Neurobiology of Pain 11 (2022) 100082

Contents lists available at ScienceDirect

Neurobiology of Pain

journal homepage: www.sciencedirect.com/journal/neurobiology-of-pain

Short Communication

Small molecule targeting NaV1.7 via inhibition of the CRMP2-Ubc9 interaction reduces and prevents pain chronification in a mouse model of oxaliplatin-induced neuropathic pain

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ARTICLE INFO

Keywords:
Oxaliplatin
Chemotherapy
Neuropathy
NaV1.7
CRMP2
SUMOylation

ABSTRACT

Treatment with anti-neoplastic agents can lead to the development of chemotherapy induced peripheral neuropathy (CIPN), which is long lasting and often refractory to treatment. This neuropathic pain develops along dermatomes innervated by peripheral nerves with cell bodies located in the dorsal root ganglia (DRG). The voltage-gated sodium channel NaV1.7 is expressed at high levels in peripheral nerve tissues and has been implicated in the development of CIPN. Efforts to develop novel analgesics directly inhibiting NaV1.7 have been unsuccessful, and our group has pioneered an alternative approach based on indirect modulation of channel trafficking by the accessory protein collapsin response mediator protein 2 (CRMP2). We have recently reported a small molecule, compound 194, that inhibits CRMP2 SUMOylation by the E2 SUMO-conjugating enzyme Ubc9 (Cai et al. , Sci. Transl. Med. 2021 13(619):eabh1314). Compound 194 is a potent and selective inhibitor of NaV1.7 currents in DRG neurons and reverses mechanical allodynia in models of surgical, inflammatory, and neuropathic pain, including spared nerve injury and paclitaxelinduced peripheral neuropathy. Here we report that, in addition to its reported effects in rats, 194 also reduces mechanical allodynia in male CD-1 mice treated with platinumcomplex agent oxaliplatin. Importantly, treatment with 194 prevented the development of mechanical allodynia when co-administered with oxaliplatin. No effects were observed on the body weight of animals treated with oxaliplatin or 194 throughout the study period. These findings support the notion that 194 is a robust inhibitor of CIPN that reduces established neuropathic pain and prevents the emergence of neuropathic pain during treatment with multiple anti-neoplastic agents in both mice and rats.

Introduction

Therapeutic agents used to treat cancers, especially those belonging to the platinum-complex, taxane, vinca alkaloid, and proteosome inhibitor classes are known to cause chronic peripheral neuropathy that can last for years following treatment (Gadgil et al., 2019; Xiao et al., 2012). These patients are left with a debilitating pain condition, known as chemotherapy induced peripheral neuropathy (CIPN), which is often refractory to treatment with available analgesics (Gordon-Williams and Farquhar-Smith, 2020). Patients that develop CIPN present to the clinic with sensory, motor, and autonomic deficits that develop in a characteristic glove and stocking pattern due to differential toxicity among

Abbreviations: CIPN, chemotherapy induced peripheral neuropathy; DRG, dorsal root ganglia; CRMP2, collapsin response mediator protein 2; NaV1.7, voltage-gated sodium channel family 1 isoform 7; TTX, tetrodotoxin; TTX-S, tetrodotoxin-sensitive; TTX-R, tetrodotoxin-resistant; SUMO, smallubiquitin like modifier; Ubc9, E2 SUMO-conjugating enzyme; SNI, spared nerve injury; t-CSM, tat-CRMP2 SUMOylation motif; PWT, paw withdrawal threshold; CRISPR, clustered regularly interspaced short palindromic repeats.

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https://doi.org/10.1016/j.ynpai.2021.100082
Received 7 December 2021; Received in revised form 21 December 2021; Accepted 21 December 2021
Available online 27 December 2021

neurons with longer axons (Starobova and Vetter, 2017). Given the high burden associated with neuropathy following treatment with anti-neoplastic agents, identification of new compounds that can prevent or reverse this type of pain is essential.

