Regular Article

Involvement of the Peripheral μ -Opioid Receptor in Tramadol-Induced Constipation in Rodents

Kana Yasufuku,^a Katsumi Koike,^a Mika Kobayashi,^b Hiroki Chiba,^b Motoji Kitaura,^c Shino Takenouchi,^d Minoru Hasegawa,^a Yasuhide Morioka,^e Hirokazu Mishima,^e Tsutomu Suzuki,^f and Masahide Fujita^{*,a}

^aLaboratory for Drug Discovery and Disease Research, Shionogi & Co., Ltd.; 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan; ^bResearch Area for Pharmacological Evaluation, Shionogi TechnoAdvance Research Co., Ltd.; 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan; ^cGeneral Administration Division, Shionogi Administration Service Co., Ltd.; 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan; ^dTransgenic and Breeding Technology, Shionogi TechnoAdvance Research Co., Ltd.; 1405 Gotanda, Koka-cho, Koka, Shiga 520-3423, Japan; ^eMedical Affairs Department, Shionogi & Co., Ltd.; 3-1-8 Doshomachi, Chuo-ku, Osaka 541-0045, Japan; and ^fDepartment of Pharmacology, School of Pharmacy, Shonan University of Medical Sciences; 16-48 kamishinano, Totsuka-ku, Yokohama 244-0806, Japan.

Received June 1, 2021; accepted August 24, 2021

Tramadol is a weak opioid that produces analgesic effect *via* both the μ -opioid receptor (MOR) and non-opioid targets. Constipation is the most common opioid-related side effect in patients with cancer and non-cancer pain. However, the contribution of MOR to tramadol-induced constipation is unclear. Therefore, we used naldemedine, a peripherally acting MOR antagonist, and MOR-knockout mice to investigate the involvement of peripheral MOR in tramadol-induced constipation using a small intestinal transit model. A single dose of tramadol (3–100 mg/kg, *per os* (*p.o.*)) inhibited small intestinal transit dose-dependently in rats. Naldemedine (0.01–10 mg/kg, *p.o.*) blocked the inhibition of small intestinal transit induced by tramadol (30 mg/kg, *p.o.*) in rats. The transition rate increased dose-dependently over the range of naldemedine 0.01–0.3 mg/kg, and complete recovery was observed at 0.3–10 mg/kg. Additionally, tramadol (30 and 100 mg/kg, subcutaneously (*s.c.*)) inhibited small intestinal transit in wild-type mice but not in MOR-knockout mice. These results suggest that peripheral MOR participates in tramadol-induced constipation.

Key words constipation; naldemedine; μ -opioid receptor; tramadol

INTRODUCTION

Opioid analgesics are widely used to manage moderate-to-severe pain, but the clinical benefit of opioids is often accompanied by side effects including constipation, nausea/vomiting, and drowsiness.^{1,2} Tramadol is one of the most frequently used weak opioids in patients with cancer and non-cancer pain, and it produces an analgesic effect by activating the μ -opioid receptor (MOR) as well as through non-opioid mechanisms including the inhibition of noradrenaline and serotonin (5-HT) transporters.^{3,4} The main metabolite of tramadol, *O*-desmethyl tramadol (M1), has weaker affinity for MOR than strong opioids such as morphine,⁵ which is associated with a lower incidence of opioid-related side effects.⁶

Constipation is the most common opioid-related side effects in patients treated with opioids, and it is persistently observed even with the concomitant use of laxatives. Meanwhile, reduced doses may be used in some patients to achieve pain relief.⁷ Constipation is reported in 3.4–45% of patients treated with tramadol.⁸ Tramadol and M1 display affinity for targets including receptors and ion channels.^{9,10} However, the mechanism of tramadol-induced constipation has not been fully revealed. The use of tramadol to treat chronic pain is continuously increasing in the U.S.A.¹¹ and Europe,^{12–14} and thus, providing a proper diagnosis and treatment for tramadol-induced constipation would benefit these patients.

