Synergistic Antinociceptive Activity of Tramadol/Acetaminophen Combination Mediated by μ-Opioid Receptors

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We investigated whether tramadol could suppress both neuropathic and inflammatory pain in mice at the same dose level. We also examined the effects of drugs metabolized by glucuronidase, such as acetaminophen (ACAP), indomethacin, probenecid, and valproate, on the antinociceptive activity of tramadol. The administration of 5.6 or 10 mg/kg tramadol suppressed cuff-induced mechanical allodynia, but 10 mg/kg tramadol did not suppress complete Freund’s adjuvant (CFA)-induced mechanical allodynia. Although neither tramadol (10 mg/kg) nor ACAP (100 mg/kg) alone produced an antinociceptive effect, their combination suppressed CFA-induced mechanical allodynia. Moreover, pretreatment naloxone, an opioid receptor antagonist, significantly attenuated the antinociceptive effects induced by the combination of tramadol and ACAP and slowed gastrointestinal transit. Similar to ACAP, the combination of tramadol and probenecid or valproate, which has the potential to inhibit uridine 5′-diphosphate (UDP)-glucuronyltransferase (UGT), also suppressed the CFA-induced mechanical allodynia and slowed gastrointestinal transit. We concluded that tramadol was more beneficial for the treatment of neuropathic pain than inflammatory pain. Furthermore, the antinociceptive effects of the tramadol and ACAP combination were mediated by the μ-opioid receptor, and were thought to be related, at least in part, to the accumulation of the active metabolite, M1.

Key words complete Freund’s adjuvant model; tramadol; acetaminophen; glucuronidation

INTRODUCTION

Tramadol, which is an atypical opioid analgesic, is used to treat moderate to severe pain. The risk of tramadol abuse is notably low; hence, the control regulations are not as stringent as those for strong opioids such as morphine. It has a potential role as a step 2 option of the WHO analgesic ladder. It is used to treat a range of pain conditions, and differs from traditional opioids as it not only acts as a μ-opioid agonist, but also has several other properties that may contribute to its analgesic effect, including serotonin and noradrenaline reuptake inhibition. It is mainly metabolized by the CYP enzyme system in the liver; the main metabolite is O-desmethyltramadol (M1), which exhibits analgesic activity and has a higher affinity for μ-opioid receptors than the parent compound. Moreover, M1 is metabolized to a non-analgesic metabolite by glucuronidation. Tramadol has reasonable efficacy in acute postoperative pain as a single agent and in combination with acetaminophen (ACAP), and has efficacy in neuropathic pain conditions. However, the data supporting the role of modified two-step analgesic ladders or oral tramadol as an alternative to codeine/acetaminophen are insufficient to permit their recommendation for routine use in cancer patients with mild-to-moderate cancer pain.

Several potential molecular mechanisms have been proposed to explain how ACAP exerts its antinociceptive effects, including the inhibition of cyclooxygenases, the activation of spinal serotonergic descending projections, and the involvement of the brain opioid system. In addition, the generation of N-arachidonoyl phenolamine (AM404) from acetaminophen through deacetylation to p-aminophenol and the subsequent conjugation with arachidonic acid by central nervous system (CNS) fatty amide hydrolase (FAAH), has indicated the possible involvement of the endocannabinoid system.

The aim of this study was to investigate whether tramadol could suppress neuropathic and inflammatory pain in mice at the same dose level. We also examined the effects of drugs metabolized by glucuronidase, such as ACAP, indomethacin, probenecid, and valproate, on the antinociceptive effects of tramadol.

MATERIALS AND METHODS

Animals  Eight-week-old male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). Six mice were housed per cage in an environment with controlled temperature (23 ± 1°C) and light cycle (lights on from 8:00–20:00), and given free access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Tokyo University of Science, and studies were conducted in accordance with the guidelines of the National Institute of Health and the Japan Neuroscience Society. All efforts were made to reduce the number of animals used and their suffering. For this study, a minimum of six animals was used for each test.

