



Synergistic symptom-specific effects of ketorolac–tramadol and ketorolac–pregabalin in a rat model of peripheral neuropathy

Wen-Ying Lin^{a,b,c}, Yu-Ting Cheng^a, Yu-Hsin Huang^a, Feng-Sheng Lin^b, Wei-Zen Sun^{b,*}, Chen-Tung Yen^{a*}

^aDepartment of Life Science, National Taiwan University, Taipei, Taiwan, ROC; ^bDepartment of Anesthesiology, National Taiwan University Hospital, Taipei, Taiwan, ROC; ^cNational Taiwan University Cancer Center, National Taiwan University College of Medicine, Taipei, Taiwan, ROC

Abstract

Background: Although current neuropathic pain treatment guidelines do not recommend the use of nonsteroidal anti-inflammatory drugs (NSAIDs), whether NSAIDs can serve as a useful adjuvant to conventional multimodal therapy remains unclear.

Methods: The spared nerve injury (SNI) rats rapidly developed profound and long-lasting spontaneous and evoked pain behaviors, including mechanical and cold allodynia of the ipsilateral hind paw. At day 5, we first characterized the nociceptive responses to ketorolac, tramadol, pregabalin, and their combinations.

Results: We found that tramadol and pregabalin exerted dose-dependent analgesic effects on both spontaneous and evoked behaviors. However, ketorolac alone did not suppress any behaviors regardless of the dose. Ketorolac–tramadol and ketorolac–pregabalin produced variable degrees of additive suppression of spontaneous and evoked behavioral responses. Cold allodynia was profoundly diminished after ketorolac was added to ineffective pregabalin or tramadol. Mechanical allodynia was markedly attenuated by ketorolac–pregabalin but less so by ketorolac–tramadol mixtures.

Conclusion: Our data demonstrated that an NSAID alone failed to relieve spontaneous or evoked pain behaviors in the rat SNI model, but when combined with a weak opioid and α -2- δ -ligand produced a profound synergistic analgesic effect on cold allodynia and discrepant efficacy for mechanical allodynia and spontaneous pain.

Keywords: Alpha-2-delta blocker; Neuropathic pain; Nonsteroidal anti-inflammatory drug; Opioid; Spared nerve injury

1. INTRODUCTION

Peripheral neuropathic pain is one of the most disastrous types of chronic pain. It causes ongoing spontaneous pain and amplifies pain responses to noxious or even innocuous stimuli.¹ Current neuropathic pain treatment guidelines do not recommend nonsteroidal anti-inflammatory drugs (NSAIDs) as an effective treatment. Instead, tricyclic antidepressants, α -2- δ subunit blockers, and serotonin–noradrenaline reuptake inhibitors are recommended as first-line medications, and tramadol is listed as a second-line medication.² However, NSAIDs are still being used worldwide in patients with neuropathic pain.³

NSAIDs, which work by inhibiting the enzyme cyclooxygenase (COX) and suppressing prostaglandin synthesis, are mostly used for treating inflammatory and acute postoperative pain.⁴ By contrast, pregabalin and tramadol are frequently prescribed to treat neuropathic pain symptoms. Tramadol hydrochloride is both a weak μ -opioid receptor agonist and an inhibitor of the reuptake of serotonin and norepinephrine.⁵ Pregabalin, an anti-convulsant, inhibits the release of excitatory neurotransmitters by selectively binding to the α -2- δ -subunit of the presynaptic calcium channel.⁶ Previous studies have confirmed that a combination of ketorolac and tramadol achieved a synergic analgesic effects on arthritic pain,⁷ and a combination of diclofenac sodium and gabapentin was also synergistically effective in a postoperative pain model.⁸ Similarly, a combination of an opioid and NSAIDs exerted a synergic analgesic effect on both neuropathic and inflammatory pain.⁹

The optimal pharmacotherapy for neuropathic pain has not been thoroughly investigated. However, monotherapy has been associated with limited efficacy and dose-related adverse effects.¹⁰ A review of the pharmacotherapy of neuropathic pain concluded that a two-drug combination frequently produces prominent central nervous system (CNS) depression when it has a similar effect on CNS.¹¹ Multimodal therapy using various mechanistic pathways can maximize the analgesic effect and minimize the adverse effects of individual monotherapy. Therefore, we hypothesized that combinations of ketorolac and tramadol or pregabalin could have a synergistic analgesic effect on neuropathic pain responses. The potential synergistic analgesic effects were tested

*Address correspondence: Prof. Wen-Zen Sun, Department of Anesthesiology, National Taiwan University Hospital, 7, Chung-Shan S Road, Taipei 100, Taiwan, ROC. E-mail address: wzsun@ntu.edu.tw (W.-Z. Sun); Prof. Chen-Tung Yen, Department of Life Science, National Taiwan University, 1, Roosevelt Road, Section 4, Taipei 106, Taiwan, ROC. E-mail address: ctyen@ntu.edu.tw (C.-T. Yen).