Naldemedine, an oral peripherally acting MOR antagonist

(PAMORA), is clinically used for the treatment of constipation.¹⁵ In a non-clinical study, naldemedine reversed morphine-induced constipation without affecting antinociceptive responses in rats,¹⁶ indicating the essential role of peripheral MOR in morphine-induced constipation. Therefore, in this study, we used naldemedine to investigate the involvement of peripheral MOR in tramadol-induced constipation.

MATERIALS AND METHODS

Animals Male Wistar and Sprague–Dawley (SD) rats weighing less than 200 g were obtained from Charles River Laboratories Japan Inc. (Kanagawa, Japan). MOR-knockout (MOR-KO) mice and control mice were used at 12 weeks old. All animals were housed in cages under 12/12-h light/dark cycles and provided standard food and tap water *ad libitum*. The study was conducted according to the guidelines for animal experimentation of Shionogi & Co., Ltd. (Osaka, Japan).

Development of MOR-KO Mice MOR-KO mice were developed by Taconic Biosciences (Rensselaer, NY, U.S.A.). A targeting vector was injected into B6-derived embryonic stem cells, and a recombinant clone was selected. Chimeric mice were developed by injecting the selected clone into B6 blastocysts. Then, chimeric mice were successively mated with CAG-Flp mice and CMV-Cre mice to remove Neo and exons 2–3 from Floxed-MOR mice, respectively. To confirm gene KO in mice, genomic DNA was isolated from the tail and ana-

*To whom correspondence should be addressed. e-mail: masahide.fujita@shionogi.co.jp

lyzed using PCR and the following primers: Primer A, 5'-CAG ACTAAGCATGGCAGTGC-3'; Primer B, 5'-GAAGACCCT GACAGTAGACC-3'; Primer C, 5'-CAGGACTTGGTAGTG AATCC-3'; internal control forward, 5'-GAGACTCTGGCT ACTCATCC-3'; and internal control reverse, 5'-CCTTCAGCA AGAGCTGGGGAC-3'.

Drugs Naldemedine tosylate was synthesized by Shionogi & Co., Ltd. and administered orally at a dose of 0.01–10 mg/kg. Tramadol hydrochloride was obtained from AK Scientific, Inc. (Union City, CA, U.S.A.) and administered orally or subcutaneously at a dose of 3–100 mg/kg. Morphine hydrochloride was manufactured by Shionogi & Co., Ltd. and administered subcutaneously at a dose of 3 or 30 mg/kg. Orally administered drugs were dissolved in 0.5% carboxymethyl cellulose, whereas injected drugs were dissolved in 0.9% saline. The administration volumes were 2 and 10 mL/kg in rats and mice, respectively. Randomized non-blinded administrations of naldemedine were performed for evaluation of drug efficacy.

Evaluation of Small Intestinal Transit Wistar rats were fasted for 18–22 h with water provided *ad libitum* before the evaluation. Rats were administered tramadol or morphine, followed by the intragastric administration of 0.5% Evans Blue dye (Wako Pure Chemical Corporation, Osaka, Japan) after 30 min. After 15 min, rats were euthanized *via* cervical dislocation, and the stomach and small intestine were quickly removed. The distance traveled by the dye relative to the total length of the small intestine was measured. Naldemedine was administered 15 min before tramadol or morphine, and the effect was calculated as the percent maximal possible effect (%MPE), as previously described.¹⁶⁾ The mouse experiment was performed using a similar method.

Evaluation of Nociceptive Responses Nociceptive responses were evaluated by the tail-flick test (Ugo-Basile, Comerio, VA, Italy) in SD rats. Thermal stimulation was applied to the ventral surface of the tail, and the latency of the tail withdrawal reflex was measured using a cutoff time of 20 s. The anti-nociceptive effect of tramadol was expressed as %MPE, which was calculated as $(T_1 - T_0) \times 100 / (T_2 - T_0)$, where T_0 and T_1 were the tail-flick latencies before and after tramadol administration, respectively, and T_2 was the cutoff time.