Drugs  The following drugs and chemicals were used in this study: tramadol hydrochloride from Nippon Shinyaku Co., Ltd. (Kyoto, Japan); ACAP from Pfizer Japan Inc. (Tokyo, Japan); probenecid and granisetron hydrochloride from Tokyo Chemical Industry (Tokyo, Japan); naloxone hydrochloride

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and sodium valproate from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and indomethacin from Sigma-Aldrich (St. Louis, MO, U.S.A.). Indomethacin was dissolved in water containing 5% dimethyl sulfoxide (Wako Pure Chemical Industries), 5% Tween 80 (Tokyo Chemical Industry), and 90% saline. Probenecid (10 mg/mL) was dissolved in 0.3 mol/L hydrochloric acid (0.065 mL), neutralized with 0.3 mol/L sodium hydroxide (0.1 mL), and diluted with saline (0.835 mL). Other drugs were dissolved in saline. All drugs were administered at a volume of 10 mL/kg, except probenecid (15 mL/kg).

**Neuropathic Pain Model** All surgeries were performed under aseptic conditions. Anesthesia was induced by the intraperitoneal administration of medetomidine (0.03 mg/mL), midazolam (0.4 mg/mL), and butorphanol (0.5 mg/mL) at a dose of 10 mL/kg. The common branch of the right sciatic nerve was exposed and a split section of polyethylene tubing (length = 2 mm, internal diameter = 0.38 mm, external diameter = 1.09 mm; PE-20, Imamura Co., Ltd., Tokyo, Japan) was placed around the nerve (the cuff group), as previously described.9,10) Sham-operated mice (the sham group) underwent the same procedure, but without cuff implantation.

**Inflammatory Pain Model** A persistent inflammatory pain model was produced by unilateral intraplantar injection of 50 µL complete Freund’s adjuvant (CFA; Mycobacterium tuberculosis; Sigma) into the plantar surface of the right hind paw of mice after anesthesia with 3% isoflurane. Probenecid (10 mg) was dissolved in 0.3 mol/L hydrochloride (0.1 mL), and neutralized with 0.3 mol/L sodium hydroxide (0.835 mL). Other drugs were dissolved in saline. All drugs were administered at a volume of 10 mL/kg, except probenecid (15 mL/kg).

**Measurement of the Tactile Threshold** The mechanical threshold for ipsilateral hind paw withdrawal was determined by using a series of von Frey filaments (0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0 g) (Aesthesio®; DanMic Global, San Jose, CA, U.S.A.).

**Nociceptive Test** A 0.16 g von Frey filament was placed in the middle of the plantar surface of the ipsilateral hind paw, and a nociceptive test was performed as previously described.9) Briefly, mice were placed individually in a plastic cage with a wire mesh bottom, and allowed to adapt to the testing environment for 60 min. The von Frey filaments were pressed perpendicularly against the mid-plantar surface of the hind paw from below the mesh floor and held for 3–5 s so that the filament buckled slightly. Lifting of the paw was recorded as a positive response. Stimulation of the same intensity was applied 10 times per mouse to the plantar surface of the ipsilateral hind paw at 5 s intervals. In the antagonism tests, mice were pretreated with the opioid receptor antagonist naloxone (10 mg/kg, subcutaneously (s.c.)), the α2-adrenergic receptor antagonist atipamezole (1 mg/kg, s.c.), or the serotonin (5-HT3) receptor antagonist granisetron (10 mg/kg, intraperitoneally (i.p.)) for 30 min before the administration of tramadol and/or ACAP.