Peripheral pain detecting neurons, known as nociceptors, have cell bodies located in the dorsal root ganglia (DRG) with bifurcated projections innervating the skin and the dorsal horn of the spinal cord (Woolf and Ma, 2007). Neurons of the DRG are particularly sensitive to damage following treatment with chemotherapeutic drugs and demonstrate accumulation of these agents following repeated administration (Jimenez-Andrade et al., 2008). This increased exposure of DRG neurons is thought to be a result of significant vascularization of these structures (Cavaletti et al., 2000). The voltage-gated sodium channel NaV1.7 is found primarily in peripheral tissues, including DRG neurons, and its expression is upregulated in multiple pain states (Herzog et al., 2003; Mukai et al., 2014; Sangameswaran et al., 1997; Siqueira et al., 2009; Toledo-Aral et al., 1997). The biophysical properties of NaV1.7 position this channel to amplify subthreshold stimuli and ultimately determine the threshold for action potential generation in nociceptors (Dib-Hajj et al., 2013). This critical role in pain sensation is exemplified by human pain conditions where NaV1.7 gain of function mutations have been linked to severe pain disorders and loss of function mutations are associated with insensitivity to pain (Cox et al., 2006; Yang et al., 2004).

In addition to its involvement in human genetic pain disorders, recent evidence has emerged suggesting that NaV1.7 is also important in the pathophysiology of CIPN. Treatment with the chemotherapeutic agent paclitaxel increases expression of NaV1.7 in rat DRG neurons, which correlated with onset of mechanical allodynia (Wang et al., 2020; Xiao et al., 2016). Cultured rat DRG neurons treated with paclitaxel displayed increased NaV1.7 membrane localization and increased vesicular transport of the channel to distal axonal terminals (Akin et al., 2021). Furthermore, silencing NaV1.7 expression using either a zinc finger protein or a CRISPR construct targeting *Scn9a*, the gene encoding NaV1.7, resulted in robust reversal of the neuropathic pain phenotype in mice with CIPN (Moreno et al., 2021). Interestingly, another report identified a single nucleotide polymorphism in a Japanese family that predisposed patients to development of CIPN following treatment with drugs in the taxane class (Tanabe et al., 2020). These findings support a crucial role for NaV1.7 in mediating development of sensory neuropathy following treatment with anti-neoplastic agents and suggests targeting NaV1.7 is a viable strategy for ameliorating CIPN associated pain.

While the essential role for NaV1.7 in pain transmission is clear and its involvement in CIPN is emerging, targeting NaV1.7 for analgesia has proven difficult. Many attempts have been made to directly inhibit channel function, leading to the development of small molecule inhibitors, as well as venom derived peptides, that are highly potent (Deuis et al., 2017; McCormack et al., 2013). This approach has produced candidate compounds displaying profound selectivity for human NaV1.7 over other channel isoforms (Alexandrou et al., 2016; Theile et al., 2016). Despite this, clinical trials of direct NaV1.7 inhibiting compounds have largely failed to demonstrate analgesic efficacy (Siebenga et al., 2020). Recent reports have suggested that failure in translation from preclinical studies to the clinic could be related to the homogeneity of pain models used to evaluate novel analgesics, which fail to capture the heterogenous disease mechanisms in patients (Berge, 2011; Eagles et al., 2020). Furthermore, a lack of diversity in the species used to test these candidate compounds in the preclinical stage could also contribute to the high attrition rates observed (Sadler et al., 2021). The failure in direct NaV1.7 inhibitors has led our group to develop a different strategy focused on disruption of protein–protein interaction networks to alter channel trafficking to relieve pain (Chew et al., 2019; Chew and Khanna, 2018).

Exploration of NaV1.7 interacting partners using a mouse expressing an epitope tagged NaV1.7 revealed an interaction between NaV1.7 and the cytosolic phosphoprotein collapsin response mediator protein 2 (CRMP2) (Chew et al., 2019; Kanellopoulos et al., 2018). Previous work from our group suggested that CRMP2 could regulate specific channel populations since knockdown of CRMP2 expression in DRG neurons, using a validated siRNA, reduced TTX-S currents but had no effect on TTX-R currents (Dustrude et al., 2016). We posited that this specificity could arise from precise control of the post-translational modification state of CRMP2. One such modification includes the covalent addition of an ~ 11 kDa protein known as a small ubiquitin like modifier (SUMO) by the E2 SUMO-conjugating enzyme Ubc9 at target lysine residues (Flotho and Melchior, 2013; Moutal et al., 2019). There is an established role for SUMOylation dependent control of potassium channel inactivation, which indicates that SUMOylation can control ion channel function (Benson et al., 2009; Benson et al., 2007). We have shown that loss of CRMP2 SUMOylation *in vitro*, by substituting lysine at position 374 with alanine, specifically reduced NaV1.7 currents without affecting currents from other voltage-gated sodium channels (Dustrude et al., 2016; Dustrude et al., 2013). This reduction in sodium current density following loss of CRMP2 SUMOylation was due to clathrin-dependent internalization of the channel via recruitment of an endocytic complex (Dustrude et al., 2016; Gomez et al., 2021; Moutal et al., 2020). In neuropathic pain states, SUMOylation of CRMP2 is increased in the spinal cord, glabrous skin, and sciatic nerve (Moutal et al., 2018). Preventing CRMP2 SUMOylation with a cell penetrant interfering peptide, corresponding to the CRMP2 SUMOylation motif (t-CSM), was sufficient to reverse spared nerve injury (SNI) induced neuropathic pain (François-Moutal et al., 2018a). To further illustrate that SUMOylation was essential for NaV1.7 regulation in vivo we generated a SUMO-null transgenic mouse harboring a knock-in $CRMP2^{K374A/K374A}$ mutation (Moutal et al., 2020). These animals had reduced peak DRG sodium current density, reduced NaV1.7 membrane localization, and were resilient to the development of neuropathic pain. Collectively, these findings led us to conclude that CRMP2 SUMOylation serves as a regulatory node that controls NaV1.7 trafficking, which has implications for the development of novel therapeutics.