Measurement of Plasma Drug Concentrations The plasma concentrations of tramadol and M1 were measured using LC (LC30AD; Shimadzu, Kyoto, Japan) with tandem mass spectrometry (API 5000TM; SCIEX, Foster City, CA, U.S.A.). Blood samples of SD rats were collected from the juvenile vein at 0.083, 0.25, 0.5, 1, 1.5, 2, and 3 h after tramadol administration and centrifuged at 3000 rpm for 10 min at 4°C. The obtained plasma samples were stored in a freezer until analysis. The concentrations were determined according to a previously described method.¹⁶⁾

Data Analysis All data are presented as the mean $\pm$ standard error of the mean (S.E.M.). Statistical significance was determined using Student's *t*-test for non-paired samples. For multiple comparisons, ANOVA was performed, followed by Dunnett's *post-hoc* test or the Tukey–Kramer test. Statistical significance in the *post-hoc* comparison was indicated by $p < 0.05$. GraphPad Prism 6.0 software (San Diego, CA, U.S.A.) was used to perform statistical analyses.

RESULTS

Pharmacological Effects of Tramadol in Rats First, we investigated the effects of tramadol on small intestinal transit and nociceptive responses in rats. An oral dose of tramadol decreased small intestinal transit dose-dependently in rats (Fig. 1A). The inhibitory effect of tramadol compared to that of vehicle was significantly better at doses of 10–100 mg/kg ($p < 0.05$ for 10 mg/kg, $p < 0.0001$ for 30 and 100 mg/kg). Tramadol also produced anti-nociceptive responses in rats (Fig. 1B). %MPE was increased dose-dependently at 60 min after administration, and significant changes were observed at doses of 10–100 mg/kg ($33.5 \pm 3.6\%$ at 10 mg/kg, $36.2 \pm 6.5\%$ at 30 mg/kg, and $57.6 \pm 11.6\%$ at 100 mg/kg).

Pharmacokinetics of Tramadol in Rats Next, we measured the plasma concentrations of tramadol and its active metabolite M1 after an oral dose of tramadol (10–100 mg/kg). The peak plasma concentrations of tramadol and M1 increased dose-dependently in rats (Table 1).

Effect of Naldemedine on the Tramadol-Induced Inhibition of Small Intestinal Transit in Rats We selected a tramadol dose of 30 mg/kg, which significantly inhibited small intestinal transit and nociceptive responses in rats, and investigated the effects of naldemedine on tramadol-induced constipation. Naldemedine (0.01–10 mg/kg) dose-dependently reversed the inhibition of small intestinal transit, and significant reversal was observed at doses of ≥ 0.03 mg/kg ($p < 0.01$ for 0.03 mg/kg, $p < 0.0001$ for 0.1–10 mg/kg, Fig. 2A). The ED₅₀ of naldemedine for tramadol-induced constipation was calculated as 0.050 ± 0.011 mg/kg, which was similar to that for

Fig. 1. Effects of Tramadol on Small Intestinal Transit and Nociceptive Responses in Rats

Small intestinal transit (A) and nociceptive responses (B) were measured in rats after an oral dose of tramadol or vehicle. Small intestinal transit was measured 45 min after administration. Data are presented as the mean and S.E.M. ($n = 8$ for small intestinal transit and $n = 6$ for nociceptive responses). Differences between the groups were compared using Dunnett's test (A) or two-way ANOVA followed by the Tukey *post-hoc* test (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1. Plasma Concentrations of Tramadol and Its Metabolite M1 after an Oral Dose of Tramadol in Rats

		Plasma concentration (ng/mL)	
		Tramadol	M1
Tramadol 10 mg/kg	$C_{\max}$ (ng/mL)	187.7 ± 115.7	111.8 ± 10.8
	$T_{\max}$ (h)	0.9 ± 0.6	0.5 ± 0.3
Tramadol 30 mg/kg	$C_{\max}$ (ng/mL)	567.7 ± 349.8	300.0 ± 89.6
	$T_{\max}$ (h)	0.7 ± 0.3	0.6 ± 0.2
Tramadol 100 mg/kg	$C_{\max}$ (ng/mL)	1194.3 ± 522.7	361.7 ± 16.3
	$T_{\max}$ (h)	1.4 ± 0.9	0.7 ± 0.2

Data are presented as the mean and S.E.M. ($n=3$). M1, *O*-desmethyl tramadol; $C_{\max}$, peak plasma concentration; $T_{\max}$, time to peak plasma concentration.