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on a spared nerve injury (SNI) rat model that included spontaneous pain and mechanical and cold allodynia.

2. METHODS

2.1. Animals

This study used 8 to 10-week-old male Sprague Dawley rats weighing 250 to 350 g. The animals were acclimatized to the colony room for at least 7 days before participating in any experiment. Rats were group-housed (two rats per cage) and kept in a standard environment with a 12-hour dark–light cycle and a temperature of 22°C. Food and water were available *ad libitum*. The Institutional Animal Care and Use Committee of National Taiwan University approved all experimental procedures and animal handling. The methods adopted in this study abided by the Codes for Experimental Use of Animals of the Council of Agriculture of Taiwan, which is based on the Animal Protection Law of Taiwan.

2.2. Sciatic nerve injury surgery

The entire surgery was performed under anesthesia through an intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (75:7.5 mg/kg). We induced peripheral nerve damage according to the methods detailed by Decosterd and Woolf.¹² Briefly, the skin on the left lateral thigh was incised and the biceps femoris muscle was sectioned and held apart by a retractor, exposing three terminal branches of the sciatic nerve: the sural, common peroneal, and tibial nerves. A ligature with a 6/0 silk thread was tied tightly around the tibial and common peroneal nerves and sectioned distal to the ligation, which was followed by the removal of 2 to 3 mm of the nerve distal to the ligation. Any contact with the intact sural nerve was carefully avoided. After surgery, the muscle was closed with a 6/0 and the skin with a 4/0 silk thread to prevent any opening of the wound. Recovery and rest of at least 5 days were allowed before performing any further experiment.

2.3. Drugs

I.p. injections were given to nonanesthetized rats. Both tramadol hydrochloride (Tramal 100 solution for injections; Grunenthal, Germany) and ketorolac tromethamine (Keto, 30 mg/mL, IM/IV; Yungshin Pharm Ind., Taiwan) were intraperitoneally injected in nonanesthetized rats at doses of 2.5, 5, and 10 mg/kg. Pregabalin (Pfizer Inc., Groton, Connecticut, USA) was injected at doses of 3, 10, and 30 mg/kg. To assess the interaction between drugs, a combination of 5 mg/kg ketorolac and 5 mg/kg tramadol or 10 mg/kg pregabalin was administered to investigate their synergistic analgesic effect.

2.4. Behavioral test and drug injection

Three behavioral indices induced by peripheral nerve damage were assessed, including spontaneous pain, mechanical allodynia, and cold allodynia. All behavioral tests were conducted in a room where the animals were routinely habituated for at least 1 h/day for 3 days before the day of the behavioral experiments. During testing, the animals were first allowed to adapt to the behavior apparatus, and then the baseline values for three behaviors were measured before drug injection. The effectiveness of the drugs in three nociceptive behaviors was analyzed up to 2 hours after their i.p. administration. Each group comprised 4 to 6 rats; each rat randomly received one of the five drugs injected from a low to high dosage with at least a 3-day interval between doses for drug washout.^{9,13,14}

2.4.1. Spontaneous pain

Spontaneous pain behavior was tested at least 5 days after SNI surgery.¹⁵ The rats were first placed on a glass plate at a

controlled temperature of 30°C ± 1°C for a 5-minute adaptation. The cumulative number of the abnormal lifting of the ipsilateral hind paw was recorded for 30 minutes before injection as a baseline value,¹⁵ and postinjection values were recorded every 30 minutes until 120 minutes after injection.

2.4.2. Mechanical allodynia (von Frey test)

Mechanical allodynia was tested at least 5 days after SNI surgery.¹⁵ The rats were acclimated to the elevated wire mesh at least 30 minutes before the test. During the test, a set of von Frey filaments of increasing force (1, 2, 4, 6, 9, and 15 g) was applied to the plantar surface of the hind paw using a modified staircase method.¹⁶ Pain response was determined when the rat briskly withdrew its foot from the bending filament. The 50% withdrawal threshold was tested before drug injection, and postinjection values were obtained every 30 minutes until 120 minutes after injection. Tests were repeated three times at each interval.