2. Methods

2.1. Animals

Pathogen-free adult male CD-1 mice (20–30 g; Envigo, USA) were used. Mice were housed 4–5 per cage in a light- and temperature-controlled room (12:12-h light–dark cycle, lights on at 6:00 am) with food and water available *ad libitum*. No animals were excluded from this study for any reason. All procedures were approved by the Saint Louis University School of Medicine Institutional Animal Care and Use Committee.

2.2. Chemotherapy induced peripheral neuropathic pain

Male ICR mice were treated daily for five consecutive days with oxaliplatin (3 mg/kg; i.p.) or vehicle (5% dextrose) between experimental days 0–4 and again between days 10–14 (Wahlman et al., 2018). The total cumulative dose was 30 mg/kg per mouse.

2.3. Drug administration

194 was prepared in the appropriate diluent [10% DMSO, (Sigma-Aldrich, St.Louis, MO) 10% tween-80 (Sigma-Aldrich) in saline (Hospira, Inc., Lake Forest, IL)] and administered by oral gavage. For prevention experiments, **194** (2 mg/kg) or vehicle control (10% DMSO, 10% Tween-80, 80% saline) was administered concurrently with oxaliplatin. For the reversal experiments, a single dose of 10 mg/kg **194** or vehicle was administered orally on day 25. An independent experimenter performed dosing to blind the behavioral experimenter to the treatment groups.

2.4. Mechanical allodynia

Mice were placed in elevated chambers (28 X 40 X 35 cm) placed on a wire mesh floor and allowed to acclimate for 30 min prior to behavioral testing. The mechanical paw withdrawal threshold in grams [PWT, (g)] was measured manually with von Frey filaments according to the up and down method (Dixon, 1980) [Stoelting, ranging from 2.36 (0.02 g) to 4.31 (2 g) bending force]. The development of mechanical allodynia is indicated by a significant ($p < 0.05$) reduction in mean absolute PWT (g) at forces that fail to elicit withdrawal responses before chemotherapy treatment (Day 0). Treatment with chemotherapeutic agents results in bilateral allodynia with no differences between the left and right paw withdrawal thresholds observed, therefore values for both paws were averaged to determine the PWT. Animals receiving chemotherapeutic agents in the presence or absence of **194** did not display signs of any toxicity: i.e., they exhibited normal posture, grooming, locomotor behavior, hair coat was normal, no signs of piloerection or ocular porphyrin discharge, and gained body weight normally, in a fashion that

