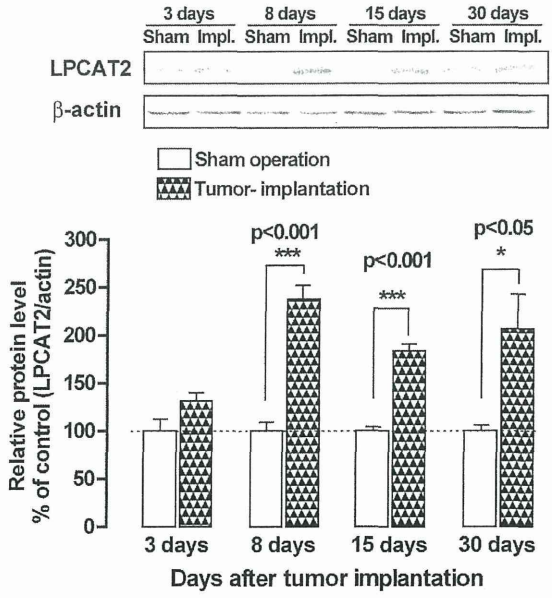


Figure 3. Changes in lysophosphatidylcholinr acyltransferase 2 (LPCAT2), an inducible PAF synthesis enzyume, in the spinal cords of mice after implantation of tumor cells. Alteration of spinal LPCAT2 expression 3, 8, 15, and 30 days after the levels of immunoreactivity were normalized to that of β-actin and represented as % induction compared with the values of sham-operated (deaden cells implanted) mice (mean ± SEM., n = 5–7). *P<0.05, ***P<0.001 versus the corresponding values in sham-operated mice (unpaired Student's *t*-test).
doi:10.1371/journal.pone.0091746.g003

Treatment with TCV-309 Prolonged the Survival of FBC Mice

The effect of treatment with TCV-309 was evaluated in FBC mice (Figure 8). Median survival was significantly prolonged by repeated treatment with TCV-309; about 50% of FBC mice receiving saline died up to 26 days after tumor implantation, while about 50% of FBC mice receiving TCV-309 at 0.3 mg/kg every 4 days died up to 50 days. Gain of body weight by tumor-bearing mice was small during the observation period and the change in body weight was similar between control and TCV-308 treated mice (Figure 8 insert).

Discussion

Bone cancer pain is often very complex; bone is highly innervated with C fibers, which are triggered by an inflammatory infiltrate secondary to cancer cells and others including acids, cytokine, growth factors, etc along with primary afferent destruction following osteoclast activation. Additionally, bone resorption weakens the bone under torsion, thus exciting mechanosensitive fibers within mineralized bone. The most intractable pain is often neuropathic in origin. However, in bone cancer pain, there is a unique neurochemical reorganization of the spinal cord, as well peripheral sensitization of afferent fibers innervating the cancerous bone, while spinal synaptic transmission mediated through Aδ and C fibers is enhanced in the substantia gelationsa across a wide area of lumbar levels following sarcoma implantation in the femur [12]. As the disease progresses, analgesics effective to treat inflammatory or neuropathic pain, even opioids, are frequently insufficient in this pain state [13,14]. It has been reported that toll-like receptor (TLR) 4, which plays an important role in glial activation in neuropathic pain increased in the spinal expression of a rat model of cancer-induced bone pain and intrathecal injection of TLR4 siRNA or TLR4 signaling pathway blockers led to a pain relieving effect at an early stage, but not at day 16 of cancer-induced bone pain [15]. The present study demonstrated that intravenous administration of PAF receptor antagonists, TCV-309, BN 50739 and WEB 2086, effectively ameliorated allodynia and pain behaviors such as guarding and limb-use abnormalities in FBC mice. The pain relieving effects of PAF receptor antagonists are long lasting. We have previously suggested that the anti-allodynia effect of PAF antagonists in sciatic nerve injured mice is at least in part mediated by spinal relief of PAF-induced dysfunction of GlyRα3 [8]. In agreement with this concept, the present results showed that the intrathecal introduction of siRNA of PAF receptor mRNA effectively improved bone cancer pain behaviors in FBC mice. DRG contains a PAF synthesis enzyme, LPCAT2, and PAF receptor mRNA was increased in the ipsilateral DRG after nerve injury [6]. LPCAT2 mRNA and PAF receptor mRNA were increased in the spinal microglia after nerve injury in a rat spared nerve injury model [7]. Several studies have showed the ability of several cancer cell types to produce PAF and express PAF receptors on their membranes [16–20]. In the present study, the amount of LPCAT2 protein increased in the spinal cord of FBC mice, although in which cells the increase occurred remains to be elucidated. Therefore, PAF signaling in the microenvironment of the spinal pain transduction system may be increased by bone cancer due to peripheral nerve injury. We have further revealed a unique mode of action of TCV-309; TCV-309 is a specific competitive inhibitor of PAF receptors [21], but the potency of TCV-309 intensified as a function of time after administration, and the mode of action changed from a competitive manner within several hours after the injection of TCV-309 to a non-competitive manner later [8]. The intensification of the anti-allodynia potency of TCV-309 and change in its mode of action to a non-competitive manner as a function of time led us to speculate about a different mechanism of action; such as down-regulation of PAF receptors by binding TCV-309 to PAF receptors in the later stage of after nerve injury. This idea may explain the long lasting pain relieving effect of TCV-309.