2.4.3. Cold allodynia (acetone test)

Acetone tests were conducted at least 10 days after SNI surgery to ensure the full development of pain response to innocuous cooling stimuli in rats.¹⁷ Using blunt plastic tubing connected with a syringe, a droplet of acetone was used to touch the plantar surface of the hind paw. The cumulative duration of the hind paw lift immediately after acetone spray was recorded to assess nociceptive response. The length of the withdrawal response was recorded with a minimum of 0.5 seconds and a maximum of 20 seconds.¹⁷ The withdrawal duration was tested before drug injection, and postinjection values were obtained every 30 minutes until 120 minutes after injection. Tests were repeated five times at each interval.

2.5. Data analysis and statistics

The analgesic effects of the drugs are presented as a percentage of the maximum possible effect (% MPE) according to the following formulas: % of MPE for spontaneous pain = (baseline value – postdrug value)/(baseline value) × 100; % of MPE for mechanical allodynia = (postdrug value – baseline value)/(cutoff value – baseline value) × 100; % of MPE for cold allodynia = (baseline value – postdrug value)/(baseline value) × 100. To analyze the whole course effect of either a single or combined agent, we present the area under the curve (AUC). The AUC is the accumulated % MPE during 2 hours obtained using the trapezoidal rule (AUC = % MPE × h). All data are presented as mean ± SEM. We evaluated the differences in AUC using one-way analysis of variance (ANOVA) followed by a *t* test between two groups. Statistical significance was set at *p* < 0.05. Statistical tests were conducted using Medcalc software version 17.9.7 (MedCalc, Ostend, Belgium).

3. RESULTS

3.1. Effect of tramadol, ketorolac, and pregabalin

The results of predrug behavioral tests showed a baseline spontaneous lifting of 0.9 ± 0.09 times (mean ± SEM; *n* = 54), a baseline mechanical threshold of 2.3 ± 0.27 g (*n* = 54), and a baseline cold withdrawal duration of 16.44 ± 0.91 seconds (*n* = 54). No significant differences were noted between the tramadol, ketorolac, and pregabalin groups (*p* = 0.46 through sphericity assumed using repeated-measures ANOVA). These results indicated no individual differences in all the groups before drug treatment.

The AUC of the maximum possible effect of high doses of pregabalin and tramadol was 128 ± 9.3 (*n* = 4) and 91 ± 11.4 (*n* = 6) in spontaneous pain, respectively; 86 ± 10.2 (*n* = 4) and 61 ± 19.6 (*n* = 6) in cold allodynia, respectively; and 64 ± 6.9

($n = 4$) and 72 ± 17.0 ($n = 5$) in mechanical allodynia, respectively. These results indicated that tramadol and pregabalin attenuated all three neuropathic pain behaviors in a dose-dependent manner (Fig. 1A, B). Specifically, they were more effective at reducing spontaneous hind paw lifting frequencies than at attenuating cold and mechanical allodynia. However, ketorolac was not effective as a sole agent for attenuating neuropathic pain responses even at a high dose of 10 mg/kg. A partial analgesic effect from ketorolac was only seen in cold allodynia with an AUC of the maximum possible effect of 45 ± 15.9 ($n = 6$) (Fig. 1C).

3.2. Effect of combination of ketorolac and tramadol or pregabalin

The synergistic analgesic effects of the coadministration of ketorolac with pregabalin or tramadol were evident and alleviated specific neuropathic pain symptoms (Fig. 2). Pregabalin

with ketorolac was more effective than tramadol-ketorolac combination in all three neuropathic pain behaviors. Adding 5 mg/kg ketorolac to 10 mg/kg pregabalin increased the AUC of the MPE value from 13 ± 24.6 ($n = 4$) to 116 ± 31.9 ($n = 5$), a nearly 9-fold increase in cold withdrawal tolerance (Fig. 3A). Similarly, the AUC of the MPE for mechanical allodynia increased from 25 ± 4.5 ($n = 4$) to 113 ± 14.1 ($n = 5$), a nearly 5-fold increase in mechanical threshold (Fig. 3B); the AUC of the MPE for spontaneous pain increased from 62 ± 8.0 ($n = 4$) to 142 ± 11.0 ($n = 4$), a 2.2-fold increase in the tolerability of spontaneous pain (Fig. 3C). Additionally, the time-effect curves showed that the onset time of pregabalin with ketorolac was faster than pregabalin only in all three neuropathic pain behaviors; however, this result was not significant (Fig. 3D-F).