Fig. 2. Effects of Naldemedine on the Tramadol-Induced Inhibition of Small Intestinal Transit in Rats

Rats were administered naldemedine (*p.o.*) followed by tramadol (*p.o.*) or morphine (*s.c.*) after 15 min. After 45 min, small intestinal transit was measured, and the effects of naldemedine are presented as the transition rate (A) and percent maximal possible effect (%MPE) (B). Data are presented as the mean and S.E.M. ($n=10$ for tramadol and $n=6$ for morphine). Student's *t*-test was used to compare the tramadol and vehicle groups (** $p<0.001$). Dunnett's test was used to compare the naldemedine and vehicle groups (## $p<0.01$, ### $p<0.001$).

morphine-induced constipation ($ED_{50} = 0.053 \pm 0.013$ mg/kg, Fig. 2B).

Effect of MOR-KO on the Tramadol-Induced Inhibition of Small Intestinal Transit in Mice To reveal the direct involvement of MOR, we established MOR-KO mice (Figs. 3A, B) and investigated the efficacy of MOR deletion on tramadol-induced constipation. Tramadol at a dose of 30 or 100 mg/kg decreased small intestinal transit in WT mice compared to the effects of vehicle, whereas the changes were completely elimi-

nated in MOR-KO mice (Fig. 3C). Morphine also decreased small intestinal transit in WT mice, but no effect was detected in MOR-KO mice (Fig. 3C).

DISCUSSION

In the present study, we investigated the involvement of peripheral MOR in tramadol-induced constipation in rodents. We found that the tramadol-induced inhibition of small intestinal transit was completely blocked by naldemedine pretreatment and MOR deletion in rats and mice, respectively. These results suggest the direct involvement of MOR in the tramadol-induced inhibition of small intestinal transit. Our previous report indicated that naldemedine improves the morphine-induced inhibition of small intestinal transit without affecting anti-nociceptive effects mediated by the central nervous system, which partly modulates gastrointestinal activity in rats.¹⁸) This study revealed that the dose-response curve of naldemedine was comparable between tramadol and morphine, suggesting that peripheral MOR is involved in the tramadol-induced inhibition of small intestinal transit.

To investigate the efficacy of naldemedine, we used a tramadol dose of 30 mg/kg, which produced significant anti-nociceptive effects in rats. The dose also induced anti-allodynic effects in rat neuropathic pain models induced by chronic constriction injury¹⁹) and by the systemic injection of reserpine.²⁰) Pharmacokinetic analysis revealed the dose-dependent increases of tramadol and M1 concentrations in rat plasma. This is consistent with previous reports.^{21,22}) The peak plasma concentration of tramadol in rats that received an oral dose of 30 mg/kg tramadol was similar to that in healthy human subjects treated with 100 mg of tramadol,²³) a dose that is widely used for pain relief in patients with cancer and non-cancer pain.²⁴) Additionally, we subcutaneously administered tramadol to mice because little research examined its oral administration. We found that 30 or 100 mg/kg tramadol inhibited small intestinal transit in mice, consistent with previous research.²⁵) A mouse pharmacokinetic study reported that the subcutaneous administration of tramadol produced a similar ratio of tramadol and M1 to that in humans, in contrast to the large difference observed between humans and mice following oral administration.^{23,26}) These findings suggest that the dose and administration route of tramadol used in this study were suitable for speculating the effects in humans.

Although several factors are involved in intestinal motility,²⁷) 5-HT is an important neurotransmitter that accelerates intestinal motility. The 5-HT₃ antagonist alosetron and 5-HT₄ agonist tegaserod are used to treat diarrhea and constipation from irritable bowel syndrome, respectively.²⁸) Tramadol may increase the levels of 5-HT in the intestine by inhibiting 5-HT transporters, indicating that 5-HT might be related with less frequent constipation in patients treated with tramadol than in those treated with strong opioids. Furthermore, tramadol has affinity for non-opioid targets including receptors and ion channels.^{9,10}) For example, tramadol, but not M1, directly inhibits the muscarinic acetylcholine receptor M₃,²⁹) which is functionally expressed in gastrointestinal tract smooth muscle.³⁰) The selective M₃ antagonist darifenacin increases the risk of constipation in patients with overactive bladder.³¹) Tramadol has weaker affinity for MOR than M1,⁵) but the ratio of tramadol per M1 in blood is higher in human than that in