The dose of morphine to block bone cancer pain was ten times that required to block inflammatory pain behaviors [12]. Morphine became less effective than oxycodone in FBC mice [22,23]. Differential attenuation of μ-opioid receptor activation between morphine and oxycodone in FBC mice was reported, the former was significant whereas the latter was limited [24]. According with the evidence, morphine at 10 mg/kg s.c. had a tiny anti-allodynia effect in the present study, and the analgesic effect of a higher dose of 30 mg/kg of morphine was underestimated due to increased locomotion.

The essential finding in the present study is the enhanced pain reliving effect by combined administration of PAF receptor antagonists and morphine. For example, the combination of TCV-309 as low as 1 μg/kg i.v. and morphine 0.3 mg/kg s.c. (each drug had no pain relieving effect by itself) produced a significant anti-allodynia effect in FBC mice. Guarding behavior and limb-use abnormality were also reversed by the combined administration (data not shown). It is interesting that the enhanced pain relieving effect of PAF receptor antagonists, TCV-309, BN 50739 and WEB 2086 on morphine still remained at 8 days after the administration of these PAF receptor antagonists.

Constipation resulting from treatment with opioids is the most common component of a more general condition – opioid-induced

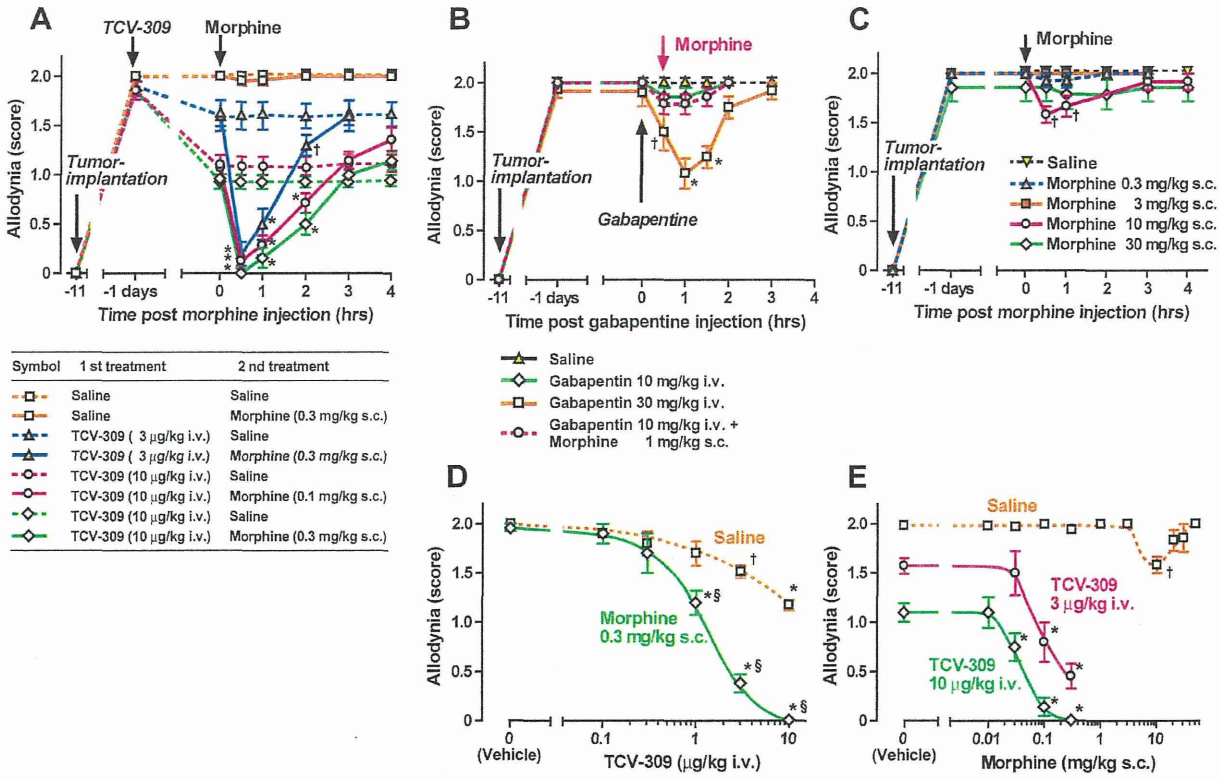


Figure 4. Enhanced pain reliving effect with a combination of TCV-309 and morphine in FBC mice. TCV-309, 3 and 10 μg/kg were injected i.v. at 10 days post tumor implantation and morphine 0.1 mg/kg and 0.3 mg/kg were injected s.c. 1 day after the injection of TCV-309 (A). Gabapentine 10 and 30 mg/kg i.v. were injected 11 days post tumor transplantation and morphine, 1 mg/kg was injected at 30 min after gabapentin injection (B). Morphine 0.3–30 mg/kg was injected at 11days post tumor transplantation (C). Pain-like behaviors were evaluated at 20 min after morphine injection. Various doses of TCV-309 were injected i.v. at 11 days post tumor implantation and morphine 0.3 mg/kg were injected s.c. 1 days after the injection of TCV-309 (D). One day after the injection of TCV-309, various doses of morphine were injected (E). Control mice received injections with a vehicle: saline. The pain-related behaviors that developed after tumor implantation in mice were not affected by vehicle treatments. Each group contained 11 mice. †P<0.05, *P<0.01 compared with the corresponding control (vehicle treated) values, as determined by analysis of variance followed by Tukey-Kramer test. §P<0.01 compared with the corresponding control (vehicle treated without morphine) values, as determined by analysis of variance followed by an unpaired Student's *t*-test.
doi:10.1371/journal.pone.0091746.g004

bowel dysfunction [25]. Tolerance commonly develops to opioids during long-term use, requiring increased doses to achieve the same analgesia. Although some tolerance develops against the effects of opioids on gastrointestinal motility, constipation often persists unless remedial measures are taken [26]. Using lower doses of opioids will not prevent constipation because the dose that produces constipation is approximately 4-fold less than the analgesic dose [25,27]. Therefore, some patients may discontinue opioids to avoid constipation. The present study showed that while a dose of morphine 10 mg/kg s.c. exhibited only a tiny pain relieving effect, 0.1 and 0.3 mg/kg of morphine in combination with PAF receptor antagonists produced a significant pain relieving effect. This result suggests that the combination use of morphine with PAF receptor antagonists may reduce the effective dose of morphine needed to below constipating dose. Activation of μ-receptors by morphine can have any of several effects depending on receptor location. Mu-opioid receptors in the central nervous system modulate pain perception and can depress respiratory function, while those in the gastrointestinal tract reduce bowel motility [26]. If μ-receptor-mediated signaling in the gastrointestinal tract was enhanced by PAF receptor antagonists, it would make defecation more difficult. The dose-response curve of morphine-induced defecation was not affected at all by 0.3 mg/kg of TCV-309, which is the dose producing the maximal analgesic effect and thus the possibility to avoid the appearance of morphine-induced constipation is suggested. On the other hand, PAF antagonists may cause side effects by interfering with the physiological roles of PAF, such as regulation of blood pressure, immunological or inflammatory responses, Ca^{2+} mobilization in polymorphonuclear leucocyte, or implantation of embryos, as shown by the creation of PAF receptor-transgenic and PAF receptor-deficient mice [28]. The possible side effects of PAF receptor antagonists could be reduced by combination with morphine. Therefore, good quality management of bone cancer pain could be achieved with a combination of morphine and PAF receptor antagonists. The long lasting effects of PAF receptor antagonists make it possible to relieve pain by repeated treatment at 4 day intervals and the results showed that these antagonists were effective from an early stage to a late stage, 30 days after tumor implantation at which point more than 50% of control mice died. The results further showed no formation of tolerance to repeated treatment with PAF receptor antagonists.