The addition of 5 mg/kg ketorolac increased the AUC of the MPE values of 5 mg/kg tramadol alone. The increase in the MPE was 3.7-fold ($n = 6$) for cold withdrawal tolerance (Fig. 4A), 5.8-fold ($n = 6$) for mechanical threshold (Fig. 4B), and 2-fold ($n = 6$) for tolerability of spontaneous paw lifting (Fig. 4C). The

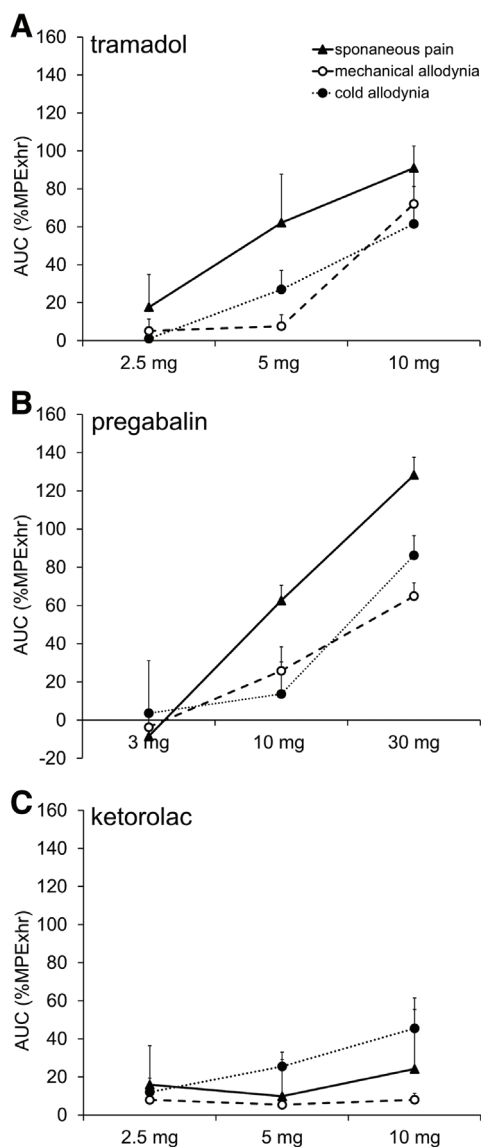


Fig. 1 Dose-response curve of the effects of tramadol (A), pregabalin (B), and ketorolac (C) on three different neuropathic pain behaviors. Tramadol and pregabalin exerted good analgesic effects at higher doses. Each line represents the group AUC (% MPE × h) mean and SEM of four to six rats for each drug dosage (mg/kg).

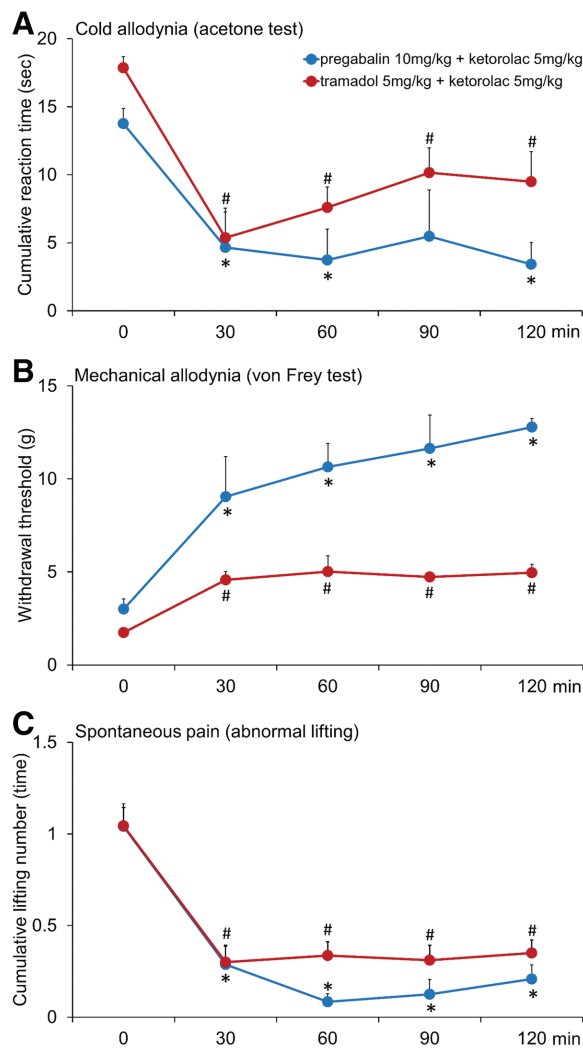


Fig. 2 Effect of combination drugs in acetone test (A), von Frey test (B), abnormal lifting of paw (C). Each line represents the group mean and SEM of four to six rats for two combination drugs. Significantly different from the baseline at * $p < 0.05$ in pregabalin with ketorolac; # $p < 0.05$ in tramadol with ketorolac by t test.

