Inhibition of α9α10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain

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Opioids are first-line drugs for moderate to severe acute pain and cancer pain. However, these medications are associated with severe side effects, and whether they are efficacious in treatment of chronic nonmalignant pain remains controversial. Medications that act through alternative molecular mechanisms are critically needed. Antagonists of α9α10 nicotinic acetylcholine receptors (nAChRs) have been proposed as an important nonopioid mechanism based on studies demonstrating prevention of neuropathology after trauma-induced nerve injury. However, the key α9α10 ligands characterized to date are at least two orders of magnitude less potent on human vs. rodent nAChRs, limiting their translational application. Furthermore, an alternative proposal that these ligands achieve their beneficial effects by acting as agonists of $GABA_B$ receptors has caused confusion over whether blockade of α9α10 nAChRs is the fundamental underlying mechanism. To address these issues definitively, we developed RgIA4, a peptide that exhibits high potency for both human and rodent α9α10 nAChRs, and was at least 1,000-fold more selective for α9α10 nAChRs vs. all other molecular targets tested, including opioid and $GABA_B$ receptors. A daily s.c. dose of RgIA4 prevented chemotherapy-induced neuropathic pain in rats. In wild-type mice, oxaliplatin treatment produced cold allodynia that could be prevented by RgIA4. Additionally, in α9 KO mice, chemotherapy-induced development of cold allodynia was attenuated and the milder, temporary cold allodynia was not relieved by RgIA4. These findings establish blockade of α9-containing nAChRs as the basis for the efficacy of RgIA4, and that α9-containing nAChRs are a critical target for prevention of chronic cancer chemotherapy-induced neuropathic pain.

pain | chemotherapy | alpha9 | nicotinic

Opioids are first-line treatments for the treatment of acute postoperative and cancer pain. However, the unprecedented and epidemic use of opioids to treat chronic nonmalignant pain has resulted in a broad range of problems, including an estimated 12 million Americans who currently abuse or are addicted to prescription opioids, with tens of thousands of overdose deaths (1, 2). To deal with this crisis, recent US Health and Human Services efforts have targeted physician-prescribing practices, medication-assisted treatments for addiction, and distribution of opioid antagonist kits to enable attempts to reverse opioid overdose, with billions of dollars expended annually in these efforts (2, 3). In the long term, however, greater impact on therapy would arise from discovery and development of medications based on nonopioid mechanisms that can effectively treat chronic pain, and that may additionally have disease-modifying actions on chronic pain. To facilitate this goal, new molecular targets are urgently needed. In the past 50 y, however, very few new pain drugs with novel nonopioid receptor-based mechanisms have been approved.

In the search for new molecular targets, a standard approach is to use molecular genetics. Thus, a subtype of the voltage-gated sodium channel $Na_v1.7$ was identified as a potential molecular target for pain therapeutics, triggering a number of large drug development programs (4). However, an alternative parallel approach is to identify compounds that are effective analgesics, and to determine the molecular targets whose functions they modulate. If the compound is a natural product, it has presumably been evolved to interact with a physiologically relevant target. Natural products with analgesic properties have always been important potential leads (e.g., both morphine and aspirin are based on natural products). One more recent example that uncovered a nonopioid mechanism is the cone snail venom peptide, ω-conotoxin MVIIA, which targets a specific voltage-gated calcium channel subtype (Ca_v 2.2). This cone snail venom peptide has become a US Food and Drug Administration-approved drug, ziconotide (5). Identification of Ca_v 2.2 as a validated molecular target has further spurred drug development programs aimed at identifying and characterizing small-molecule compounds targeted to Ca_v 2.2.

Another potential molecular target that was identified using this approach is the α9α10 nicotinic acetylcholine receptor (nAChR). The pioneering work of Livett et al. (6) and Livett and coworkers (7, 8) demonstrated the analgesic activity of Vc1.1, a peptide in the α-conotoxin family, a group of venom peptides from cone snails that are generally targeted to nAChRs. Vc1.1 later entered phase II human clinical trials as a drug for chronic pain, but positive efficacy data were not reported. When the

Significance

This study addresses the need to phase out opioids as the major analgesic drugs for moderate to severe chronic pain. We establish that a highly selective and potent inhibitor of the α9α10 nicotinic acetylcholine receptor (nAChR) subtype prevents the expression of chemotherapy-induced neuropathic pain. Thus, selective antagonists of the α9α10 nAChR are potential leads for nonopioid analgesic drug development. The effects of inhibitors of the α9α10 receptor, together with genetic studies, suggest a key role for the α9α10 nAChR subtype in an intercellular signaling network that can be activated by diverse insults (e.g., chemotherapy, nerve injury, and diabetes).

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Conflict of interest statement: α-Conotoxins, including the α-conotoxins referenced in this paper, have been patented by the University of Utah, with J.M.M. and B.M.O. listed as inventors.

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human clinical trials were initiated, the molecular target of Vc1.1 was unknown; it was later established that the peptide was a potent antagonist of the α9α10 nAChR (9). This targeting specificity provided a plausible mechanistic rationale for the failed clinical development: a striking "affinity gap" of the Vc1.1 peptide for the human vs. rodent α9α10 nAChR, with the human receptor having orders of magnitude lower potency (10–12).

The studies carried out on Vc1.1 also suggested that the α9α10 nAChR was a molecular target for pain based on a unique mechanism: Inhibition of this receptor not only alleviated pain but actually altered the pathophysiology of the disease state (7, 8). This disease progression prevention was recently more thoroughly characterized using a second peptide targeted to the α9α10 nAChR, α-conotoxin RgIA from the venom of *Conus regius*. A peripheral nerve trauma and neuroinflammation model, chronic constriction injury (CCI), was used to demonstrate that RgIA attenuated not only the pathophysiological alterations to the injured nerve but also maladaptive CNS changes (13). Thus, the data with Vc1.1 and RgIA suggest that inhibition of the α9α10 nAChR provides an entirely novel nonopioid mechanism that involves alleviating pain symptoms not only acutely but also by possibly altering disease progression.

Recently, however, several investigators have questioned whether Vc1.1 and RgIA act through the α9α10 nAChR to alleviate pain; instead, they suggest that these peptides exert their effects by stimulating $GABA_B$ receptors (14–19). The series of papers published by these investigators therefore confuses the issue of whether or not the α9α10 nAChR is a validated molecular target for pain. Thus, whether antagonism of this α9α10 receptor is efficacious and may prevent progression to pathological chronic pain states needs to be definitively addressed.