**RESULTS**

**Antinociceptive Effects of Tramadol, ACAP, and Indomethacin in Neuropathic and Inflammatory Pain**

Cuff-implanted mice experienced ipsilateral mechanical allodynia that persisted for at least 4 weeks (Fig. 1a); two-way ANOVA (column factor, $p < 0.0001$; row factor, $p = 0.0001$; interaction, $p = 0.0919$) with Bonferroni’s test, **$p < 0.01$, ***$p < 0.005$ vs. sham or saline group.**

**Gastrointestinal Transit** Naive mice were orally administered blue ink (0.3 mL/mouse; Pilot Co., Ltd., Tokyo, Japan) 40 min after administration of tramadol (10 mg/kg, i.p.), ACAP (100 mg/kg, i.p.), probenecid (150 mg/kg, i.p.), or valproate (100 mg/kg, i.p.). Twenty minutes after the ink was administered, the animals were killed by cervical dislocation and the small intestines were removed. The length from the pylorus to the cecum and the distance traveled by the ink were measured. The percentage inhibition of gastrointestinal transit was calculated from the equation: (distance traveled by the ink/length from the pylorus to the cecum) × 100. In the antagonist tests, mice were pretreated with naloxone (10 mg/kg, s.c.) for 30 min before the co-administration of tramadol and ACAP.

**Data Analysis** The data were expressed as the mean ± standard errors of the mean (S.E.M.) and were evaluated by one- or two-way ANOVA followed by the Bonferroni multiple comparisons test. All statistical analyses were computed by using Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.).

Each point represents the mean ± S.E.M. of the 50% withdrawal threshold (n = 6 mice per group); **$p < 0.01$, ***$p < 0.005$ vs. sham or saline group.

Fig. 1. Changes in the Withdrawal Threshold after Mechanical Stimulation of the Ipsilateral Hind Paw Induced by Sciatic Nerve Cuffing (a) or Injection of CFA (b) in Mice

![Fig. 1](image-url)
acute tramadol treatment at either 5.6 or 10 mg/kg suppressed cuff-induced mechanical allodynia (Fig. 2a); two-way ANOVA (column factor, $p < 0.0001$; row factor, $p < 0.0001$; interaction, $p = 0.0002$) with Bonferroni’s test, **$p < 0.01$, ***$p < 0.005$ vs. saline. However, at the same doses, there was no effect on the CFA-induced mechanical allodynia (Fig. 2b); two-way ANOVA (column factor, $p = 0.3194$; row factor, $p = 0.6459$; interaction, $p = 0.1709$) with Bonferroni’s test, not significant.

Unexpectedly, ACAP suppressed both cuff- and CFA-induced mechanical allodynia. Compared with the acute injection of saline, acute ACAP treatment significantly suppressed cuff-induced mechanical allodynia in a dose-dependent manner (Fig. 2c); two-way ANOVA (column factor, $p < 0.0001$; row factor, $p = 0.0016$; interaction, $p = 0.001$) with Bonferroni’s test, *$p < 0.05$, ***$p < 0.005$ vs. saline. It also significantly suppressed the CFA-induced mechanical allodynia compared with the saline (Fig. 2d); two-way ANOVA (column factor, $p < 0.0001$; row factor, $p < 0.0001$; interaction, $p < 0.0001$) with Bonferroni’s test, ***$p < 0.005$ vs. saline.

Unlike tramadol, acute indomethacin treatment did not suppress cuff-induced mechanical allodynia compared with the vehicle (Fig. 2e); two-way ANOVA (column factor, $p = 0.3035$;
row factor, $p = 0.6918$; interaction, $p = 0.9455$) with Bonferroni’s test, not significant. In addition, in a dose-dependent manner, acute indomethacin treatment significantly suppressed the CFA-induced mechanical allodynia compared with the vehicle (Fig. 2f); two-way ANOVA (column factor, $p < 0.0001$; row factor, $p < 0.0001$; interaction, $p < 0.0001$) with Bonferroni’s test, **$p < 0.01$, ***$p < 0.005$ vs. saline or vehicle.