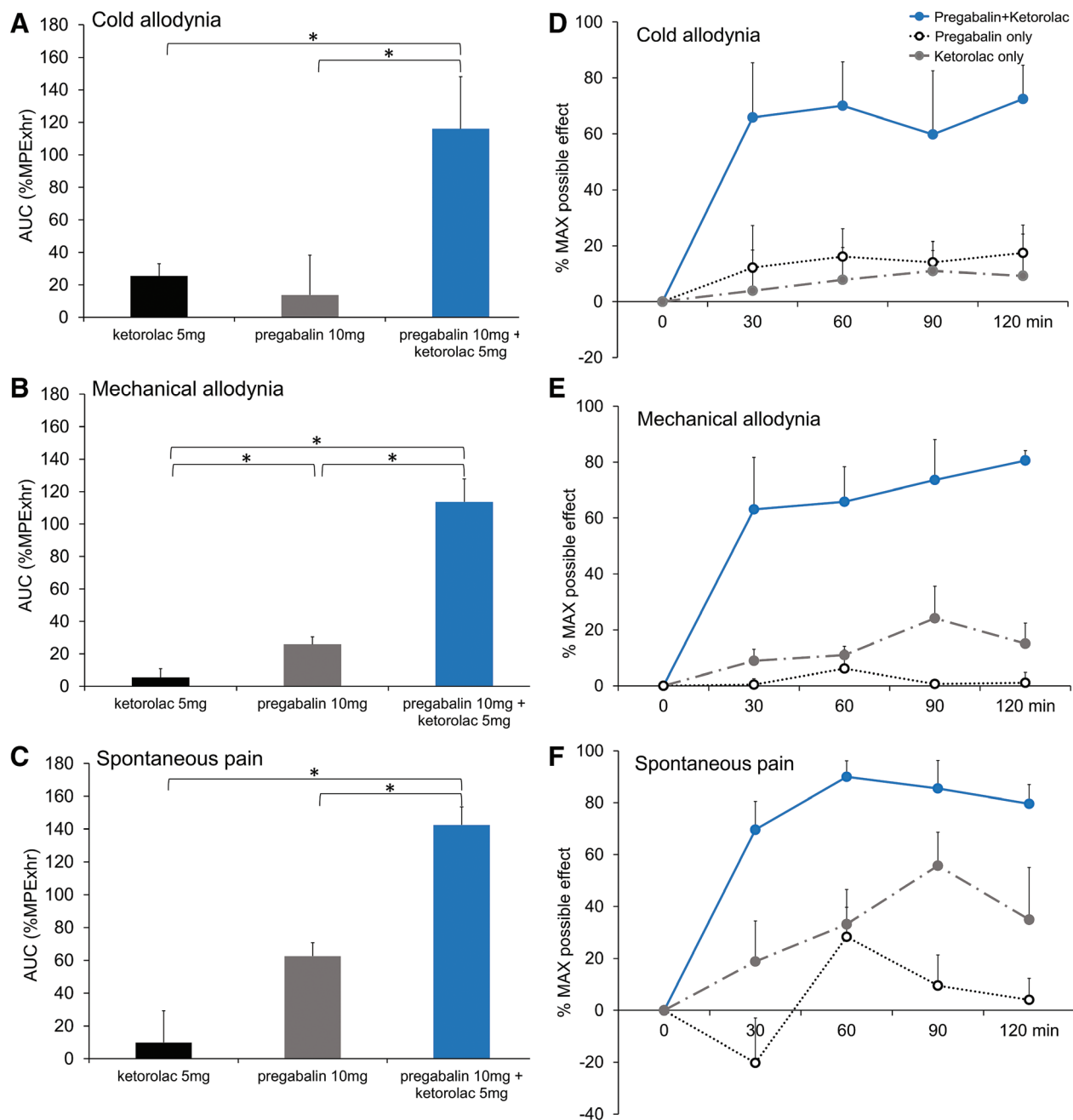


Fig. 3 Effect of combination of ketorolac with pregabalin. AUC values of cold allodynia (A), mechanical allodynia (B), and spontaneous pain (C) for pregabalin, ketorolac, and the pregabalin and ketorolac combination. Time–effect curve of cold allodynia (D), mechanical allodynia (E), and spontaneous pain (F) for pregabalin, ketorolac, and the pregabalin and ketorolac combination. Each bar represents the group AUC (% MPE × h), and each line represents the group % MPE of mean and SEM of four to six rat for each drug dosage (mg/kg). **p* < 0.05 by *t* test.

onset time of tramadol with ketorolac was not faster than that of tramadol or ketorolac alone (Fig. 4D–F).