To clarify these important issues, we have developed an analog of α-conotoxin RgIA that has suitable properties for investigating such mechanistic questions. In contrast to the affinity gap of both Vc1.1 and RgIA for the human α9α10 nAChR, the analog developed in this study, RgIA4, has high affinity for both the rodent and human α9α10 nAChRs while retaining highly selective targeting. Importantly, the analog has no activity on the $GABA_B$ receptor. We used RgIA4 to address two questions. First, is this peptide active on a very different model of pathological chronic pain other than traumatic nerve injury, and if so, does it prevent disease progression to the chronic pain state? Second, does this peptide exert its biological activity by inhibition of the α9α10 nAChR? The results of these experiments show that this α9α10-selective peptide, RgIA4, does indeed prevent expression of chronic pain in a chemotherapy-induced neuropathic pain model, and that activity at the α9α10 nAChR is sufficient for these effects. Thus, our results establish that the α9α10 nAChR is a valid molecular target for analgesic compounds, and that inhibition of this receptor is a unique mechanism to prevent the progression to a neuropathic pain state.

Results

Development of RgIA4, an Analog of α-Conotoxin RgIA. We have carried out a broad program to identify and develop peptides that target α9α10 nAChRs, and five diverse structural families of conotoxins that inhibit α9α10 receptors have been characterized (20–25). Two α-conotoxins from cone snail venoms, Vc1.1 and RgIA, are particularly promising in terms of their analgesic properties and the prevention of neuropathology; however, as detailed in the Introduction, these peptides exhibited a striking decline in affinity for the human receptor compared with the rodent α9α10 nAChR. The weak affinity of Vc1.1 for the human receptor is likely responsible for the failure of phase II human clinical trials. We therefore sought to develop analogs that had high affinity and selectivity for the human α9α10 nAChR.

Our approach was to assess the structure activity relationships of a small library of mutant α-conotoxins that were produced by chemical synthesis. We systematically mutated non-Cys residues of RgIA, the smallest of the α9α10 nAChR-targeted conotoxins, and we found that residues in both the first and second disulfide loops of RgIA could be changed to shift potency favorably (Fig. 1). By combining these mutations to create second-generation

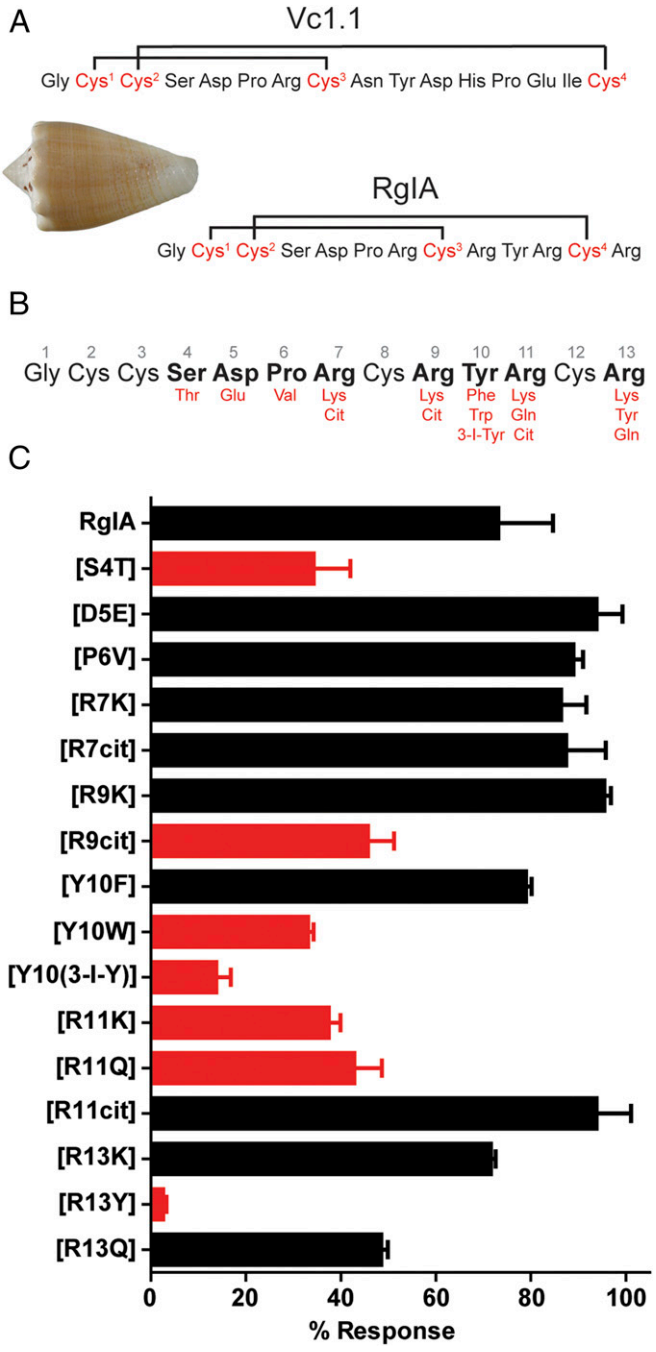

Fig. 1. Peptide sequence of α-conotoxins Vc1.1 and RgIA, as well as RgIA point mutation analogs. (*A*) Amino acid sequences of α-conotoxin Vc1.1 from *Conus victoriae* and α-conotoxin RgIA from *C. regius* (shell shown) indicating disulfide bridges between the first and third cysteines and between the second and fourth cysteines. (*B*) Single substitutions (red) were made for several RgIA amino acids (bold). In general, conservative mutations (similar hydrophobicity, size, or charge) were chosen. We also used monoido-Tyr based on our prior experience that substituting this residue for Tyr in the second disulfide loop increased the potency of α-conotoxin MI (57). (*C*) Effect (100 nM peptide) on blocking ACh-induced current on human α9α10 nAChR currents expressed in *X. laevis* oocytes. Error bars represent the mean ± SEM from three to seven separate oocytes. Red bars indicate a significant difference from RgIA ($P < 0.05$).

