \(\mu\)M Z123212, \(V_{1/2SlowInact} = -80 \pm 2\) mV, \(n = 5\)). Z123212 also caused a significant (\(P < .05\)) enhancement of the extent of Na\(_V\) channel slow inactivation at potentials of \(-80\), \(-70\), \(-60\), and \(-50\) mV. (D) Z123212 (10 \(\mu\)M) significantly (\(P < .005\), \(n = 4–5\)) slowed the recovery from CaV3.2 channel slow inactivation. Recordings in both (C) and (D) were unpaired and time-matched between control and Z123212 treatment groups to eliminate potential time-dependent changes in parameters. (E) Representative traces demonstrating that Z123212 selectively inhibited slow-inactivated T-type currents (P2 traces) in dissociated dorsal root ganglia (DRG) neurons. Scale bar: \(x = 5\) ms, \(y = 1000\) pA. (F) Average time course of the ratio of P2 peak current P1 peak current demonstrating that Z123212 increases the extent of T-type channel slow inactivation in DRG neurons (\(n = 4\)). Insets illustrate voltage step waveforms.

**Fig. 1.** Structural features of Z123212. Z123875 and Z123212 are derivatives of the high-affinity piperazine T-type antagonist, Z121912. The dipeptide backbone of Z123212 is highlighted in red.
parameter for CaV3.2 channels has been linked to changes in neuronal membrane excitability [33]; thus, we tested the effect of Z123212 on CaV3.2 recovery from slow inactivation. Application of Z123212 significantly (P < .01) slowed the recovery from CaV3.2 channel slow inactivation at concentrations of 3 μM and greater (control, τrecov = 510 ± 30 ms, n = 4; 3 μM: Z123212, τrecov = 650 ± 20 ms, n = 4; 10 μM: Z123212, τrecov = 680 ± 20 ms, n = 5; Fig. 2D). The CaV3.2 T-type channel isoform mediates the majority of whole cell T-type current within DRG neurons [1,6,8,42]; thus, we also tested the effects of Z123212 on T-type currents within isolated DRG neurons. Consistent with the recombinant CaV3.2 results, Z123212 (10 μM) was found to selectively stabilize the slow-inactivated state of T-type currents in isolated DRG neurons (Fig. 2E and F).

3.3. Z123212 inhibits recombinant NaV and CaV channels by modulating slow inactivation

Both TTX-sensitive NaV1.7 and TTX-resistant NaV1.8 channels are highly expressed in DRG neurons and exhibit overlapping distributions and functional roles with CaV3.2 T-type channels [10,19]. Hypothesizing that Z123212 may also alter NaV channel activity, we tested whether Z123212 could inhibit slow-inactivated recombinant NaV channels. In order to induce slow inactivation, sweeps were elicited every 30 s that included a 10-s conditioning pulse to −20 mV, followed by a short hyperpolarizing step to remove fast inactivation and then a depolarizing test pulse (P2; as described in [40]). We found that perfusion of Z123212 caused a robust inhibition of both recombinant NaV1.7 and NaV1.8 P2 currents that was even greater than that observed for CaV3.2 channels (Fig. 3A and B). Concentration–dependence response studies revealed that Z123212 inhibited NaV1.7 channels with an IC50 = 17 μM and NaV1.8 channels with an IC50 = 9.2 μM (Fig. 3C).

Because Z123212 stabilized the slow-inactivated state of CaV3.2 T-type and NaV1.7/NaV1.8 channels, it was of interest to test whether it also acted on other NaV and CaV channel classes. Similar to that for CaV3.2 T-type and NaV1.7/NaV1.8 channels, application of 10 μM Z123212 did not cause significant tonic block of other CaV and NaV isoforms tested (Suppl. Fig. 2). In contrast, 10 μM Z123212 was shown to selectively stabilize the putative slow-inactivated states of exogenously expressed CaV1.2 (L-type), CaV3.1 (T-type), CaV3.3 (T-type), and NaV1.5 channels, with an inhibition of P2 currents (after 10-s depolarizing conditioning pulses) ranging from ~30% to 60%. However, Z123212 did not uniformly alter slow inactivation states as the compound had no effect (~15% P2 inhibition) on the CaV2.1 (P/Q-type) and CaV2.2 (N-type) isoforms (Suppl. Fig. 2). Taken together, Z123212 selectively stabilizes the slow-inactivated states of a subset of ion channel types and does not seem to act as a tonic channel blocker. The selective action of Z123212 on channel slow inactivation may be of particular relevance to the putative hyperexcitable processes associated with various pain states compared to ion channel functioning during normal physiological processes. For example, cardiac NaV1.5 channels are adapted to have reduced slow inactivation during the repetitive (>1 Hz) and prolonged (~200 ms) depolarizations that occur during AP firing of cardiac myocytes [36]. In support, although Z123212 is able to stabilize the slow-inactivated state of NaV1.5 channels under certain experimental conditions (involving step depolarization to −20 mV for 10 s; Suppl. Fig. 3A), its effects on NaV1.5 channels activated by simulated cardiac AP waveforms is greatly reduced (Suppl. Fig. 3B).