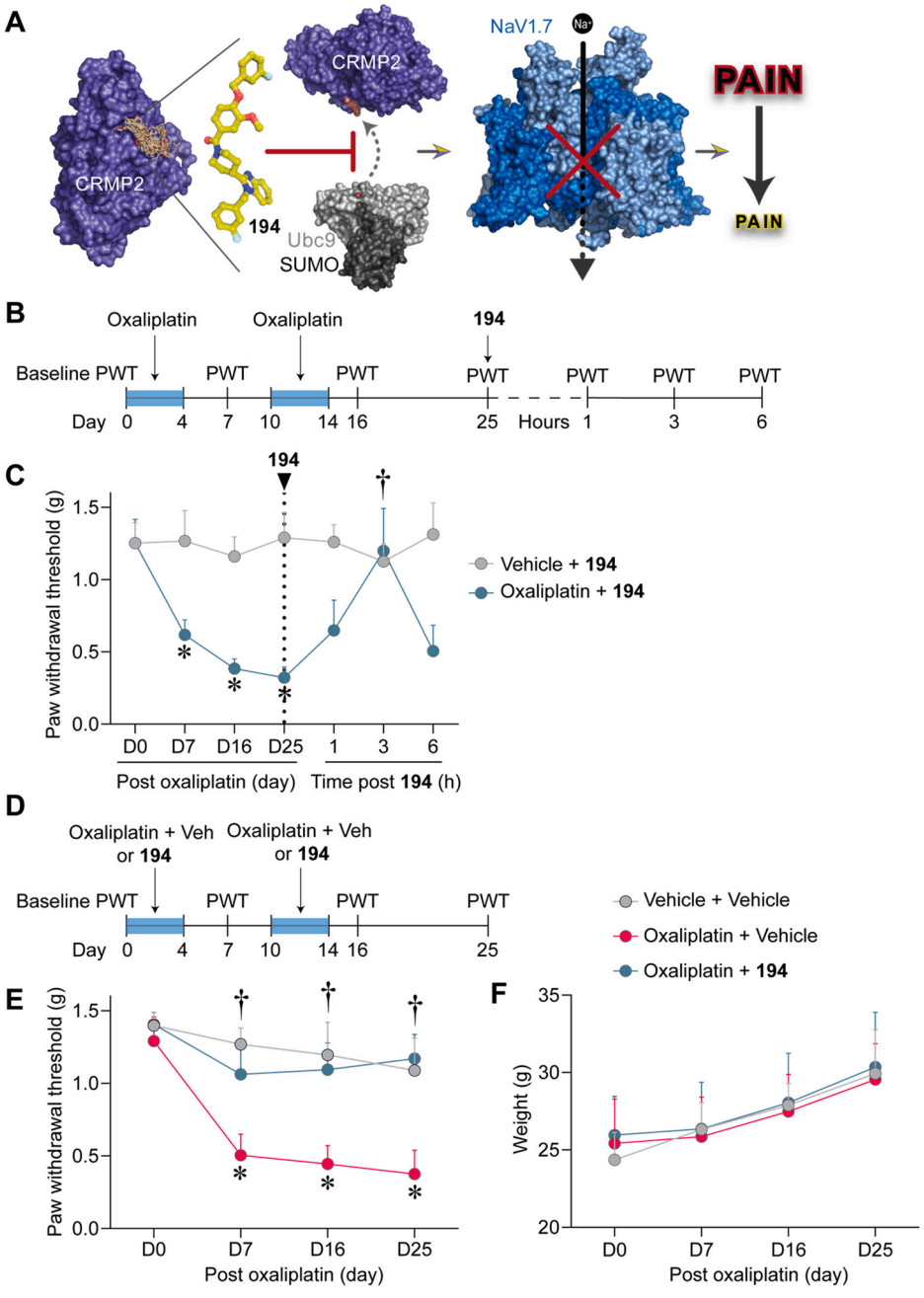

Fig. 1. 194 reduces and prevents the development of mechanical allodynia in male CD-1 mice with oxaliplatin induced peripheral neuropathy. (**A**) Compound **194** discovery and validation. **194** was designed using the structure–activity relationships of compounds obtained from a virtual screen against a pocket encompassing the SUMOylation target on CRMP2 (PDB 2GSE (Stenmark et al., 2007)). **194** effectively blocks SUMOylation of CRMP2 by the E2 SUMO-conjugating enzyme Ubc9 (PDB 5D2M (Cappadocia et al., 2015)) to reduce cell-surface trafficking of NaV1.7 (PDB 6J8H (Shen et al., 2019)). This results in dramatically reduced sodium currents and amelioration of pain in animal models. Graphic generated with BioRender. (**B**) Experimental design for behavioral assessment of oral administration of **194** on reversal of oxaliplatin induced CIPN. Baseline paw withdrawal threshold (PWT, in grams) was established at Day 0, followed by induction of CIPN with administration of oxaliplatin between Day 0–4 and Day 10–14 as indicated by the blue box. Mechanical allodynia was evaluated by measuring PWTs at regular intervals as indicated in the timeline. On day 25, mice were orally administered **194** and paw withdrawal thresholds were tested over six hours as indicated. (**C**) Mice treated with Vehicle and **194** displayed stable PWTs over the experimental time course (*gray*). Those treated with oxaliplatin developed robust mechanical allodynia that peaked at Day 25 (*blue*). Treatment with **194** completely reversed the oxaliplatin induced mechanical allodynia to vehicle treated levels 3 h following oral administration. Data are mean ± SD; Two-Way ANOVA with Bonferroni's multiple comparison *$p < 0.05$ vs D0; †$p < 0.05$ vs D25; Oxaliplatin + **194**n = 4; Vehicle + **194**n = 5. (**D**) Experimental outline of prevention experiments performed in male CD-1 mice. Baseline PWTs was first established at Day 0, followed by treatment with Vehicle + Vehicle, Oxaliplatin + Vehicle, or Oxaliplatin + **194**. PWTs were then assessed at regular intervals as indicated. Blue boxes indicate periods of treatment with oxaliplatin. (**E**) Mice treated with Vehicle + Vehicle had stable PWTs across the experimental time course (*gray*). Treatment with Oxaliplatin + Vehicle demonstrated robust mechanical allodynia in male mice (*pink*). Treatment with Oxaliplatin + **194** prevented the development of mechanical allodynia in these mice (*blue*). (**F**) Evaluation of the body weight of mice across the experimental period revealed no significant differences between groups of animals. Data are mean ± SD; Two-Way ANOVA with Bonferroni's multiple comparison †$p < 0.05$ vs Oxaliplatin + Vehicle; *$p < 0.05$ vs D0; Vehicle + Vehicle n = 4; Oxaliplatin n = 5 per group. Experimenters were blinded to the treatment conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was comparable to vehicle-treated mice.

2.5. Statistical analyses