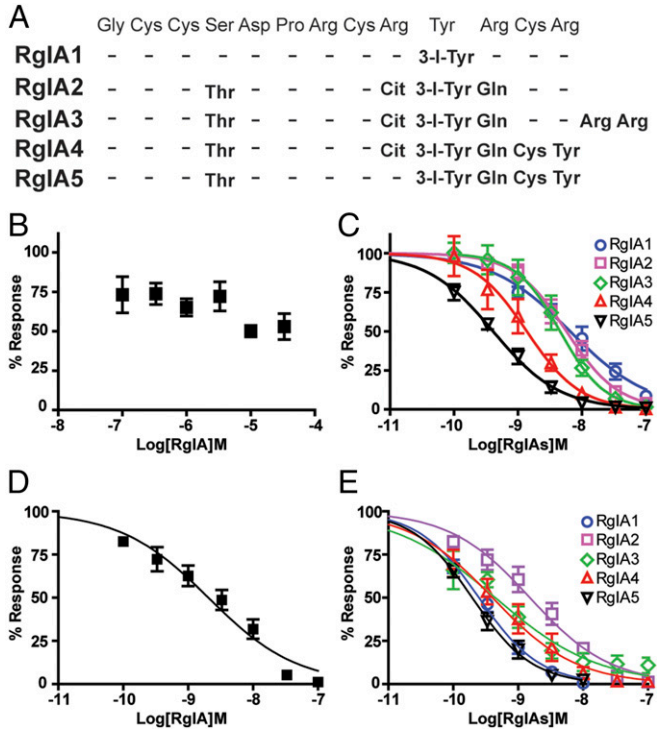

Fig. 2. Concentration response of RgIA analogs on human and Rat α9α10 nAChRs. (*A*) Amino acid sequences. Concentration responses of RgIA (*B*) and analogs (*C*) on human α9α10 nAChRs. Concentration responses of RgIA (*D*) and analogs (*E*) on rat α9α10 nAChRs. Oocytes expressing α9α10 nAChRs were voltage-clamped at −70 mV and subjected to a 1-s pulse of 10 μM ACh every minute as described in *Materials and Methods*. The IC_{50}s and Hill slopes are depicted in Table 1. Data points are the mean ± SEM from three to six separate oocytes.

analogs, 13 amino acid peptides were generated that, unlike the native RgIA, had high affinity for the human α9α10 nAChR (Fig. 2 and Table 1). The parent peptide, RgIA, produced only partial blockade of the human α9α10 nAChR at concentrations up to 33 μM. In contrast, analogs RgIA4 and RgIA5 produced complete blockade of the nAChR with low nanomolar potencies. Although RgIA4 was not the most potent analog identified, it was the most selective for the human α9α10 nAChR (Fig. S1 and Table 2). In addition, we selected peptides that retained high affinity and selectivity for the rat α9α10 nAChR (Table 1) to facilitate mechanistic studies. RgIA4 was the most selective of these analogs, and when further tested against opioid receptor subtypes and a broad panel of other receptors and ion channels,

Table 1. IC_{50} and Hill slope values for blockade of human and rat α9α10 nAChRs by RgIA and analogs

	Human			Rat		
Analog	IC_{50}, nM	Hill slope	*n*	IC_{50}, nM	Hill slope	*n*
RgIA	>10,000*	ND	4	2.4 ± 0.7	0.67 ± 0.03	4
RgIA1	8.4 ± 2.8	0.81 ± 0.1	4	0.3 ± 0.05	0.9 ± 0.02	3
RgIA2	6.1 ± 1.8	1.10 ± 0.1	3	1.7 ± 0.5	0.73 ± 0.05	3
RgIA3	4.7 ± 1.6	1.37 ± 0.3	3	0.5 ± 0.3	0.67 ± 0.03	3
RgIA4	1.5 ± 0.5	1.14 ± 0.2	3	0.9 ± 0.6	0.82 ± 0.07	6
RgIA5	0.44 ± 0.09	0.86 ± 0.1	4	0.2 ± 0.06	1.05 ± 0.12	6

IC_{50} and Hill coefficient values are expressed as the mean ± SEM. ND, not determined.
*Partial blockade at all concentrations.

Table 2. RgIA4 selectively blocks α9α10 nAChRs

nAChR	IC_{50}, human	IC_{50}, rat
α9α10	1.5 nM	0.9 nM
α2β2	>10 μM	>10 μM
α2β4	>10 μM	>10 μM
α3β2	>10 μM	>10 μM
α3β4	>10 μM	>10 μM
α4β2	>10 μM	>10 μM
α4β4	>10 μM	>10 μM
α7	1.8 μM	>10 μM
α1β1δε	>10 μM	>10 μM

micromolar levels of the peptide showed low or no activity as summarized in Tables 3 and 4. When the RgIA4 peptide was tested on the $GABA_B$ receptor using three different assays, it elicited no activity; these results are shown in Fig. S2. Thus, RgIA4 is 1,000- to 10,000-fold selective for the α9α10 nAChR compared with all other tested receptors.

RgIA4 Relieves Chemotherapy-Induced Neuropathic Pain. Oxaliplatin is a first-line chemotherapeutic agent used to treat colorectal cancers. In humans, side effects include neurotoxicity, and prominent symptoms of acute neuropathy include sensitivity to touching cold items, discomfort in swallowing cold items, throat discomfort, and muscle cramps. Cold-related sensitivity is rated as the most severe symptom. Oxaliplatin-induced peripheral neuropathic pain in rats is a commonly used model of human chemotherapy-induced neuropathic pain (26). We therefore assessed the pain-relieving effects of RgIA4, which potently blocks α9α10 nAChRs but lacks activity on $GABA_B$ receptors. Oxaliplatin administration in rats produced cold allodynia (i.e., pain due to a cold stimulus that does not usually provoke pain). Repeated daily s.c. injections (5 d each week) of RgIA4 at doses of 0.128–80 μg/kg prevented chemotherapy-induced cold allodynia; assessments were performed 24 h after the last RgIA4 dose on days 8, 15, and 22 ($P < 0.01$ for all measures; Fig. 3*A*). Repeated oxaliplatin administration in rats also induced a low pain threshold in response to mechanical noxious stimuli (Fig. 3 *B* and *C*). Administration of RgIA4 (0.128–80 μg/kg) prevented mechanical hypersensitivity at 30 min and 24 h post-RgIA4 dosing at all concentrations tested (Fig. 3 *B* and *C*). No behavioral, neurological, or autonomic effects were observed in rats administered RgIA4 (100 μg/kg) daily for 21 d or in rats administered a single dose of RgIA4 (5 mg/kg) (Tables S1–S3).

Comparison of Wild-Type and α9 nAChR KO Mice. To determine the role of α9-containing nAChRs for chemotherapy-induced cold allodynia and RgIA4-mediated pain relief further, we compared wild-type and α9 nAChR KO mice using the oxaliplatin model. Four groups of mice were treated daily (5 d each week) with (*i*) vehicle (i.p.) + vehicle (s.c.), (*ii*) vehicle (i.p.) + RgIA4 (40 μg/kg s.c.), (*iii*) oxaliplatin (i.p.) + vehicle (s.c.), or (*iv*) oxaliplatin (i.p.) + RgIA4 (s.c.). Mice were assessed for oxaliplatin-induced cold allodynia using a cold-plate test on day 1 and again on days 8, 15, and 22.