A role for PAF in tumor development has been suggested by the spontaneous development of skin tumors in transgenic mice

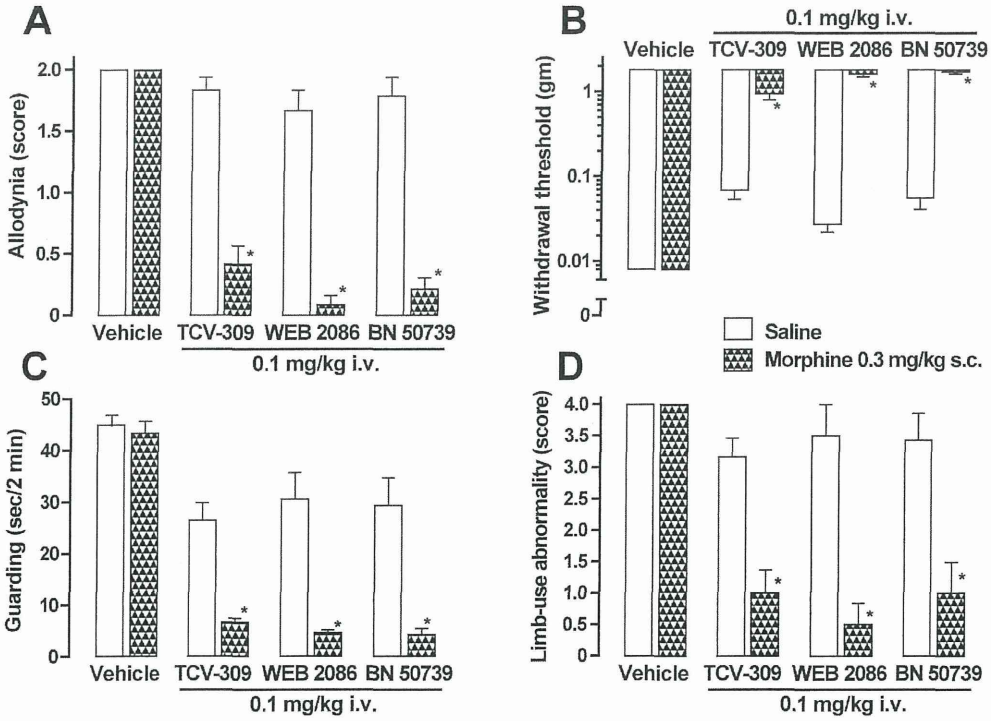


Figure 5. Enhanced pain reliving effect of TCV-309, WEB 2086, BN 50739 and morphine in FBC mice. TCV-309, WEB 2086 and BN 50739 were administered at 11 days post tumor implantation. Morphine 0.3 mg/kg s.c. was injected at 8 days after the injection of PAF receptor antagonists. Allodynia (A, B), guarding behavior (C) and limb-use abnormality (D) were evaluated at 20 min after the injection of morphine. Values represent the mean ± SEM. n = 11 mice per group. *P<0.01 compared with the corresponding control values, as determined by analysis of variance followed by an unpaired Student's *t*-test.
doi:10.1371/journal.pone.0091746.g005

overexpressing PAF receptors [29]. PAF also induced an autocrine proliferative loop in an endometrial cancer cell line HEC-1A [30], induced migration of Kaposi's cells [31], promoted migration and proliferation of tumor cells and neo-angiogenesis [17], acted as a promoter of melanoma metastasis [32], while PAF receptor-dependent pathways control tumor growth [33]. In addition, PAF receptor antagonists suppress cancer growth, proliferation and metastasis [20,32–34]. The improvement in the survival of femoral bone tumor-bearing mice with TCV-309 may be due to its tumor suppressive action. As an another possibility, pain is an exquisite stressor and a cause for potential immune dysfunction, as shown in immunosuppression during the perioperative period, and thus poor pain control is assumed to promote tumor growth. Lilleme et al. [35] reported that patients with preexisting pain who received chemical splanchnicectomy with alcohol showed a significant improvement in survival. The authors reported that the achievement of better pain control with chemical splanchnicectomy may prolong life. Whether the improvement in survival by PAF receptor antagonist may include its indirect effect via suppression of pain is remained to be clarified.

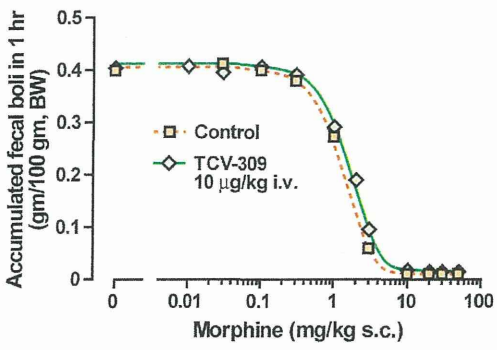


Figure 6. Morphine-induced constipation with or without TCV-309. Normal mice received with various doses of morphine and the accumulated feces on the floor over 60 min were weighed. TCV-309 was injected 1 day before morphine administration. Data are expressed as the mean. n = 10 mice per group.
doi:10.1371/journal.pone.0091746.g006

Although the possibility could not be entirely ruled out that the tumor suppressive action of PAF antagonists may partly participate in the pain relieving effect of the repeated treatment of PAF antagonists in the FBC model, the acute pain relieving effect of PAF antagonists may be independent from anti-tumor action because the effect developed shortly after the intravenous injection and even by intrathecal injection.