4. DISCUSSION

Our results demonstrated that tramadol and pregabalin could attenuate SN1-induced spontaneous pain, mechanical allodynia, and cold allodynia in a dose-dependent manner in a rat model. Meanwhile, ketorolac was barely effective against these neuropathic pain symptoms. However, adding ketorolac to tramadol or pregabalin yielded strong synergistic analgesic effects that

alleviated all three observed neuropathic pain behaviors. Table presents a summary of the included studies on the combination pharmacotherapy of NSAIDs with tramadol or gabapentinoid for neuropathic pain.

In the first study, the efficacy of celecoxib and pregabalin for the treatment of chronic low back pain of various origins was studied; data were analyzed on the basis of pain quality, which was assessed using the Leeds Assessment of Neuropathic Symptoms and Signs (LANSS) pain scale. This study showed that a combination of pregabalin and celecoxib caused a significant reduction in patient-reported pain scores when the

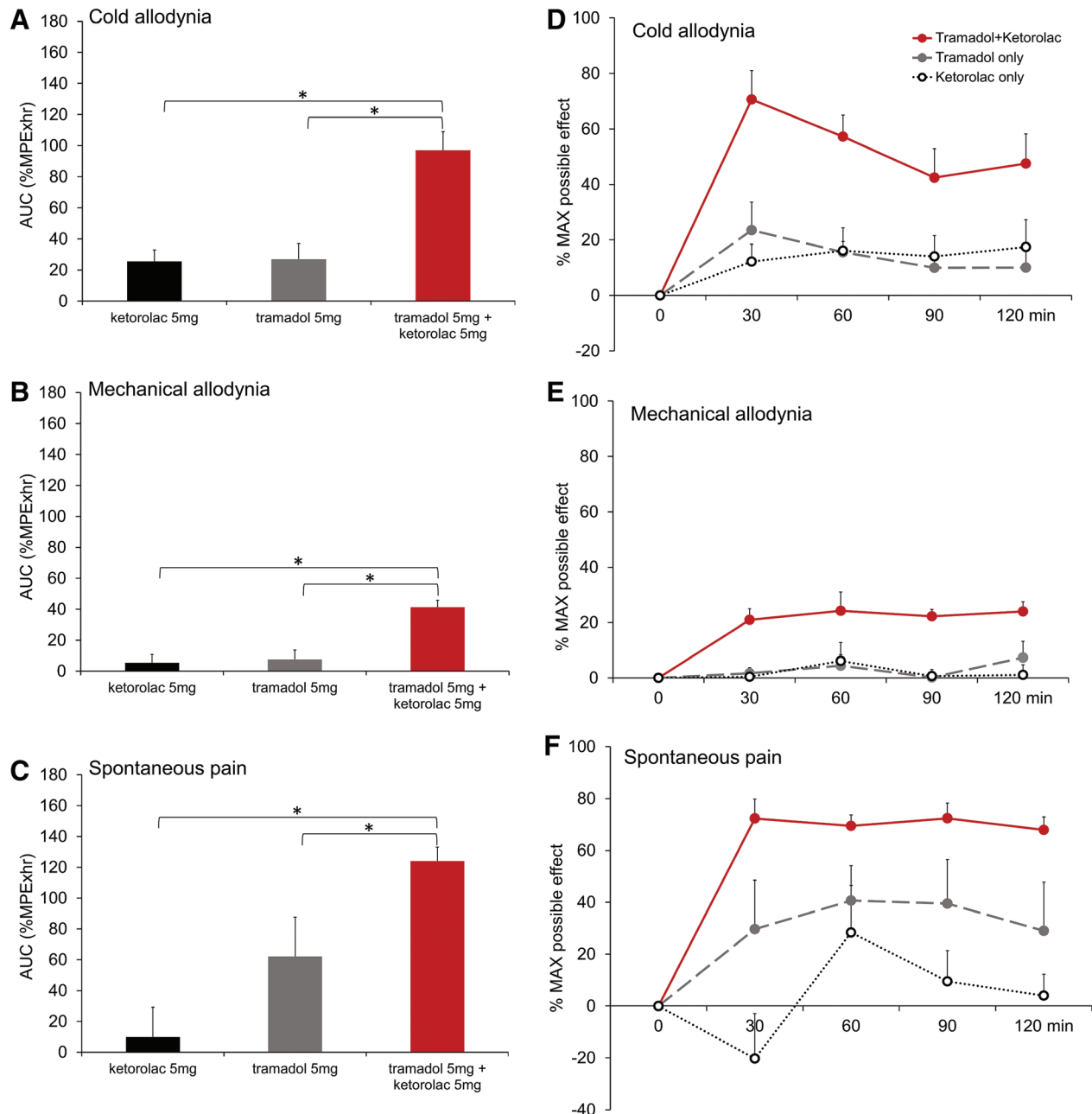


Fig. 4 Effect of combination of ketorolac and tramadol. AUC values of cold allodynia (A), mechanical allodynia (B), and spontaneous pain (C) for tramadol, ketorolac, and the tramadol and ketorolac combination. Time–effect curve of cold allodynia (D), mechanical allodynia (E), and spontaneous pain (F) for tramadol, ketorolac, and the tramadol and ketorolac combination. Each bar represents the group AUC (% MPE × h), and each line represents the group % MPE of mean and SEM of four to six rats for each drug dosage (mg/kg). * $p < 0.05$ by t test.