In the first group of animals, mice were assessed 30 min and 24 h after the last injection. At 30 min after injection, oxaliplatin-treated wild-type mice differed significantly in paw withdrawal latency from vehicle-injected controls on day 15 ($P < 0.001$) and day 22 ($P < 0.001$) (Fig. 4). At 30 min after the last injection, oxaliplatin-treated α9 KO mice did not differ significantly from vehicle-injected α9 KO controls on any given day ($P > 0.05$). At 24 h after the last injection, oxaliplatin-treated wild-type and α9 KO mice both differed significantly from vehicle-treated controls on day 22 ($P < 0.001$ for wild-type mice and $P < 0.01$ for α9 KO mice).

Table 3. Binding activity of RgIA4 on other ion channels, receptors, and transporters

Target	Radioligand	Inhibition, %
5HT1A	[^{3}H]Way100635	10.3
5-HT1B	[^{3}H]5-CT	7.3
5-HT1D	[^{3}H]5-CT	1.6
5-HT1E	[^{3}H]5-HT	−0.3
5-HT2A	[^{3}H]Ketanserin	1
5-HT2B	[3H]LSD	2.7
5-HT2C	[^{3}H]Mesulergine	3.9
5-HT3	[^{3}H]GR65630	−8.8
5-HT5A	[^{3}H]LSD	29.3
5-HT6	[^{3}H]LSD	3.1
5-HT7	[^{3}H]LSD	0.2
Sigma 1	[^{3}H]Pentazocine	−9.2
Sigma 2	[^{3}H]DTG	61.6 (2.6 μM)
$GABA_A$ (rat brain)	[^{3}H]Muscimol	−7.6
$GABA_A$/BZP (rat brain)	[^{3}H]Flunitrazepam	10.9
$GABA_A$/PBR (rat brain)	[^{3}H]PK11195	5.6
α1A adrenergic	[^{3}H]Prazosin	−17.2
α1B adrenergic	[^{3}H]Prazosin	15.7
α1D adrenergic	[^{3}H]Prazosin	−0.5
α2A adrenergic	[^{3}H]Rauwolscine	26.9
α2B adrenergic	[^{3}H]Rauwolscine	50 (>10 μM)
α2C adrenergic	[^{3}H]Rauwolscine	19.4
β1 adrenergic	[^{3}H]CGP12177	−10.3
β2 adrenergic	[^{3}H]Pindolol	1.8
β3 adrenergic	[^{3}H]CGP12177	−10.8
M1	[^{3}H]QNB	9.5
M2	[^{3}H]QNB	−1.4
M3	[^{3}H]QNB	−6.1
M4	[^{3}H]QNB	−2.4
M5	[^{3}H]QNB	1.7
DOR	[^{3}H]DADLE	7
KOR	[^{3}H]U69593	61.6 (2.4 μM)
MOR	[^{3}H]DAMGO	25.7
D1	[^{3}H]SCH23390	3.4
D2	[^{3}H]N-methylspiperone	20.6
D3	[^{3}H]N-methylspiperone	−2.9
D4	[^{3}H]N-methylspiperone	4.3
D5	[^{3}H]SCH23390	1.8
H1	[^{3}H]Pyrilamine	−1.5
H2	[^{3}H]Cimetidine	−44.6
H3	[^{3}H]Tiotidine	−6.9
H4	[^{3}H]Histamine	−10.5
DAT	[^{3}H]WIN35428	−19.3
SERT	[^{3}H]Citalopram	2
NET	[^{3}H]Nisoxetine	−3.3

RgIA4 was tested in triplicate or quadruplicate at 10 μM in a primary screening binding assay. K_d concentrations of indicated radioligands were used. Stably or transiently transfected cell lines expressing human channel, receptors or transporters were used unless otherwise indicated. A secondary, concentration response analysis was conducted when the primary screening assay indicated ≥50% inhibition by RgIA4. Results for secondary concentration-response analysis are shown in parenthesis. 5-HT, 5-hydroxytryptamine; D, dopamine; DAT, dopamine transporter; DOR, δ-opioid receptor; GABA, γ-aminobutyric acid; KOR, κ-opioid receptor; M, muscarinic; MOR, μ-opioid receptor; NET, norepinephrine transporter; SERT, serotonin reuptake transporter.

In the second group of animals, mice were assessed 72 h after the last injection. At 72 h after the last oxaliplatin injection, oxaliplatin-treated wild-type mice significantly differed in paw withdrawal latency from vehicle-treated controls on all test days: day 8 ($P < 0.05$), day 15 ($P < 0.05$), and day 22 ($P < 0.01$). In contrast, oxaliplatin-treated α9 KO mice did not differ from vehicle-treated control mice on any treatment day. Overall, the data suggest that compared with wild-type mice, α9 KO mice do not develop chronic cold allodynia in response to oxaliplatin treatment.

We also compared the effects of oxaliplatin treatment in wild-type and α9 KO mice that had been treated with RgIA4 (40 μg/kg). When measured at 30 min or 24 h after the last injection, RgIA4 reversed oxaliplatin-induced cold allodynia in wild-type mice by day 22 ($P < 0.001$ at both time points) (Fig. 4). When measured at 72 h after the last injection, RgIA4 reversed oxaliplatin-induced cold allodynia in wild-type mice by day 15 ($P < 0.05$) and on day 22 ($P < 0.01$). In contrast, RgIA4 did not have a significant effect on the attenuated oxaliplatin-induced cold allodynia in α9 KO mice at any time point on any test day (all days, $P > 0.05$). Thus, daily s.c. injection of RgIA4 prevented chemotherapy-induced cold allodynia in wild-type mice but not the transient and attenuated cold allodynia in α9 KO mice.

Discussion

The results of this study demonstrate that the α9α10 nAChR is a valid molecular target for drug development. Using a chemotherapy-induced animal model of pain, we have shown that when α9α10 nAChR function is inhibited, either by pharmacological manipulation or by genetic manipulation, the expression of neuropathic pain is attenuated. We eliminated α9α10 nAChR function genetically using α9 KO mice. The pharmacological blocker developed for this study was RgIA4, an analog of α-conotoxin RgIA, a native peptide expressed in cone snail venom. The analog has been extensively characterized: It has high affinity for both rodent and human α9α10 nAChRs (in contrast to the native venom peptide, which has low affinity for the human receptor), and is highly selective (>10^3-fold) for the α9α10 nAChRs vs. all other tested receptors, including opioid and $GABA_B$ receptors.