Opiates in which their mechanisms of analgesia include the enhancement of the descending inhibitory pathway connected to the inhibitory inter-neurons such as glycinergic and GABAergic neurons. Recent findings emphasize that a reduction in the $GABA_A$ receptor– and glycine receptor-mediated synaptic inhibition; ie., disinhibition of inhibitory neurotransmission within the dorsal horn, is implicated in the generation of neuropathic pain and may be a cause for insufficiency of morphine analgesia. Thus, reinforcement of the glycinergic neurotransmission by glycine transporter inhibitors are proposed as a novel drug discovery

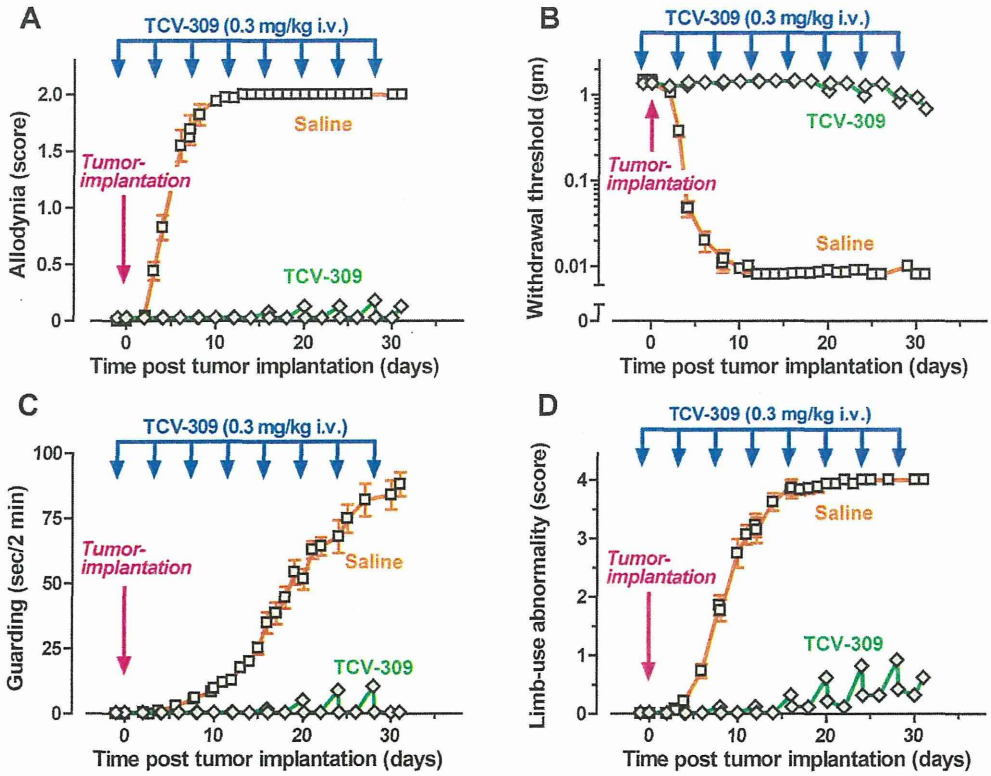


Figure 7. Effect of the repeated administration of TCV-309 on the pain-like behaviors in FBC mice. The administration of TCV-309 0.3 mg/kg i.v. was started 6 hr before the tumor implantation, given once a day and continued every 4 days up to 28 days. Allodynia (A, B), guarding behavior (C) and limb-use abnormality (D) were evaluated at 3 hr and 1, 2, 3 days after TCV-309 injection. Data are expressed as the mean ± SEM. n = 15 mice per group.
doi:10.1371/journal.pone.0091746.g007