subpopulations were divided according to LANSS scores. The highest pain reduction (51.8%) was observed in patients with a LANSS score > 12 .¹⁸

In the second study, the interaction of five combinations of meloxicam and gabapentin were studied in a rat neuropathy model. This study showed that the AUC of the antihyperalgesic effects produced by the two-drug combination were generally similar to the theoretical sum of effects produced by each drug alone. However, 1.0 mg/kg meloxicam with 10 mg/kg gabapentin exerted synergistic anticold allodynic effects. Such a result may indicate that the interaction of the drug combinations depends on the proportion of each compound used in the dosing.¹⁹

In the third study, the effects of preoperative and postoperative administration of a combination of tramadol and meloxicam were studied after sciatic nerve ligation in rats. In the pre-emptive analgesic group, the combination of tramadol and meloxicam induced statistically significant reversal of hyperalgesia and reduced serum IL-6 production compared with postoperative treatment. In the chronic postoperative group, treatment with 6 mg/kg meloxicam plus 15 mg/kg tramadol caused a maximum significant decrease of serum PGE-2 compared with the use of each drug alone. However, the thermal pain latency test showed that the combination treatment achieved no significant difference in efficacy compared with treatment with a single agent.²⁰

Table**Studies on NSAIDs with tramadol or gabapentinoid therapy for neuropathic pain**

Reference	Trial design	Inclusion and exclusion criteria	Pain type	Intervention and dose	Outcome
Carlo Luca Romanò (2009) ¹⁸	12-week, prospective, randomized, three-way cross-over study	Age, 18–75 y; chronic low back pain for >6 mos due to disc prolapse, lumbar spondylosis and/or spinal stenosis. Minimum visual analogue scale > 40 mm (0–100 mm). Patients with neurological disease excluded.	Mix	Celecoxib 3–6 mg/kg/d + placebo (n = 36) and pregabalin 1 mg/kg/d for the first week, then 2–4 mg/kg/d + placebo (n = 36) and celecoxib 3–6 mg/kg/d + pregabalin 1 mg/kg/d for the first week, then 2–4 mg/kg/d (n = 36).	Combination therapy was more effective than either monotherapy for mean pain reduction.
Francisco Javier López-Muñoz (2016) ¹⁹	Preclinical research Male Wistar rats	Mechanical (von Frey) hyperalgesia and cold (acetone) allodynia tests were conducted 10 d after right sciatic nerve chronic constriction injury.	Neuropathic	First phase: meloxicam (0.1, 0.3, 1, 3.2, 10, or 31.6 mg/kg, p.o.) or gabapentin (3.2, 10, 31.6, 100, or 177.8 mg/kg, p.o.) were administered (n = 6 per dose). Second phase: meloxicam 0.1, 0.31, 1.0, 3.2, or 10 mg/kg was administered with gabapentin 10 mg/kg p.o. (n = 6 per dose).	The AUC of the antiallodynic effect produced by one combination (meloxicam 1.0 mg/kg + gabapentin 10 mg/kg) was greater than the theoretical sum of the effects produced by each drug alone.
Awad Rizk (2014) ²⁰	Preclinical research Male Wistar rats	Tail withdrawal latency and hot plate tests were assessed 14 d after left sciatic nerve ligation. The serum concentration of IL-6 and PGE-2 was determined at 5, 10, and 14 d.	Neuropathic	Tramadol 15 mg/kg, meloxicam 6 mg/kg, tramadol 15 mg/kg + meloxicam 6 mg/kg or saline intramuscular injected (n = 15 for each treatment) from 5 until 11 d postoperatively.	Combination drug (meloxicam 6 mg/kg + tramadol 15 mg/kg) caused a maximum significant decrease (p < 0.05) of serum PGE-2 compared with the use of each drug alone.
Tatsuo Yamamoto (2015) ⁹	Preclinical research Male Sprague Dawley rats	Mechanical allodynia and the neuroma pain were observed between 7 and 21 d after TNT surgery.	Neuropathic	The dose ratio of tramadol and indomethacin was determined using ED50 of each drug. Indomethacin:tramadol = 1:2, p.o. Naloxone (1 mg/kg, i.p.) was administered when the effect of tramadol was maximum.	ED50 tramadol > ED50 tramadol + indomethacin. The antiallodynic effect of tramadol with indomethacin was completely antagonized by naloxone.