The well-characterized rodent model of oxaliplatin-induced neuropathy was used to assess the effects of blockade of α9α10 nAChRs. Oxaliplatin is a first-line chemotherapeutic agent for colorectal cancer and also is used to treat pancreatic, gastric, and testicular cancers (27, 28). It is an organoplatinum complex that is thought to produce cytotoxicity via the formation of DNA adducts that interfere with DNA replication and transcription, leading to the induction of apoptosis. Neurotoxicity is dose-dependent, and symptoms (typically, increased sensitivity to cold) often continue to worsen for several months after completing chemotherapy, suggesting an ongoing disease process (26). There are currently no agents approved for prevention of the chemotherapy-induced peripheral neuropathy, often leaving clinicians to decrease the dose or duration of the medication treatment, thus diminishing the effectiveness of the intended cancer therapy (29).

The results of the present study establish a key role for the α9α10 nAChR in the progression of chemotherapy-induced neuropathic pain. This conclusion is illustrated in Fig. 5, which summarizes the 72-h time points obtained in the experiment with wild-type and α9-KO mice described in *Results* (also Fig. 4). These data indicate that oxaliplatin produces an increasing severity of cold sensitivity, similar to the syndrome observed in human chemotherapy patients. The cold allodynia, assessed in rodents by paw withdrawal latency, becomes more intense with successive doses of oxaliplatin; in contrast, the cold allodynia is not observed if RgIA4 is injected concurrently with oxaliplatin administration or if the experiment is carried out on α9 KO mice.

The extended duration (24–72 h) of efficacy of RgIA4 in the rodent model is remarkable, in that RgIA4 is rapidly metabolized in vivo; the plasma half-life in rodents is less than 20 min.* The analgesia data shown in Figs. 3 and 5 are 24-h and 72-h points, respectively; the lack of detectable cold allodynia suggests

*Mercado J, et al. (2014) 15th World Congress on Pain, International Association for the Study of Pain, October 6–11, 2014, Buenos Aires, abstr 2026.

that application of RgIA4 may have disease-modifying effects. Because of the high specificity of RgIA4 for the α9α10 nAChR, it is reasonable to conclude that the absence of cold allodynia is a direct consequence of α9α10 nAChR inhibition. The results obtained using α9 KO mice are consistent with this mechanism. Thus, this nAChR subtype appears to play a key signaling role in preventing a pathological pain state.

Blockade of the α9α10 nAChR did not appear to be analgesic per se (i.e., the baseline thresholds were not elevated). Thus, the utility of the mechanism of action is appropriate for prevention of chronic pain, such as in cancer chemotherapy and, as detailed in a previous study, in prevention of development of trauma-induced neuropathic pain (13). However, acute pain (e.g., acute surgical pain) may not be affected. Nevertheless, single doses of other conotoxins that block α9α10 nAChRs produce analgesia in models of established neuropathic pain, including CCI and partial sciatic nerve ligation, suggesting that this mechanism may also have utility in these settings (8, 9, 24).

The occurrence of a species affinity gap between homologous human and rodent molecular targets is a potentially important general issue in drug development, particularly if the molecular target has not been identified. Preclinical pain studies are most often carried out on rodent models, and a large affinity gap could lead to failure to demonstrate efficacy in human clinical trials (such as was observed for Vc1.1). For the present studies, α-conotoxin RgIA, a venom peptide from *C. regius*, was used as a template for engineering highly selective peptide antagonists of α9α10 nAChRs. The final analog, RgIA4, has high affinity (low nanomolar) for both the human and rodent α9α10 nAChR; RgIA4 retains high selectivity, with no activity detected on the $GABA_B$ receptor. Low affinity for the rodent receptor can also be problematic. Lead compounds with high affinity only for human transient receptor potential (TRP) channels were abandoned as analgesic drug leads because the low affinity for rodent TRP channels precluded validation using animal models of pain (30, 31). Because the α-conotoxin RgIA analogs developed in the current study have high affinity for both human and rodent α9α10 nAChRs, they are useful both for mechanistic studies using rodent models of pain as well as for potential drug leads for human therapy. The specific peptide chosen for the animal studies, although not the most potent analog on the human receptor, was the most selective, and was therefore suitable for assessing the role of α9α10 nAChRs. The approach we used to obtain selective analogs of native RgIA that also addressed the affinity gap between rodent and human α9α10 nAChRs provides a paradigm that should be generally applicable whenever species-specific differences in affinity exist.

The α9α10 nAChR represents a mechanistically distinctive target for modifying the disease course of chronic pain. Presently available pain medications act on a limited number of pharmacological targets that treat, but do not prevent, disease progression. Commonly used medications include opioids, COX inhibitors, N-type Ca_V channel blockers, and catecholamine reuptake inhibitors. Each of these medications generally provides only partial pain relief. For treatment of severe chronic pain, medications from multiple classes are often required, and despite combination therapy, millions of patients worldwide are left to suffer from treatment-resistant symptoms. Whereas morphine and opioid derivatives are the gold standard for the treatment of moderate to severe acute pain, long-term use is problematic. Opioid tolerance rapidly develops, leading to the need for dose increases to obtain the same analgesic effect (32). RgIA4 lacks activity at opioid receptors, and multiple doses of RgIA4, rather than causing tolerance, produced gradual and sustained pain relief. Whether the short course of treatment produces permanent beneficial effects requires further study.

Conus venoms have been a rich source of peptides that target neuronal receptors and ion channels. The ω-conotoxin MVIIA (ziconotide) from the fish-eating *Conus magus* was approved for treatment-refractory severe chronic pain, such as in patients with cancer or AIDS. Ziconotide is a positively charged peptide that does not readily cross the blood–brain barrier and, consequently, must be delivered intrathecally to produce its centrally acting effects (5). RgIA4 differs substantially from ω-conotoxin MVIIA in structure (two vs. three disulfide loops), function (nAChR antagonist vs. Ca_V channel antagonist), effective route of administration (s.c. vs. intrathecal), and side-effect profile (no neurological effects in Irwin testing for RgIA4 vs. 25% or more of patients receiving ziconotide reporting dizziness, nausea, confusion, somnolence, or nystagmus) (33).