### Table 1. Summary of Antinociception (expressed as Mean ± S.E.M.) in Mice Following Administration of Saline, Tramadol/ACAP, or ACAP with and without Pretreatment with Naloxone, Granisetron, or Atipamezole

| Pain model | Compounds | Antagonists | Response times/10 times (administration of compounds after 30 min) |
|------------|-----------|-------------|-------------------------------------------------------------|
| Control    | Saline    | None        | $1.7 ± 0.33$                                                |
| CFA        | Saline    | None        | $8.0 ± 0.37$                                                |
|            | Tramadol (10 mg/kg, i.p.)/ACAP (100 mg/kg, i.p.) | None | $3.8 ± 0.53$                                                |
|            | ACAP (200 mg/kg, i.p.) | None | $7.3 ± 0.40^{***}$                                          |
|            | ACAP (200 mg/kg, i.p.) | Naloxone (10 mg/kg, s.c.) | $3.7 ± 0.33$                                                |
|            | ACAP (200 mg/kg, i.p.) | Atipamezole (1 mg/kg, s.c.) | $3.2 ± 0.31$                                                |
|            | ACAP (200 mg/kg, i.p.) | Granisetron (10 mg/kg, i.p.) | $2.8 ± 0.37$                                                |
|            | ACAP (200 mg/kg, i.p.) | None | $3.3 ± 0.56$                                                |
|            | ACAP (200 mg/kg, i.p.) | Naloxone (10 mg/kg, s.c.) | $7.5 ± 0.43^{***}$                                          |
|            | ACAP (200 mg/kg, i.p.) | Atipamezole (1 mg/kg, s.c.) | $7.5 ± 0.43^{***}$                                          |
|            | ACAP (200 mg/kg, i.p.) | Granisetron (10 mg/kg, i.p.) | $7.5 ± 0.43^{***}$                                          |

Each point represents the mean ± S.E.M. of the paw withdrawal scores ($n = 6$ mice per group); **$p < 0.01$, ***$p < 0.005$ vs. tramadol/ACAP combination group; $^{***}p < 0.005$ vs. ACAP alone group.

Comparison of the Antinociceptive Effects of Each Single Agent and Their Combinations in Inflammatory Pain The combination of tramadol (10 mg/kg) and ACAP (100 mg/kg) better enhanced the antinociceptive effects than tramadol or ACAP alone (Fig. 3a) in the inflammatory pain-like state; two-way ANOVA (column factor, $p < 0.0001$; row factor, $p = 0.0124$; interaction $p < 0.0001$) with Bonferroni’s test, **$p < 0.01$, ***$p < 0.005$ vs. saline.

In contrast, the combination of tramadol (10 mg/kg) and indomethacin (3 mg/kg) did not exert altered antinociceptive effects compared with tramadol or indomethacin alone (Fig. 3b) in the inflammatory pain model; two-way ANOVA (column factor, $p = 0.1398$; row factor, $p = 0.7872$; interaction,
Antagonism Test of Tramadol and/or ACAP

**Antinociception**

The results of the antagonism tests are shown in Table 1. Pretreatment with naloxone significantly attenuated the tramadol/ACAP combination-induced antinociceptive effects, whereas atipamezole and granisetron had no effect on the antinociceptive effects in the inflammatory pain model (one-way ANOVA, F[3, 20] = 15.78, p < 0.0001; Bonferroni’s test, ***p < 0.005 vs. tramadol/ACAP combination). Pretreatment with granisetron significantly attenuated ACAP-induced antinociceptive effects, whereas naloxone and atipamezole had no effect on the antinociceptive effects in the inflammatory pain model; one-way ANOVA, F[3, 20] = 28.44, p < 0.0001; Bonferroni’s test, ***p < 0.005 vs. ACAP alone.

**Antinociceptive Effects of the Combination of Tramadol and Glucuronosyltransferase Inhibitors in Inflammatory Pain**

Neither probenecid (150 mg/kg) nor valproate (100 mg/kg) suppressed CFA-induced mechanical allodynia compared with saline. However, the combination of tramadol (10 mg/kg) and probenecid (150 mg/kg) significantly suppressed CFA-induced mechanical allodynia compared with saline (Fig. 3c; two-way ANOVA (column factor, p < 0.0001; row factor, p = 0.0009; interaction, p < 0.0001)) with Bonferroni’s test, ***p < 0.005 vs. saline.