All data was first tested for a Gaussian distribution using a D'Agostino-Pearson test (Prism 9 Software, Graphpad, San Diego, CA). All data was analyzed using the two-way ANOVA test followed by the Bonferroni test for multiple comparisons. Differences were considered significant if $p \leq 0.05$. Error bars in the graphs represent mean ± SD. All data were plotted in Prism 9.

3. Results and discussion

Encouraged by the findings from our studies using the t-CSM peptide (François-Moutal et al., 2018a) and the CRMP2$^{K374A/K374A}$ transgenic mouse (Moutal et al., 2020), we embarked upon a campaign to design a small molecule to disrupt the interaction between CRMP2 and Ubc9. We had previously identified key residues surrounding K374 in CRMP2, specifically R440 and V371, that were critical for interaction with Ubc9 and were therefore suitable for small molecule targeting (François-Moutal et al., 2018b). In silico screening of the CRMP2 interface that facilitates SUMO conjugation by Ubc9 identified a series of compounds that could potentially disrupt this interaction. Subsequent *in vitro* characterization of the top scoring compounds revealed a benzoylated 2-(4-piperidinyl)-1,3-benzimidazole analog capable of interfering with CRMP2 SUMOylation, which we refer to as compound **194** (Fig. 1**A**) (Cai et al., 2021; Kingwell, 2021). A recent report from our group outlines the promising characteristics of **194**, which shows excellent specificity for inhibiting NaV1.7 currents in cultured DRG neurons (Cai et al., 2021). Treatment with **194** reduced membrane localization of NaV1.7 in a clathrin-dependent manner and reduced presynaptic NaV1.7 in the spinal cord. Furthermore, treatment with compound **194** reversed mechanical allodynia following spared nerve injury (SNI) and after treatment with paclitaxel in male rats (Cai et al., 2021). Since vesicular trafficking and membrane localization of NaV1.7 are increased in CIPN, it is possible that treatment with **194** could normalize this dysregulated trafficking to restore normal levels of nociception.

Since multiple anti-neoplastic agents are employed in the clinic, it is important to establish that new analgesic candidates are effective in treating neuropathy induced by agents from multiple therapeutic classes. Considering the need for therapies for CIPN, and the importance of cross species validation, we asked whether **194** could reverse mechanical allodynia associated with CIPN in a similar fashion to what we observed previously in paclitaxel treated rats (Cai et al., 2021). Male mice were injected with oxaliplatin, between days 0–4 and 10–14, followed by assessment of paw withdrawal thresholds (Fig. 1**B**). These mice developed robust mechanical allodynia compared to mice that were not given oxaliplatin. On day 25, both groups of mice were given oral **194**, which completely reduced oxaliplatin induced mechanical allodynia 3 h after treatment (Fig. 1**C**). Treatment with **194** had no effect on animals treated with vehicle instead of oxaliplatin, suggesting no effect of **194** on basal mechanical sensation, which is in line with our previous observations (Cai et al., 2021).