The pathophysiology of oxaliplatin-induced chronic pain remains poorly understood. T cells and macrophages infiltrate damaged neuronal tissue, and neuroimmune-mediated mechanisms may play a role in disease progression (34, 35). Although the tissue target of RgIA4 is unknown, it is noteworthy that the parent compound, RgIA, reduces neural immune cell accumulation (T cells, macrophages, and acetylcholine-synthesizing lymphocytes) after CCI (9, 13). The α9 and α10 subunit transcripts have been identified in immune cells, including lymphocytes, neutrophils, and monocytes (36). It has also recently been shown that functional α9α10 nAChRs regulate ATP-dependent cytokine release in monocytes (37) and that α9-containing nAChRs modify the function of proinflammatory monocytes (38). The α9-containing nAChRs also modulate the influx of monocytes and neutrophils in experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis (39, 40). Thus, it is possible that the restorative properties of RgIA4 are related to neuroprotective modulation of immune function.

An additional indication that inhibition of α9α10 nAChRs may be disease-modifying comes from studies of the parent compound, RgIA, in rats that have undergone CCI of the sciatic nerve. Morphological analysis indicates that RgIA administration prevents CCI-induced decreases of axon diameter, loss of myelin sheath, and decreases in fiber number (13). In addition, CCI causes secondary pathological changes along the ascending pain pathway, including edema and infiltration of macrophages in dorsal root ganglia (DRG) and activation of microglia and astrocytes in the dorsal horn of the spinal cord. RgIA administration also prevents these ascending pathway pathologies, indicating protective effects against disease progression (13). Additionally, another conotoxin that preferentially inhibits the α9α10 receptor, Vc1.1, was previously shown (41) to prevent neuropathic pain in a rodent model of diabetes. In humans, chronic neuropathic pain is a highly prevalent complication of diabetes. Established neuropathy is irreversible, even after subsequent improvements in metabolic parameters (42). Remarkably, the analgesic effects of Vc1.1 persisted for 10 d after cessation of Vc1.1 administration, consistent with a disease-modifying effect (41).

Thus, compounds that have a pharmacological profile that includes α9α10 antagonist activity prevent or attenuate the expression of pain in several rodent models, including neuropathic pain induced by chemotherapy, traumatic nerve injury, or diabetes (8, 13,

Table 4. RgIA4 activity on hERG K^+ channels and $GABA_B$ receptors

Ion channel	Assay	Inhibition, %	Receptor	Assay	EC_{50}, μM
hERG	Patch clamp	0	$GABA_B$	cAMP	>10
				Cellular dielectric spectroscopy	>10
				DRG Ca_V channel current	>1

RgIA4 was tested in quadruplicate on the hERG K^+ channel at 10 μM as described in *Materials and Methods*. RgIA4 was also tested on $GABA_B$ receptors using the indicated assays (see *Materials and Methods*; see also Fig. S2).

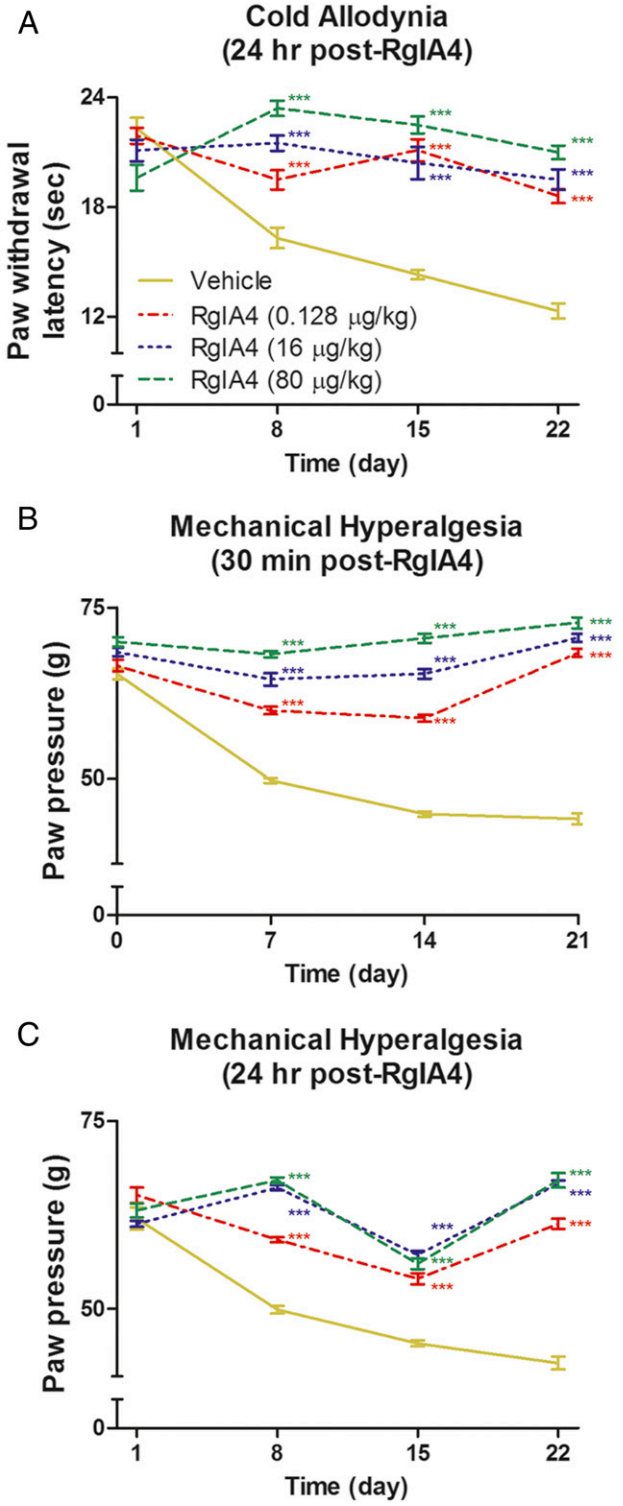

Fig. 3. RgIA4 prevents chemotherapy-induced neuropathic pain in rats. Chemotherapy-induced peripheral neuropathy in rats was induced by repeated i.p. injection of oxaliplatin as described in *Materials and Methods*. Control animals were treated with vehicle. RgIA4 was dissolved in saline and injected s.c. daily. Withdrawal latency (*A*) and paw pressure (*B*) were used as a measure of cold allodynia and mechanical hyperalgesia, respectively. The cold allodynia test was performed on days 8, 15, and 22 at 24 h after RgIA4 administration (*A*), and the mechanical hyperalgesia test was performed at 1-wk time points 30 min (*B*) and 24 h (*C*) after RgIA4 administration. Values are expressed as the mean ± SEM from eight rats for each experimental determination. ***$P < 0.001$ significantly different from vehicle.