3.4. Z123212 selectively stabilizes the slow-inactivated state of TTX-sensitive NaV channels in lamina I/II spinal cord neurons

We next set out to determine whether Z123212 could alter native NaV channels implicated in the nociceptive signaling pathway selectively through its effect on slow inactivation. Voltage-clamp recordings on lamina I/II neurons from spinal cord slices were performed to examine the effects of Z123212 on TTX-sensitive NaV currents in nociceptive spinal cord neurons. In order to ensure adequate voltage-clamp of NaV currents, the ESI technique pioneered by Safronov et al. [38] was used to remove healthy lamina I/II neurons from the slice surface (see Section 2.6 and Suppl. Fig. 4). In this recording configuration, NaV currents in lamina I/II neurons were completely blocked by TTX (data not shown; see [38]). Similar to that for recombinant NaV and CaV channels, application of 10 μM Z123212 did not result in tonic block of native TTX-sensitive NaV currents during depolarizations from a hyperpolarized state (Vhold = −100 mV; Fig. 4A and B). The perfusion of 10 μM Z123212 did result in a small (~2.4 ± 0.5 mV, n = 5) but significant (P < .01) hyperpolarizing shift in the voltage dependence of NaV channel activation (Fig. 4A); however, time-dependent negative shifts in the voltage dependence of activation were also observed during control recordings (data not shown). Thus, this small hyperpolarizing shift was likely not mediated by Z123212.

Application of 10 μM Z123212 also had no effect on the voltage dependence of NaV channel fast inactivation (100-ms conditioning pulses, Fig. 4C). The voltage dependence of NaV channel slow inactivation could not be directly assayed by using the native recording system because the neurons would not tolerate the highly hyperpolarized holding potential (Vhold = −120 mV) required to allow recovery from slow inactivation between pulses. However, during slow inactivation-inducing sweeps, perfusion of 10 μM Z123212 caused a robust reduction in the amplitude of lamina I/II neuron NaV currents during P2 pulses by 45 ± 7% (n = 5; P < .02; Fig. 4D). Analysis of NaV current amplitudes in P1 control pulses versus P2 test pulses further demonstrated that Z123212 selectively stabilized the slow-inactivated state of native TTX-sensitive NaV.
channels (Suppl. Fig. 5). The inhibition by Z123212 of NaV channels reaching the slow-inactivated state (P2) was significant \((P < .05)\) at concentrations of 3 \(\mu M\) and higher, with an \(IC_{50} = 13 \mu M\) \((\text{Fig. 4E})\).

3.5. Z123212 selectively stabilizes the slow-inactivated state of TTX-resistant NaV channels in peripheral nociceptors

The majority of TTX-resistant NaV current in nociceptive DRG neurons is composed of the NaV1.8 channel isoform \([10]\). We next evaluated the effects of Z123212 on pharmacologically isolated TTX-resistant currents in dissociated small diameter DRG neurons. Similar to that for TTX-sensitive NaV currents, perfusion of 10 \(\mu M\) Z123212 did not result in tonic block of native TTX-resistant NaV currents when depolarized from a relatively hyperpolarized potential \((V_{\text{hold}} = -70 \text{ mV}); \text{Fig. 5A} \text{ and B}) and also had no significant \((P > .05)\) effect on the voltage dependence of NaV channel activation \((\text{Fig. 5A})\). Further, while application of 10 \(\mu M\) Z123212 did not affect the voltage dependence of fast channel inactivation \((\text{Fig. 5C})\), it significantly \((P < .05)\) enhanced the fraction of NaV channels that reached the slow-inactivated state during conditioning pre-pulses of \(-20 \text{ mV}\) (as well as more depolarized potentials; \text{Fig. 5D}).