We next asked whether **194** could prevent mechanical allodynia in mice treated with the platinum-complex agent, oxaliplatin. To answer whether **194** could prevent CIPN, male CD-1 mice were treated with oxaliplatin and co-administered oral **194** or a vehicle control followed by assessment of paw withdrawal thresholds (Fig. 1**D**). Animals co-administered oxaliplatin and vehicle, during days 0–4 and 10–14, developed robust mechanical allodynia, which was prevented in animals co-administered **194** (Fig. 1**E**). There were no differences observed between groups in the body weight of the animals assessed over the experimental time course (Fig. 1**F**).

Interpreting these findings in the context of our previous report we can conclude that **194** is a potent inhibitor of mechanical allodynia in preclinical models of CIPN. We have previously shown that **194** reversed mechanical allodynia in rats treated with paclitaxel (Cai et al., 2021). Here, we observed that **194** can reduce mechanical allodynia in male mice treated with oxaliplatin, indicating that this effect is not species specific nor is it restricted to a single class of anti-neoplastic agent. That the reduction lasted three hours can perhaps be attributed to the dose and/or the short half-life of 194. In contrast, we found that **194**, when given concurrently with oxaliplatin, prevented the development of mechanical allodynia, which points to a potential role as an adjuvant therapy in the clinic. Patients undergoing treatment for cancer with a neuropathy causing agent could be given **194** to decrease the possibility of developing CIPN. Furthermore, the fact that this compound is effective orally is promising for translation of these findings to the clinic (Yusof and Segall, 2013). It is important to note that we previously assessed the effects of **194** in male rats and here we used male mice. However, it is unlikely that a sex difference exists in response to treatment with **194** because we have previously shown that inhibition of CRMP2 SUMOylation is equally effective in both sexes (Moutal et al., 2020).

A imitation of our study is that we did not evaluate oxaliplatin-induced cold allodynia, which is regarded as an important clinical marker of oxaliplatin neurotoxicity and can be recapitulated in rodents. Among the ion channels contributing to cold perception include TREK1, TRAAK, Kv1.1, NaV1.8, and HCN (Descoeur et al., 2011), while NaV1.7 is thought to be dispensable for cold sensing (MacDonald et al., 2021). However, it remains to be determined whether **194** can block or reduce cold allodynia. This will be evaluated in future studies.

Conclusions

There is an emerging role for NaV1.7 in the CIPN literature supporting dysregulation of this channel in the pathogenesis of CIPN (Akin et al., 2021; Gordon-Williams and Farquhar-Smith, 2020; Li et al., 2018). Our previous work has shown that **194** is highly selective for NaV1.7, which means the effects we observed here are likely mediated by inhibition of NaV1.7 in affected DRG sensory neurons. This evidence adds weight to the conclusion that NaV1.7 is critically involved in CIPN and should be considered in future studies investigating the pathophysiology of CIPN. The findings reported here, as well as those of our prior work, positions **194** to make the transition to the clinic as a novel therapeutic candidate for the prevention and treatment of established CIPN.

Ethics approval and consent to participate

Not applicable.

Ethics approval for use of animals

Saint Louis University School of Medicine Institutional Animal Care and Use Committee sanctioned all experiments.

Consent for publication

Not applicable.

Availability of data and materials

Please contact author for data requests.

Funding

This work is supported by startup funds from St. Louis University to D.S. and grants from the National Institutes of Health awards to R.K. (NINDS (NS098772 and NS120663) and NIDA (DA042852).

Author Contributions

R.K. developed the concept of indirectly targeting NaV1.7.; D.S., and K.B. designed the experiments; K.B. collected and analyzed the data; R. K. provided funding; K.B., H.J.S., D.S., and R.K. wrote the manuscript; and D.S. supervised all experiments. All authors had the opportunity to discuss results and comment on the manuscript.

Declaration of Competing Interest

R. Khanna is the co-founder of Regulonix LLC, a company developing non-opioids drugs for chronic pain. In addition, R. Khanna 481 has patents US10287334 (Non-narcotic CRMP2 peptides targeting sodium channels for chronic 482 pain) and US10441586 (SUMOylation inhibitors and uses thereof) issued to Regulonix LLC. R. Khanna is also a co-founder of ElutheriaTx Inc., a company developing gene therapy approaches for chronic pain.

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