Fig. 3. Effect of *MOR* Deletion on the Tramadol-Induced Inhibition of Small Intestinal Transit in Mice

(A) Schematic diagram of the targeting strategy. Exons 2 and 3 of *MOR* were placed into a PGK-Neo cassette. The white boxes indicate the exon numbers of *MOR* (ex). (B) Specific signals of a targeted allele by genomic PCR. PCR was performed using primer sets indicated by arrows in Fig. 3A (left: primer A/B, right: primer A/C). The samples isolated from heterogeneous (+/-), homogenous (-/-), wild-type (+/+), positive control (P), and wild-type control (C) were used. P indicates the wild-type and floxed allele in the left panel and gene knockout (KO) in the right panel. M, DNA marker. (C) Effect of tramadol on small intestinal transit in *MOR*-KO mice and Floxed-*MOR* mice. Mice were subcutaneously administered tramadol or morphine, and small intestinal transit was measured after 45 min. Data are presented as the mean and S.E.M. ($n = 6-8$). Two-way ANOVA followed by the Tukey *post-hoc* test was used to compare the tramadol or morphine group with the vehicle group (### $p < 0.001$) or *MOR*-KO group with Floxed *MOR* group (*** $p < 0.001$).

rodents.^{21-23,26} These reports indicate that the anti-cholinergic effects of tramadol might be partly associated with tramadol-induced constipation in patients. Further research is required to reveal the involvement of other mechanisms in humans.

MOR is expressed in submucosal ganglia, myenteric ganglia, the myenteric plexus, and the lamina propria.³² Opioids bind to MOR in enteric excitatory or inhibitory motor neurons in the myenteric plexus and inhibit the release of neurotrans-

mitters such as acetylcholine, decreasing smooth muscle contraction.³³ As a limitation in this study, we evaluated small intestinal transit to investigate the involvement of MOR in tramadol-induced constipation, but other groups reported a role of MOR in the large intestine in opioid-induced constipation.^{34,35} Our previous report also found that naldemedine blocked the morphine-induced inhibition of large intestinal transit in isolated guinea pig tissue,¹⁶ revealing that MOR ex-

pression in the large intestine might be involved in tramadol-induced constipation. Furthermore, we did not evaluate the defecation, one of common clinical symptoms of constipation, in this study. Further studies are required to examine the gastrointestinal effects of tramadol by using other experimental methods.

In summary, many patients treated with weak opioids experience constipation, which is treated with laxatives and PAMORAs. We revealed the direct involvement of peripheral MOR in the tramadol-induced inhibition of small intestinal transit in rodents, suggesting the usefulness of naldemedine as a mechanism-based therapy for constipation induced by weak opioids including tramadol.

Acknowledgments We thank Joe Barber Jr., PhD, for editing a draft of this manuscript.

Conflict of Interest KY, KK, MH, YM, HM and MF are employees of Shionogi & Co., Ltd. MK, HC and ST are employees of Shionogi TechnoAdvance Research Co., Ltd. MK is employee of Shionogi Administration Service Co., Ltd. TS has received funding from Shionogi & Co., Ltd.