3.6. Z123212 reduces the excitability of peripheral nociceptors and second order spinal cord neurons

Because Z123212 can modulate the activity of several NaV and CaV channel isoforms linked to neuronal excitability, we used current-clamp recordings to test for possible direct effects of Z123212 on the overall excitability of both DRG and spinal cord lamina I/II neurons. Current-clamp recordings were initially performed on dissociated small diameter \((25 \pm 3 \text{ pF, } n = 6)\) DRG neurons from neonatal rats. A 350-ms depolarizing current injection step \((-220 \pm 120 \text{ pA, } n = 6)\) was used to elicit 4–6 APs (\text{Fig. 6A}), and this sweep was repeated every 30 s to ensure that a stable baseline number of APs was reached before applying compound \((\text{Fig. 6B})\). Subsequent perfusion of 10 \(\mu M\) Z123212 was found to significantly \((P < .01)\) reduce the number of APs elicited during the depolarizing pulse by \(60 \pm 9\%\) \((n = 6; \text{Fig. 6C})\).
Current-clamp recordings were also performed on intact tonic firing lamina I/II neurons in parasagittal lumbar spinal cord slices from juvenile rats [34]. A current–voltage relationship was repeated every 2 min with 1200 ms hyperpolarizing/dépolarizing current injection steps ranging from −50 pA to +80 pA in +10 pA increments. From this IV relationship, the effect of Z123212 on the number of elicited APs was analyzed for a moderate depolarizing current injection step (40 ± 6 pA, n = 7) that caused only a minor decay in AP amplitude (15 ± 2%, n = 5) over the entire train but still elicited a robust number of APs (16 ± 2, n = 7). Control recordings demonstrated no time-dependent changes in the number of APs during the moderate depolarizing steps, whereas the number of APs decreased with time for depolarizing current injection steps of greater magnitude (data not shown). Application of 10 μM Z123212 resulted in a 47 ± 12% (n = 7) decrease in AP firing during the moderate depolarizing steps (Fig. 7A), while membrane properties including input resistance (R\text{m}) and V\text{rest} remained unchanged (control, R\text{m} = 490 ± 60 MΩ, n = 5, V\text{rest} = −63 ± 2 mV, n = 7; 10 μM Z123212, R\text{m} = 460 ± 50 MΩ, n = 5, V\text{rest} = −63 ± 2 mV, n = 7). The inhibition of AP firing by Z123212 was concentration dependent with an IC\text{50} = 480 nM (Fig. 7C).

Lacosamide is an anticonvulsant that shares some structural features with Z123212 and has been reported to specifically stabilize the slow inactivation of Na\text{v} channels [4,12,40]. When tested in the spinal cord slice preparation, lacosamide inhibited lamina I/II neuron AP firing approximately 300 times less potently (IC\text{50} = 150 μM) than Z123212 (Fig. 7C). Lacosamide did reach a higher level of AP reduction than Z123212, but only at concentrations (>100 μM) well above therapeutic plasma levels for this agent (see Fig. 7C and [4]).

Z123212 reduced AP firing in lamina I/II neurons under conditions where V\text{rest} remained unaltered by tonic current injection and the depolarizing current injection steps under analysis followed depolarizing current injection steps using an IV protocol. Thus, Z123212 may exert its inhibitory effects on AP firing by stabilizing accumulated slow inactivation of native Na\text{v} and Ca\text{v} channels. In support, repeating the above experiments during tonic hyperpolarizing current injection to elicit a more hyperpolarized V\text{rest} (−86 ± 3 mV, n = 3) completely eliminated the inhibitory effect of Z123212 (Fig. 7B).

3.7. Z123212 effectively reverses multiple pain modalities

We next tested whether the highly state-dependent mechanism of action of Z123212 on nociceptor/spinal cord neuron inhibition translated to an ability to reverse behavioral hypersensitivity in animal models of pain. Oral administration of Z123212 (30 mg/kg) was shown to attenuate both acute thermal hypersensitivity by using the hot-plate test and to a greater extent, chronic mechanical and thermal hypersensitivity assessed using the SNL-induced model of neuropathic pain (Fig. 8). More specifically, Z123212 significantly (P < .05) reversed both tactile allodynia
**4. Discussion**

We report the design, synthesis and functional characterization of a novel small organic agent (Z123212) that uniquely stabilizes the slow-inactivated state of a subset of NaV and CaV channels. The data suggest that by enhancing the slow inactivation of a combination of TTX-sensitive and TTX-resistant NaV and T-type CaV currents, Z123212 reduces AP firing in peripheral DRG and lamina I/II spinal cord neurons. We predict that this highly state-specific mechanism underlies the ability of orally administered Z123212 to significantly attenuate thermal and mechanical hypersensitivity in rodent models of both chronic neuropathic pain and acute pain.