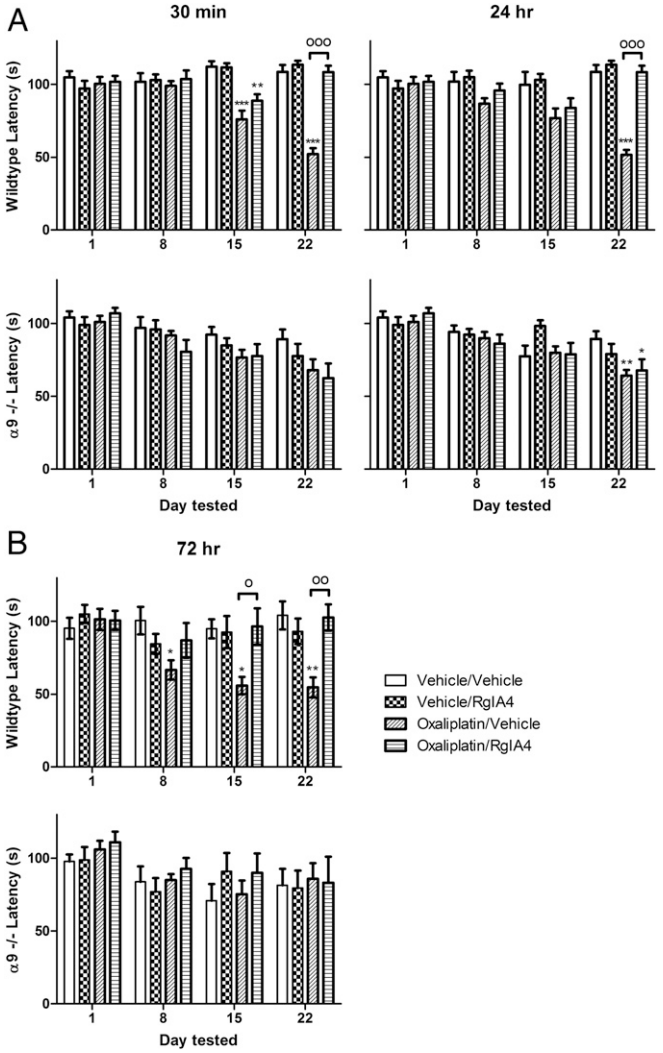

Fig. 4. RgIA4 prevents chemotherapy-induced neuropathic pain in wild-type but not α9 KO mice. Chemotherapy-induced peripheral neuropathy in mice was induced by repeated i.p. injection of oxaliplatin. Control animals were treated with vehicle. RgIA4 was dissolved in saline and injected s.c. daily. Mice were assessed in a cold-plate chamber in which the temperature was decreased from room temperature at rate of 10 °C per minute. Time to response is indicated on the *y* axis. (*A*) Mice were injected i.p. 5 d each week with vehicle or oxaliplatin, and injected s.c. with RgIA4 (40 μg/kg) or vehicle 5 d each week. Cold-plate testing was performed 30 min after and 24 h after the last injection on indicated days. (*B*) In a separate experiment, mice were injected as described in *A* but assessed at 72 h after the last injection. Values are expressed as the mean ± SEM (n= 7–9) for each experimental determination. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from vehicle/vehicle control. °$P < 0.05$, °°$P < 0.01$, °°° $P < 0.001$ significantly different compared with oxaliplatin/vehicle-treated mice.

35, 43, 44). The results of the present study establish that it is the blockade of the α9α10 nAChR that enables the prevention of chemotherapy-induced neuropathic pain. Together, these findings strongly suggest that there is a common molecular pathway involved in the expression of pain following a diverse array of triggering insults, a pathway that apparently includes signaling via α9α10 nAChRs.

Materials and Methods

Conotoxin Synthesis. α-Conotoxins were synthesized using an AAPPTec Apex 396 automated peptide synthesizer as previously described (45). The peptide masses were verified by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry.

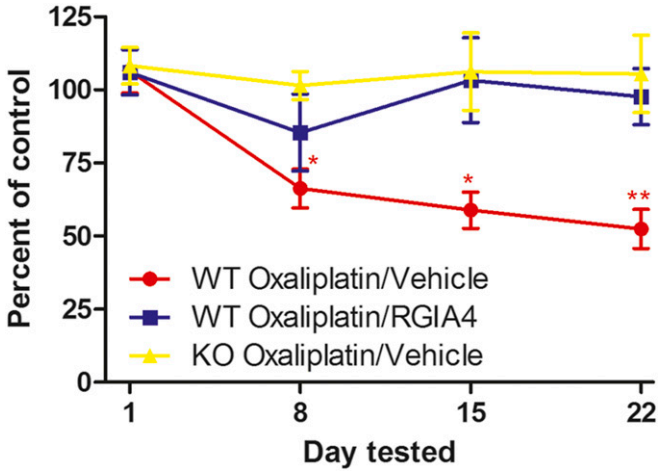

Fig. 5. RgIA4 and α9 KO prevent chronic cold allodynia. Repeated oxaliplatin injection induces progressive cold allodynia in wild-type mice. Cold allodynia is not detected 72 h after oxaliplatin administration either in wild-type mice that received RgIA4 or in α9 KO mice (additional details are provided in Fig. 4). Data shown are 72 h after the last injection. The control was i.p. saline administered five times per week + s.c. saline administered five times per week (vehicle/vehicle) at the indicated time points; i.p. oxaliplatin was administered five times per week; and s.c. saline or RgIA4 was administered five times per week. $*P < 0.05$, $**P < 0.01$ significantly different from vehicle/vehicle controls at indicated time points for respective genotypes.

Two-Electrode Voltage-Clamp Recording. *Xenopus laevis* oocytes were used to express cloned rat or human nAChR subtypes heterologously. Recordings were made 1–5 d postinjection. Oocytes were voltage-clamped at −70 mV at room temperature and exposed to acetylcholine, RgIA, or analogs as described previously (20).