Similarly, the combination of tramadol (10 mg/kg) and valproate (100 mg/kg) also significantly suppressed CFA-induced mechanical allodynia compared with saline (Fig. 3d; two-way ANOVA (column factor, p < 0.0001; row factor, p < 0.0001; interaction p < 0.0001)) with Bonferroni’s test, **p < 0.01, ***p < 0.005 vs. saline.

**Comparison of Gastrointestinal Transit Inhibition by Each Agent Individually and Their Combinations**

The administration of tramadol (10 mg/kg) or ACAP (100 mg/kg) alone did not slow gastrointestinal transit. The tramadol/ACAP combination significantly slowed gastrointestinal transit compared with saline; one-way ANOVA, F[4, 25] = 5.78, p < 0.002; Bonferroni’s test, *p < 0.05 vs. saline; Fig. 4a. Furthermore, pretreatment with naloxone (10 mg/kg) attenuated the tramadol/ACAP-induced effect; Bonferroni’s test, *p < 0.05 vs. tramadol/ACAP combination; Fig. 4a.

Similar to ACAP, neither the administration of probenecid (150 mg/kg) nor valproate (100 mg/kg) alone slowed gastrointestinal transit. The tramadol/probenecid (one-way ANOVA, F[3, 20] = 23.34, p < 0.0001; Bonferroni’s test, ***p < 0.005 vs. saline; Fig. 4b) and tramadol/valproate (one-way ANOVA, F[3, 20] = 22.32, p < 0.0001; Bonferroni’s test, ***p < 0.005 vs. saline; Fig. 4c) combinations significantly slowed gastrointestinal transit compared with saline.

**DISCUSSION**

In this study, we found that different dose levels of tramadol were required to exert antinociceptive effects on inflammatory pain and neuropathic pain. The administration of 10 mg/kg tramadol suppressed cuffed mechanical allodynia, but not CFA-induced mechanical allodynia. It has been reported that the intraperitoneal administration of tramadol produces a dose-dependent antinociceptive activity, as measured by the hot-plate test, with an ED₉₀ value of 25.2 ± 1.3 mg/kg in CFA-treated mice. Together with previous reports, these results indicated that tramadol was more beneficial for the treatment of neuropathic than inflammatory pain.

The tramadol/ACAP combination is the result of antinociceptive (analgesic) synergy between both compounds, as demonstrated in rodent models and companion human studies. Tramadol/acetaminophen fixed-dose combination capsules were introduced as pain medication and have been shown to be effective against many pain conditions, such as osteoarthritis and low back pain. In the present study, we also observed the synergistic antinociceptive effects of the tramadol/ACAP combination in a neuropathic pain model (data not shown), but the synergistic antinociceptive effects in the inflammatory pain model were remarkable. Although the administration of tramadol (10 mg/kg) or ACAP (100 mg/kg) alone did not suppress CFA-induced mechanical allosthy, they suppressed CFA-induced mechanical allodynia when administered together. In contrast, tramadol/indomethacin combination failed to suppress CFA-induced mechanical allosthy. These results were consistent with the results of a previous report, in which the co-administration of tramadol with acetaminophen, but not indomethacin, produced a synergistic antinociceptive effect in the carrageenan test. In an inflammatory pain...
model in mice, the antinociceptive effects of the tramadol/ACAP combination were inhibited by the blockade of the opioid receptors, whereas the antinociceptive activity of ACAP alone was inhibited by blockade of the 5-HT3 receptors. This synergistic antinociceptive effect was completely antagonized by naloxone, which suggested that the antinociceptive effects of tramadol were mediated by μ-opioid receptor activation. Although tramadol is known to inhibit noradrenalin and serotonin reuptake, in this study, its antinociceptive effects were mediated only by μ-opioid receptor activation. In the present study, the contribution of its noradrenalin and serotonin reuptake inhibitor properties may be relatively smaller than that of μ-opioid receptor activation properties to its synergistic antinociceptive effects.