**4.1. Mechanism underlying pain-attenuating effects of Z123212**

Z123212 inhibits certain NaV and CaV channels by selectively stabilizing the slow-inactivated state over the fast inactivated state. As channel slow inactivation is significantly enhanced during prolonged depolarizations (eg, −50 mV for 10 s; Fig. 4), Z123212 is predicted to cause pronounced inhibition of NaV and CaV channels in neurons that are either tonically depolarized or firing in bursts that create sustained depolarizations. In this regard, we observed a Z123212-mediated reduction in AP firing in lamina I/II neurons during prolonged depolarizations from rest (in slices where both excitatory and inhibitory synaptic inputs are blocked) but not when the neurons were tonically hyperpolarized (Fig. 7). During chronic neuropathic pain states, dorsal horn spinal cord neurons can become tonically depolarized and/or exhibit prolonged periods...
of fast depolarized firing due to changes in inhibitory and excitatory inputs [41,48], the remodeling of ion channel expression [26], and alteration of electrical gradients [22]. Z123212 exhibited somewhat greater antinociceptive effects in models of neuropathic pain compared to acute pain (Fig. 4), and we predict that this may be due to the ability of Z123212 to preferentially attenuate hyperexcited neurons within the peripheral and central pain pathways. Future in vivo experiments could verify this by examining the effects of Z123212 on peripheral and spinal cord neurons from animals with enhanced sensitivity for pain [3].

4.2. Molecular targets of Z123212 action

Relevant to nociceptive signaling, Z123212 was found to stabilize the slow inactivation of recombinant TTX-sensitive NaV1.7 channels as well as recombinant TTX-resistant NaV1.8 channels. Both NaV isoforms are highly expressed and involved in modulating excitability within peripheral nociceptors [10], and we directly demonstrated that Z123212 inhibits endogenous TTX-resistant NaV current within DRG neurons. Slow inactivation is induced at potentials near neuronal resting membrane potentials (Figs. 3 and 5; [40]) for both NaV1.7 and NaV1.8 isoforms. In this regard, Z123212 likely reduces DRG excitability by acting on multiple NaV channel isoforms, which may also include other prominently expressed DRG NaV isoforms such as NaV1.1, NaV1.6, and NaV1.9 [10].

To determine how Z123212 influences neurons downstream of nociceptors in the pain pathway, we evaluated the effects of Z123212 on uncontaminated TTX-sensitive NaV currents from spinal cord lamina I/II neurons using the ESI recording technique [38]. Z123212 inhibited lamina I/II NaV currents via their slow-inactivated state with a similar potency compared to both recombinant NaV1.7/NaV1.8 currents and DRG TTX-resistant NaV currents (IC50 values between 9 and 17 μM). Lamina I/II neurons have been shown to contain several functional components of TTX-sensitive NaV currents [38], although the exact NaV channel isoforms involved have not been thoroughly explored. As Z123212 stabilizes the slow-inactivated state of functionally distinct NaV isoforms, the observed reduction of lamina I/II neuronal excitability may be mediated by one or multiple NaV isoforms. It is known that the expression of NaV1.3 channel protein is upregulated within dorsal horn neurons in both peripheral and central neuropathic pain models [14,15] and that this isoform (along with NaV1.2) is localized within lamina I/II of the spinal cord [13] (our unpublished observations). In this regard, Z123212 may in part reduce neuropathic pain signaling by enhancing the natural brakes (slow inactivation) of aberrantly expressed NaV1.3 channels in dorsal horn neurons. This could be tested in future studies by examining the effects of Z123212 on recombinant NaV1.3 channels as well as endogenous NaV currents from rats with enhanced sensitivity to pain.