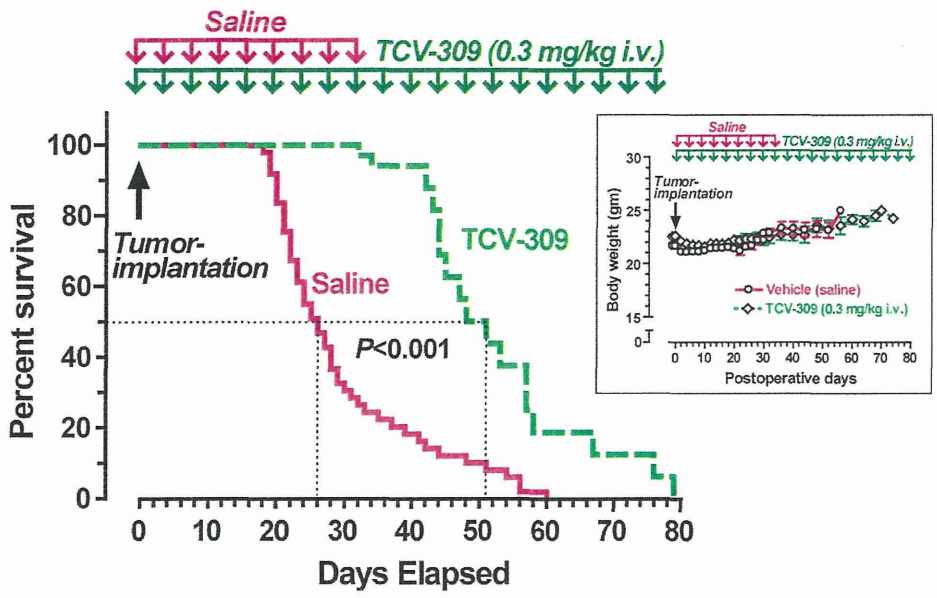


Figure 8. The Kaplan-Mayer survival curve of FBC mice and the change of body weight (insert). For the survival experiments, TCV-309 and saline were given once a day and continued every 4 days until the animals died (n = 17 and 50, respectively). Control mice received saline for 32 days. Days for 50% of mice died after receiving TCV-309 were significantly prolonged compared to the saline-treated control, P<0.001. Statistical analysis was performed by log-rank and Gehan–Breslow–Wilcoxon tests.
doi:10.1371/journal.pone.0091746.g008

strategy for neuropathic pain [36]. Taking that PAF via an increase in nitric oxide/cyclic GMP cascade reduces GlyRα3 function in the spinal cord [5], the combination of PAF receptor antagonists and opioids, the former protects from disfunction of PAF-induced inhibitory neurotransmission and the latter enhances descending inhibitory pathway may represent a new strategy for the treatment of persistent cancer pain and the quality/quantity of life of patients.

Acknowledgments

The authors wish to thank Dr. Z. Terashita (Takeda Pharmaceutical Co. Kyoto, Japan) and Dr. P. Braquet (Institute Henri Beaufour, Le Plessis Robinson, France) for generously donating TCV-309 and BN 50739, respectively. This work was carried out with equipment from the Natural Science Center for Basic Research and Development, Hiroshima University, and the National Cancer Center Research Institute.

Author Contributions

Conceived and designed the experiments: KM TD NM SS. *Performed the experiments:* KM NM T. Kitayama SS. Analyzed the data: KM NM T. Kanamatsu SS YU. Contributed reagents/materials/analysis tools: NM KM T. Kanematsu. Wrote the paper: TD KM NM.