Tramadol may be safely combined with non-opioids, especially with ACAP, to yield an improvement in analgesia with no increase in toxicity. One major advantage of tramadol is that it causes less constipation than morphine and oxycodone. In this study, the optimal antinociceptive dose of tramadol for neuropathic pain did not slow gastrointestinal transit, whereas tramadol and ACAP combination slowed gastrointestinal transit via opioid receptors. It is therefore likely that the interaction of ACAP and tramadol was sufficient to lead to an effective concentration of M1. Similar to ACAP, the combination of tramadol and the uridine 5’-diphosphate (UDP)-glucuronosyltransferase (UGT) inhibitors, such as probenecid and valproate, suppressed inflammatory pain and slowed gastrointestinal transit. Although probenecid and valproate inhibit glucuronidation, they also have other targets. It has been reported that morphine is a substrate for the probenecid-sensitive transporters at the blood-brain barrier, consequently, co-administration of probenecid decreased the brain efflux clearance of morphine. Valproate acts on sodium and calcium channels, as well as gamma aminobutyric acid (GABA) receptors (by virtue of inhibiting GABA transaminase). The antinociceptive effects of valproate are possibly related to its action on the GABAergic system, and would therefore be able to potentiate the activity of opioid analgesics, as previously shown. Our present findings suggest that the co-administration of a UGT inhibitor (probenecid or valproate) potentiates the antinociceptive effects of tramadol in mice, but that this may not be specific to the inhibition of glucuronidation. That is to say, the antinociceptive effects of the tramadol/probenecid or tramadol/valproate combinations may be related, at least in part, to the inhibition of glucuronidation.

M1 is excreted renally, but also further metabolized to the inactive M1-glucuronide via glucuronidation by UGT2B7 and UGT1A8 in the liver. In contrast, studies in human liver microsomes and cultured hepatocytes have shown that UGT1A1, UGT1A6, UGT1A9, and UGT2B15 are involved in ACAP glucuronidation. From these previous reports, it is unlikely that ACAP inhibits M1 metabolism. However, it has been reported that recombinant expressed UGT1A8 was also found to catalyze activity towards acetaminophen, but at a 25-fold lower rate than UGT1A7. In addition, UGTs 1A7-10 exhibited strict stereoselectivity, exclusively glucuronidating the 1R,2R-enantiomer, (+)-M1. It is well known that (+)-M1 is a high-affinity ligand that produces more potent analgesic effects than the parent compound, which is a low-affinity opioid agonist. Thus, ACAP may have inhibited (+)-M1 glucuronidation mediated by the UGT1A families. In contrast, it has been reported that UGT2B7 plays a predominant role in indomethacin glucuronidation in the human liver, with the partial involvement of UGT1A9.

In this study, the indomethacin/tramadol combination failed to suppress CFA-induced mechanical allodynia. Although the mechanisms of action were not determine, it is likely that indomethacin did not competitively inhibit M1 glucuronidation mediated by UGT2B7. In this study, the serum concentrations of the M1 metabolite of tramadol were not recorded. Thus, it is unknown whether M1 serum concentrations were higher in the tramadol/ACAP combination group than in the tramadol group. This is a limitation of this study.

In conclusion, the administration of tramadol alone suppressed neuropathic pain, but not inflammatory pain, at the same dose level. Furthermore, the combination of tramadol and ACAP suppressed inflammatory pain at lower doses of tramadol than required for the administration of tramadol alone. The antinociceptive effect of the tramadol/ACAP combination was mediated by the μ-opioid receptor, and was thought to be related, at least in part, to the accumulation of the active metabolite M1.

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Conflict of Interest The authors declare no conflict of interest.

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