REFERENCES

- 1) Swegle JM, Logemann C. Management of common opioid-induced adverse effects. *Am. Fam. Physician*, **74**, 1347–1354 (2006).
- 2) Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R. Opioid complications and side effects. *Pain Physician*, **11** (Suppl.), S105–S120 (2008).
- 3) Raffa RB, Friderichs E, Reimann W, Shank RP, Codd EE, Vaught JL. Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an 'atypical' opioid analgesic. *J. Pharmacol. Exp. Ther.*, **260**, 275–285 (1992).
- 4) Desmeules JA, Piguet V, Collart L, Dayer P. Contribution of monoaminergic modulation to the analgesic effect of tramadol. *Br. J. Clin. Pharmacol.*, **41**, 7–12 (1996).
- 5) Gillen C, Haurand M, Kobelt DJ, Wnendt S. Affinity, potency and efficacy of tramadol and its metabolites at the cloned human mu-opioid receptor. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **362**, 116–121 (2000).
- 6) Avouac J, Gossec L, Dougados M. Efficacy and safety of opioids for osteoarthritis: a meta-analysis of randomized controlled trials. *Osteoarthritis Cartilage*, **15**, 957–965 (2007).
- 7) Christensen HN, Olsson U, From J, Breivik H. Opioid-induced constipation, use of laxatives, and health-related quality of life. *Scand. J. Pain*, **11**, 104–110 (2016).
- 8) Langley PC, Patkar AD, Boswell KA, Benson CJ, Schein JR. Adverse event profile of tramadol in recent clinical studies of chronic osteoarthritis pain. *Curr. Med. Res. Opin.*, **26**, 239–251 (2010).
- 9) Minami K, Ogata J, Uezono Y. What is the main mechanism of tramadol? *Naunyn Schmiedeberg's Arch. Pharmacol.*, **388**, 999–1007 (2015).
- 10) Minami K, Uezono Y, Ueta Y. Pharmacological aspects of the effects of tramadol on G-protein coupled receptors. *J. Pharmacol. Sci.*, **103**, 253–260 (2007).
- 11) Bigal LM, Bibeau K, Dunbar S. Tramadol Prescription over a 4-Year Period in the USA. *Curr. Pain Headache Rep.*, **23**, 76 (2019).
- 12) Hider-Mlynarz K, Cavalié P, Maison P. Trends in analgesic consumption in France over the last 10 years and comparison of patterns across Europe. *Br. J. Clin. Pharmacol.*, **84**, 1324–1334 (2018).
- 13) Kalkman GA, Kramers C, van Dongen RT, van den Brink W, Schellekens A. Trends in use and misuse of opioids in the Netherlands: a retrospective, multi-source database study. *Lancet Public Health*, **4**, e498–e505 (2019).
- 14) Hurtado I, García-Sempere A, Peiró S, Sanfeliix-Gimeno G. Increasing trends in opioid use from 2010 to 2018 in the region of Valencia, Spain: a real-world, population-based study. *Front. Pharmacol.*, **11**, 612556 (2020).
- 15) Blair HA. Naldemedine: a review in opioid-induced constipation. *Drugs*, **79**, 1241–1247 (2019).
- 16) Kanemasa T, Koike K, Arai T, Ono H, Horita N, Chiba H, Nakamura A, Morioka Y, Kihara T, Hasegawa M. Pharmacologic effects of naldemedine, a peripherally acting μ -opioid receptor antagonist, in *in vitro* and *in vivo* models of opioid-induced constipation. *Neurogastroenterol. Motil.*, **31**, e13563 (2019).
- 17) Nakamura A, Hasegawa M, Ito H, Minami K, Koike K, Habu-Tomita N, Nanba K, Hamaguchi K, Noshi E, Hashimoto H, Nishino I, Okabayashi Y, Koyabu K, Kihara T, Iwamoto Y, Inoue Y, Narita M, Suzuki T, Kato A. Distinct relations among plasma concentrations required for different pharmacological effects in oxycodone, morphine, and fentanyl. *J. Pain Palliat. Care Pharmacother.*, **25**, 318–334 (2011).
- 18) Thörn SE, Wattwil M, Lindberg G, Säwe J. Systemic and central effects of morphine on gastroduodenal motility. *Acta Anaesthesiol. Scand.*, **40**, 177–186 (1996).
- 19) Corona-Ramos JN, De la O-Arciniegua M, Déciga-Campos M, Medina-López JR, Domínguez-Ramírez AM, Jaramillo-Morales OA, Espinosa-Juárez JV, López-Muñoz FJ. The antinociceptive effects of tramadol and/or gabapentin on rat neuropathic pain induced by a chronic constriction injury. *Drug Dev. Res.*, **77**, 217–226 (2016).
- 20) Kaneko K, Umehara M, Homan T, Okamoto K, Oka M, Oyama T. The analgesic effect of tramadol in animal models of neuropathic pain and fibromyalgia. *Neurosci. Lett.*, **562**, 28–33 (2014).
- 21) Parasrampur R, Vuppugalla R, Elliott K, Mehvar R. Route-dependent stereoselective pharmacokinetics of tramadol and its active O-demethylated metabolite in rats. *Chirality*, **19**, 190–196 (2007).
- 22) Godoy AL, De Moraes NV, Martinez EZ, Carvalho TM, Marques MP, Lanchote VL. Simultaneous analysis of tramadol, O-desmethyltramadol, and N-desmethyltramadol enantiomers in rat plasma by high performance liquid chromatography-tandem mass spectrometry: application to pharmacokinetics. *Chirality*, **23**, 287–293 (2011).
- 23) Ardakani YH, Rouini MR. Improved liquid chromatographic method for the simultaneous determination of tramadol and its three main metabolites in human plasma, urine and saliva. *J. Pharm. Biomed. Anal.*, **44**, 1168–1173 (2007).
- 24) Leppert W. Tramadol as an analgesic for mild to moderate cancer pain. *Pharmacol. Rep.*, **61**, 978–992 (2009).
- 25) Dürsteler C, Mases A, Fernandez V, Pol O, Puig MM. Interaction between tramadol and two anti-emetics on nociception and gastrointestinal transit in mice. *Eur. J. Pain*, **10**, 629–638 (2006).
- 26) Evangelista Vaz R, Draganov DI, Rapp C, Avenel F, Steiner G, Arras M, Bergadano A. Preliminary pharmacokinetics of tramadol hydrochloride after administration via different routes in male and female B6 mice. *Vet. Anaesth. Analg.*, **45**, 111–122 (2018).
- 27) Bharucha AE, Lacy BE. Mechanisms, evaluation, and management of chronic constipation. *Gastroenterology*, **158**, 1232–1249.e3 (2020).
- 28) Ford AC, Brandt LJ, Young C, Chey WD, Foxx-Orenstein AE, Moayyedi P. Efficacy of 5-HT 3 antagonists and 5-HT 4 agonists in irritable bowel syndrome: systematic review and meta-analysis. *Am. J. Gastroenterol.*, **104**, 1831–1843, quiz, 1844 (2009).
- 29) Shiga Y, Minami KMD, Shiraishi M, Uezono YMD, Murasaki O, Kaibara M, Shigematsu A. The inhibitory effects of tramadol on muscarinic receptor-induced responses in xenopus oocytes expressing cloned M3 Receptors. *Anesth. Analg.*, **95**, 1269–1273 (2002).
- 30) Eglen RM, Hegde SS, Watson N. Muscarinic receptors and smooth