References

1. Mundy GR (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2: 584–593.
2. Portenoy RK, Lesage P (1999) Management of cancer pain. Lancet 353: 1695–1700.
3. de Wit R, van Dam F, Loonstra S, Zandbelt L, van Buuren A, et al. (2001) The amsterdam pain management index compared to eight frequently used outcome measures to evaluate the adequacy of pain treatment in cancer patients with chronic pain. Pain 91:339–349.
4. Morita K, Morioka N, Abdin J, Kitayama S, Nakata Y, et al. (2004) Development of tactile allodynia and thermal hyperalgesia by intrathecally administered platelet-activating factor in mice. Pain 111: 351–359.
5. Morita K, Kitayama T, Morioka N, Dohi T (2008) Glycinergic mediation of tactile allodynia induced by platelet-activating factor (PAF) through glutamate-NO-cyclic GMP signalling in spinal cord in mice. Pain 138: 525–536.
6. Hasegawa S, Kohro Y, Shiratori M, Ishii S, Shimizu T, et al. (2010) Role of PAF receptor in proinflammatory cytokine expression in the dorsal root ganglion and tactile allodynia in a rodent model of neuropathic pain. PLoS One 5, e10467.
7. Okubo M, Yamanaka H, Kobayashi K, Kanda H, Dai Y, et al. (2012) Up-regulation of Platelet-activating factor synthases and its receptor in spinal cord contribute to development of neuropathic pain following peripheral nerve injury. Mol Pain 8: 8.
8. Motoyama N, Morita K, Kitayama T, Shiraishi S, Uezono Y, et al. (2013) Pain-releasing action of Platelet-activating factor (PAF) antagonists in neuropathic pain animal models and the mechanisms of action. Eur J Pain 17: 1156–1167.
9. Honore P, Mantyh PW (2000) Bone cancer pain: from mechanism to model to therapy. Pain Med 1: 303–309.
10. Luger NM, Sabino MA, Schwei ML, Mach DB, Pomonis JD, et al. (2002) Efficacy of systemic morphine suggests a fundamental difference in the mechanisms that generate bone cancer vs inflammatory pain. Pain 99: 397–406.
11. Minami T, Nishihara I, Ito S, Sakamoto K, Hyodo M, et al. (1995) Nitric oxide mediates allodynia induced by intrathecal administration of prostaglandin E_2 or prostaglandin $F_{2\alpha}$ in conscious mice. Pain 61: 285–290.
12. Yanagisawa Y, Furue H, Kawamata T, Uta D, Yamamoto J, et al. (2010) Bone cancer induces a unique central sensitization through synaptic changes in a wide area of the spinal cord. Mol Pain 6: 38.
13. Portenoy RK, Rayne D, Jacobsen P (1999) Breakthrough pain: characteristics and impact in patients with cancer pain. Pain 81: 129–134.
14. Becker R, Jakob D, Uhle EI, Riegel T, Bertalanffy H (2000) The significance of intrathecal opioid therapy for the treatment of neuropathic cancer pain conditions. Stereotact Funct Neurosug 75: 16–26.
15. Li X, Wang X-W, Feng X-M, Zhou W-J, Wang Y-Q, et al. (2013) Stage-dependent anti-allodynia effects of intrathecal Toll-like receptor 4 antagonists in a rat model of cancer induced bone pain. J Physiol Sci DOI 10.1007/s12576-D12-0244-5.
16. Bussolino F, Arese M, Montrucchio G, Barrs L, Primo L, et al. (1995) Platelet-activating factor produced in vitro by Kaposi's Sarcoma cells induces and Sustains in vivo angiogenesis. J Clin Invest 96: 940–952.
17. Bussolati B, Biancone L, Cassoni P, Russo S, Rola-Pleszczynski M, et al. (2000) PAF produced by human brest cancer cells promotes migration and proliferation of tumor cells and neo-angiogenesis. Am J Pathol 157: 1713–1725.
18. Bussolati B, Russo S, Deambrosis I, Cantaluppi V, Volpe A, et al. (2002) Expression of CD154 on renal cell carcinomas and effect on cell proliferation, motility and platelet-activating factor synthesis. Int J Cancer 100: 654–661.