Receptor Pharmacology. Synthetic RgIA4 was tested on non-nAChRs by the National Institute of Mental Health Psychoactive Drug Screening Program using previously described methods (46–48). Compounds were initially tested in quadruplicate at 10 μM in primary binding assays. Compounds that blocked more than 50% of the radioligand binding were then tested in secondary binding assays to determine a concentration–response curve. Patch-clamp electrophysiology was used for assessing the human ether-a-go-go–related gene (hERG) function (48). G_i-coupled cAMP modulation was assessed in CHO-K1 cells expressing $GABA_{B1a/B2}$ with $G_{i/o}$ coupling; assays were performed by DiscoverX. Compounds were incubated with cells for 30 min at 37 °C in the presence of 15 μM forskolin, and cellular cAMP was detected using the HitHunter cAMP XS assay detection method according to the manufacturer's protocol. Effects were calculated as a percentage of maximal GABA effect (GABA EC_{50} = 188 nM). Values were obtained in duplicate, and the experiment was repeated once. In a separate experiment, cellular dialectric spectroscopy (49) was used to assay changes in electrical impedance in CHO cells expressing human $GABA_{B1a/B2}$ (50). Values were obtained in duplicate. The assay was performed by Eurofins Cerep according to the manufacturer's protocol. Compounds were incubated with cells for 10 min at 28 °C. Cellular agonist effect was calculated as a percentage of 100 μM 3-aminopropyl(methyl)phosphinic acid agonist control.

Animals. All experimental procedures on animals were performed in accordance with the NIH guidelines for the care and use of laboratory animals and were performed under Institutional Animal Care and Use Committees' approved protocols at the University of Utah, University of Florence, A. T. Still University Kirksville College of Osteopathic Medicine, or Kineta, Inc. Male or female Sprague–Dawley rats, weighing ∼200–250 g at the beginning of the experimental procedures, were used. Wild-type mice were CBA/CaJ (The Jackson Laboratory). Germline α9 KO mice (28), originally on a 129Sv/Ev and CBA/CaJ background, were crossed using an accelerated backcrossing program (The Jackson Laboratory) until 99.5% identity with wild-type CBA/CaJ mice was achieved. Mice were then further backcrossed with wild-type CBA/CAJ mice for an additional three generations. Experiments involving animals have been reported according to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (51). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Irwin Test. A modified Irwin screen was used to test the potential adverse behavioral, neurological, and autonomic effects of RgIA4 treatment. Groups of two to three rats (Charles River Laboratories) were used. Two independent blinded studies were conducted, each with a different route of administration, RgIA4 concentration, adverse effect readouts, and times of assessments. In one study, RgIA4 was administered s.c. at a concentration of 5 mg/kg. Observations were conducted at 0, 0.25, and 2 h postdosing. In a second study, RgIA4 was administered i.m. for 14 d at a concentration of 100 μg/kg. Observations were conducted daily at 0, 0.5, 1, 2, 4, and 24 h postdosing. Each sign or effect was noted either for its presence or absence, or was assigned a score ranging from 0 to 3. In this scoring scheme, 0 represents no visible change, whereas 1 represents mild changes and 3 represents marked changes. The animals were also observed for vehicle- or compound-induced mortality during the observation period.

Voltage-Dependent Calcium Current Recording in Rat DRG Neurons. L_4 and L_5 DRG neurons were isolated from Sprague–Dawley rats, enzymatically dissociated, plated onto polylysine-coated glass coverslips, and maintained overnight at 37 °C in a 5% (vol/vol) CO_2 incubator in minimum essential media with 10% FBS and 1% penicillin/streptomycin (52). DRG neurons were recorded in a solution containing 145 mM *N*-methyl-D-glucosamine (NMG)•Cl, 5 mM $BaCl_2$, 10 mM Na•Hepes, and 5 mM glucose, with pH 7.4 and osmolarity of 320 mOsm. The pipet solution contained 104 mM NMG•Cl, 14 mM creatine•PO_4, 6 mM $MgCl_2$, 10 mM NMG•Hepes, 5 mM Tris•ATP, 10 mM NMG_2•EGTA, and 0.3 mM $Tris_2$•GTP with pH 7.4 and osmolarity of 300 mOsm. Test solutions were applied, and ionic currents were recorded and analyzed as previously described (24, 53). Recordings were carried out at room temperature, and the holding potential was −80 mV. Group data were calculated as the mean ± SD, and Student's *t* test was used to determine significant differences ($P < 0.05$).

Oxaliplatin-Induced Neuropathic Pain in Rats. Neuropathy in rats (Harlan) was induced as described by Cavaletti et al. (54). Briefly, rats were treated with 2.4 mg/kg i.p. oxaliplatin (Sequoia Research Products) for 5 consecutive days every week for 3 wk (15 i.p. injections). Throughout the study period, experimenters were blinded as to the identity of the injected compounds. Data were analyzed with one-way ANOVA using Dunnett's multiple comparison test (GraphPad Prism).

Rat Cold Plate Test. As previously described (55), rats were placed in a stainless steel box (12 cm × 20 cm × 10 cm) with a cold plate as the floor. The temperature of the cold plate was kept constant at 4 ± 1 °C. Pain-related behaviors (i.e., lifting and licking of the hind paw) were observed, and the time of the first sign was recorded. The cutoff time of the latency of paw lifting or licking was set at 60 s.

Rat Paw Pressure Test. The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile) as previously described (56). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred. Vocalization or withdrawal reflex thresholds were expressed in grams. For analgesia measures, mechanical pressure application was stopped at 120 g.

Oxaliplatin-Induced Neuropathic Pain in Mice. Oxaliplatin (MedChem Express) was dissolved at 0.6 μg/μL in 0.9% NaCl. RgIA4 was dissolved at 0.01 μg/μL in 0.9% NaCl. For each experiment, mice were divided into four equally sized groups (n = 7–9 animals) and injected with saline (i.p.) + saline (s.c.), saline (i.p.) + RgIA4 (40 μg/kg, s.c.), oxaliplatin (2.4 mg/kg i.p.) + saline (s.c.), or oxaliplatin (2.4 mg/kg, i.p.) + RgIA4 (40 μg/kg, s.c.).

In the first study, mice were injected once per day on Wednesday, Thursday, and Friday, and again on Monday and Tuesday. On Wednesday, mice were tested before injection (24-h time point) and 30 min after injection. This pattern was repeated for an additional 2 wk. In the second set of experiments, mice were injected once per day on Monday through Friday and tested on Monday before injection (72-h time point). This pattern was repeated for an additional 2 wk.

For mice, testing was conducted using a cold-plate test chamber (IITC, Inc. Life Science). Animals were allowed to acclimate in the chamber at room temperature (23 °C) for 5 min. Temperature was then lowered at a rate of 10 °C per minute.

The testing was stopped when the animal lifted both forepaws and shaking or licking occurred. Alternating lifting of forepaws was not scored.

Throughout the study period, experimenters were blinded as to the identity of the injected compounds. Data were analyzed with one-way ANOVA using Dunnett's multiple comparison test (GraphPad Prism).

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