T-type CaV channels are functionally expressed and implicated in modulating the excitability of both DRG and lamina 1 spinal cord neurons [20,31,37,39]. More specifically, CaV3.2 T-type channels have been directly linked to hyperalgesia and allodynia in various pain animal models [1,6,9,21,29]. We characterized the slow inactivation properties of recombinant CaV3.2 channels for the first time and found properties consistent with those previously described for CaV3.1 channels (Fig. 2C; [171]). Z123212 significantly altered the voltage dependence and extent of CaV3.2 slow inactivation as well as the recovery from slow inactivation (Fig. 2). Given the contributions of CaV3.2 T-type channels toward nociceptive signaling, the effects of Z123212 observed on AP firing, hyperalgesia and allodynia may be partly mediated by interactions with CaV3.2 channels. A definitive role for functionally expressed CaV3.3 channels in peripheral and central nociception pathways remains to be elucidated. In contrast, attenuation of central CaV3.1 channel activity has actually been shown to be pronociceptive [23]; thus, in the central nervous system at least Z123212 is unlikely to induce antinociceptive effects through this T-type isoform.

It has been previously shown that NaV and CaV currents act synergistically to prolong subthreshold depolarizations within lamina I neurons [35], which may account for the greater effect of Z123212 on lamina I/II neuron excitability compared to its individual effects on specific NaV and CaV3.2 channel isoforms. Taken together, we predict that Z123212 exerts its effects on neuronal excitability and nociceptive signaling by enhancing the combined slow inactivation of multiple pronociceptive NaV and CaV channel isoforms.

Z123212 shares some structural features with lacosamide (the dipeptide backbone highlighted in Fig. 1), an antiepileptic drug shown to attenuate chronic pain and enhance slow inactivation of NaV1.3, NaV1.7, and TTX-resistant DRG NaV currents [40]. To date, the effects of lacosamide on in situ neuronal firing patterns have only been characterized for cultured neocortical neurons [12]. We find that lacosamide reduces the AP firing of lamina I/II spinal cord neurons at high micromolar concentrations (IC50 = 150 μM; Fig. 7). In the lamina I/II neuron preparation, Z123212 inhibits AP firing approximately 300 times more potently than lacosamide (IC50 = 0.48 μM; Fig. 7). The concentration of lacosamide shown to alter neuronal excitability and affect NaV channel slow inactivation (predominantly 100 μM and above; Suppl. Fig. 6) are generally beyond therapeutic plasma levels achieved by oral dosing (10–60 μM) [4,12,40]. Of note, a recent study showed that direct systemic injection of lacosamide could reduce evoked dorsal horn neuronal responses in vivo [3]. Lacosamide also binds to the signaling protein collapsin-response mediator protein 2 (affinity ~5 μM) involved in neuroprotection and axonal remodeling, and it is currently unclear whether the effects of lacosamide on NaV channel slow inactivation are directly linked to its antinociceptive properties [4]. We find that Z123212 reduces lamina I/II neuronal excitability and enhances NaV channel slow inactivation at concentrations (1–3 μM) that are within both therapeutic plasma and brain tissue levels (10–17 μM and 3–5 μM, respectively). The ability of Z123212 to target multiple mechanistic elements that contribute to neuronal hyperexcitability by stabilizing the slow-inactivated state of both NaV and CaV channels might create an additive effect not previously demonstrated for lacosamide.

4.3. Potential development of novel mixed NaV/CaV channel therapeutics

We have identified Z123212 as the first dual modulator of NaV/CaV channel slow inactivation and have shown that it is efficacious in reversing mechanical and thermal hypersensitivity in animal models of pain. A number of currently marketed therapeutics nonspecifically inhibit T-type CaV channel isoforms (eg, phenytoin and ethosuximide), and mechanistically, blockade occurs through the channel resting state [2,43]. These compounds also nonspecifically block NaV channels and act on other molecular targets [27,28]. Z123212 represents a novel class of small organic blocker that acts across the NaV and CaV ion channel families but specifically targets the slow-inactivated state. We predict that the specificity for affecting channel slow inactivation could enable the preferential targeting of channels associated with pathophysiological states linked to hyperexcitability (eg, epilepsy and neuropathic pain). In support, although Z123212 also affects NaV1.5 and CaV3.2 slow inactivation under certain experimental conditions, we did not find off-target cardiovascular effects in isolated rabbit hearts. Further, we did not observe any adverse effects of high doses of Z123212 in regards to motor coordination.
Conflict of interest statement

Zalics Pharmaceuticals is a subsidiary of Zalics Inc, Cambridge, MA. Paula Smith, Cyprus Eduljee, Janette Mezeyova, Molly Fee-Maki, Yongbao Zhu, Francesco Belardetti, Hassan Pajouhesh, David Parker, Manjeet Parmar, Elizabeth Tringham, Gerald Zamponi and Terrance P. Snutch all hold shares and/or options in Zalics Inc.

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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.pain.2010.12.035.

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