- muscle function. *Pharmacol. Rev.*, **48**, 531–565 (1996).
- 31) Chapple C, Steers W, Norton P, Millard R, Kralidis G, Glavind K, Abrams P. A pooled analysis of three phase III studies to investigate the efficacy, tolerability and safety of darifenacin, a muscarinic M3 selective receptor antagonist, in the treatment of overactive bladder. *BJU Int.*, **95**, 993–1001 (2005).
- 32) Imam MZ, Kuo A, Ghassabian S, Smith MT. Progress in understanding mechanisms of opioid-induced gastrointestinal adverse effects and respiratory depression. *Neuropharmacology*, **131**, 238–255 (2018).
- 33) Holzer P. Opioid receptors in the gastrointestinal tract. *Regul. Pept.*, **155**, 11–17 (2009).
- 34) Mori T, Shibasaki Y, Matsumoto K, Shibasaki M, Hasegawa M, Wang E, Masukawa D, Yoshizawa K, Horie S, Suzuki T. Mechanisms that underlie μ -opioid receptor agonist-induced constipation: differential involvement of μ -opioid receptor sites and responsible regions. *J. Pharmacol. Exp. Ther.*, **347**, 91–99 (2013).
- 35) Matsumoto K, Umemoto H, Mori T, Akatsu R, Saito S, Tashima K, Shibasaki M, Kato S, Suzuki T, Horie S. Differences in the morphine-induced inhibition of small and large intestinal transit: Involvement of central and peripheral μ -opioid receptors in mice. *Eur. J. Pharmacol.*, **771**, 220–228 (2016).