NSAIDs = nonsteroidal anti-inflammatory drugs; TNT = tibial neuroma transposition.

In the last study, the carrageenan and tibial neuroma transposition (TNT) models were used for evaluating inflammatory and neuropathic pain. In this study, tramadol, morphine, acetaminophen, indomethacin, and their combination were orally administered. In the TNT model, both acetaminophen and indomethacin reduced the 50% effective dose (ED50) of tramadol and morphine compared with the ED50s of the single-drug study. The efficacy of these combinations for neuropathic pain was similar to that for inflammatory pain.⁹

Because tramadol and pregabalin are the recommended pharmacotherapy drugs under the current treatment guidelines for peripheral neuropathic pain but have limited efficacy,² coadministration of ketorolac may substantially enhance the efficacy of tramadol and pregabalin.

Ketorolac, a nonspecific COX inhibitor, has been shown to reduce the local inflammatory responses of injured tissue.²¹ In another report, spontaneous foot lifting was associated with spontaneous activity rate in intact C-nociceptors, which may result from cumulative neuroinflammation.²² Furthermore, spontaneous foot lifting may involve hyperpolarization-activated cyclic nucleotide-gated channels, which have active resting membrane potential and produce an excitatory action potential.²³ However, NSAIDs are still inadequate to effectively alleviate neuropathic pain.²

NSAID combination pharmacotherapy has been shown to be effective for neuropathic pain treatment.^{9,11} In the present study, tramadol or pregabalin combined with ketorolac exerted synergistic effects on all three neuropathic pain symptoms. A possible mechanism behind the potent synergistic analgesic effects of opioids and NSAIDs is the opioid inhibition of GABAergic synaptic transmission, which is potentiated by COX inhibitors due to the enzymatic conversion to pain-relieving 12-lipoxygenase products; therefore, the GABA interneurons might be a possible site

for the interaction of opioids and NSAIDs.²⁴ The antihyperalgesic effects of gabapentin and pregabalin result from action at the $\alpha_2\delta_1$ subunits of voltage-dependent Ca^{2+} channels in the dorsal root ganglia (DRG) and spinal cord. Gabapentin and pregabalin may decrease Ca^{2+} influx through the presynaptic inhibition of glutamate release through Ca^{2+} channel inhibition.²⁵ NSAIDs cause presynaptic neurons to reduce glutamate release and the excitability of postsynaptic dorsal horn neurons through inhibition of PGE_2 synthesis.^{26,27}

In the present study, when tramadol or pregabalin was combined with ketorolac, various analgesic patterns were noted in three different neuropathic pain symptoms. Neuropathic pain behaviors involve different basic mechanisms.^{28,29} For example, spontaneous and shooting pain may be produced by sodium channels that generate ectopic impulses and oscillations in DRG neurons, whereas the targets for mechanical hypersensitivity are presynaptic mu-receptors and Ca-channels. The current study demonstrated different sensory profiles. These might indicate that different classes of neurobiological mechanisms are involved, and different neuropathic pain behaviors might respond differently to treatment.^{30,31} This new pathophysiological approach may offer an additional treatment option for neuropathic pain patients.

A limitation of this study is the lack of various dose ratios used to study the neuropathic pain effect. We used effective doses of tramadol and pregabalin, as reported in previous neuropathic pain studies,^{32,33} and ineffective doses of ketorolac, as reported in a mixed pain study.¹⁴ We assumed that 25% of the ketorolac dosage might have a synergistic effect. Other combinations must be explored in further research.

This study is a first step to exploring whether combining pregabalin and tramadol with an ineffective dose of ketorolac could provide symptom-specific efficacy and mitigate neuropathic pain

in a clinical setting. Our strategy was to identify the pharmacological responses to relieve distinct sensory phenotypes. Thus, we did not quantify any molecular marker in the DRG or in the spinal cord dorsal horn in the present study. With such promising findings in behavioral observation, we intend to investigate and quantify the immunohistochemistry of chondroitin sulfate proteoglycans and brain-derived neurotrophic factor together with neuronal activity markers, such as c-fos expression³⁴ and mitogen-activated protein kinase p38 activation,³⁵ an approach that has been extensively used in our previous studies.

In conclusion, this study is the first report of synergistic analgesic effects achieved in a symptom-specific manner through the interaction of ketorolac with tramadol and pregabalin in an SNI neuropathy model. Our results provide a preclinical reference for the management of neuropathic pain, enabling more successful screening of analgesic compounds.

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