19. Fallani A, Grieco B, Barletta E, Mugnai G, Giorgi G, et al. (2002) Synthesis of platelet-activating factor (PAF) in transformed cell lines of a different origin. Prostaglandins Other Lipid Mediat 70: 209–226.
20. Tsoupras AB, Iatrou C, Frangia C, Demopoulos CA (2009) The implication of platelet-activating factor in cancer growth and metastasis: Potent beneficial role of PAF-inhibitors and antioxidants. Infectious Disorders-Drug Targets 9: 390–399.
21. Terashita Z-I, Kawamura M, Takatani M, Tsushima S, Imura Y, et al. (1992) Beneficial effects of TCV-309, a novel potent and selective platelet-activating factor antagonist in endotoxin and anaphylaxis shock in rodents. J Pharmacol Exp Ther 260: 748–755.
22. Narita M, Nakamura A, Ozaki M, Imai S, Miyoshi K, et al. (2008) Comparative Pharmacological profiles of morphine and oxycodone under a neuropathic pain-like state in mice: evidence for less sensitivity to morphine. Neuropsychopharmacology 33: 1097–1112.
23. Minami K, Hasegawa M, Ito H, Nakamura A, Tomii T, et al. (2009) Morphine, oxycodone, and fentanyl exhibit different analgesic profiles in mouse pain models. J Pharmacol Sci 111:60–72.
24. Nakamura A, Hasegawa M, Minami K, Kanbara T, Tomii T, et al. (2013) Differential activation of the μ-opioid receptor by oxycodone and morphine in pain-related brain regions in a bone cancer pain model. Br J Pharmacol 168: 375–388.
25. Yuan C-S (2005) Handbook of opioid bowel syndrome. New York: Haworth Medical Press: Haworth Reference Press. 256 p.
26. Akil H, Owens C, Gutstein H, Taylor L, Curran E, et al. (1998) Endogenous opioids: overview and current issues. Drug Alcohol Depend 51: 127–140.
27. Yuan C-S, Foss JF (2000) Antagonism of gastrointestinal opioid effects. Reg Anesth Pain Med 25: 639–642.
28. Honda Z, Ishii S, Shimizu T (2002) Platelet–activating factor receptor. J Biochem 131: 773–779.
29. Ishii S, Nagase T, Tashiro F, Ikuta K, Sato S, et al. (1997) Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor. EMBO J 16: 133–142.
30. Maggi M, Bonaccorsi L, Finetti G, Carioni V, Muratori M, et al. (1994) Platelet-activating factor mediates an autocrine proliferative loop in the endometrial adenocarcinoma cell line HEC-1A. Cancer Res 54: 4777–4784.
31. Biancone L, Cantaluppi V, Boccellino M, Bussolati B, Del Sorbo L, et al. (1999) Motility induced by human immunodeficiency virus-1 Tat on Kaposi's sarcoma cells requires platelet-activating factor synthesis. Am J Pathol 155: 1731–1739.
32. Melnikova V, Bar-Eli M (2007) Inflammation and melanoma growth and metastasis: The role of platelet-activating factor (PAF) and its receptor. Cancer Metastasis Rev 26: 359–371.
33. de Oliveira SI, Andrade LNS, Onuchic AC, Nonogaki S, Fernandes PD, et al. (2010) Platelet-activating factor receptor (PAF-R)-dependent pathways control tumor growth and tumor response to chemotherapy. BMC Cancer 10:200
34. Im SY, Ko HM, Kim JW, Lee HK, Ha TY, et al. (1996) Augmentation of tumor metastases by platelet-activating factor. Cancer Res 56: 2662–2665.
35. Lillemoe KD, Cameron JL, Kaufman HS, Yeo CJ, Pitt HA, et al. (1993) Chemical splanchnicectomy in patients with unresectable pancreatic cancer. A prospective randomized trial. Annals Surgery 217: 447–457.
36. Dohi T, Morita K, Kitayama T, Motoyama N, Morioka N (2009) Glycine transporter inhibitors as a novel drug discovery strategy for neuropathic pain. Pharmacol Ther 123: 54–79.

ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

Novel Delta Opioid Receptor Agonists with Oxazatricyclodecane Structure

Hideaki Fujii,*[,†] Kohei Hayashida,[†] Akiyoshi Saitoh,[‡] Akinobu Yokoyama,[§,∥] Shigeto Hirayama,[†] Takashi Iwai,[†] Eriko Nakata,[⊥] Toru Nemoto,[†] Yuka Sudo,[§,∥] Yasuhito Uezono,[§] Mitsuhiko Yamada,[‡] and Hiroshi Nagase[†,#]

[†]School of Pharmacy, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

[‡]Department of Neuropsychopharmacology, National Institute of Mental Health, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashimachi, Kodaira, Tokyo 187-8553, Japan

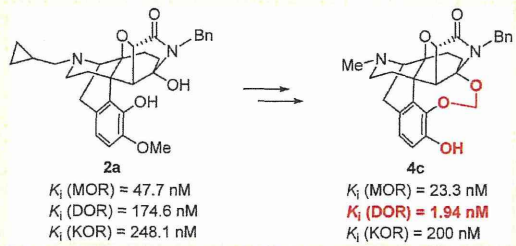
[§]Division of Cancer Pathophysiology, National Cancer Center Research Institute, 5-1-1 Tsukiji, Tokyo 104-0045, Japan

[∥]Faculty of Pharmaceutical Science, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 274-8510, Japan

[⊥]Discovery Research Laboratories, Nippon Chemiphar Co., Ltd., 1-22 Hikokawado, Misato, Saitama 341-0005, Japan

Supporting Information

ABSTRACT: We synthesized compounds **4a,c−f,h,i** containing the oxazatricyclodecane structure from a novel rearrangement reaction product **2a**. All the prepared compounds **4a,c−f,h,i** exhibited full agonistic activities for the δ opioid receptor (DOR). Among them, the *N*-methyl derivative **4c** was highly selective, and the most effective DOR agonist in functional assays. Subcutaneous administration of **4c** produced dose-dependent and NTI (selective DOR antagonist)-reversible antinociception lacking any convulsive behaviors in the mice acetic acid writhing tests. The *N*-methyl derivative **4c** is expected to be a promising lead compound for selective DOR agonists with a novel chemotype.



KEYWORDS: *Opioid, DOR, oxazatricyclodecane structure, CellKey*

The δ opioid receptor (DOR) is one of the three opioid receptor types (μ (MOR), DOR, and κ (KOR)), and activation of this receptor is associated with various pharmacological effects such as antinociceptive, antidepressive, anxiolytic, and cardioprotective effects.[1−3] In contrast to the undesirable effects mediated by the MOR such as dependence, constipation, emesis, and respiratory depression or the aversive effects mediated by the KOR,[4,5] the DOR is a promising medical target because it seems to induce neither addictive nor aversive effects. Since the first nonpeptidic DOR agonist TAN-67[6,7] (Figure 1) emerged,[3] various nonpeptidic DOR agonists have been reported.[1−3] Several investigations revealed that the DOR agonists like BW373U86[8] and SNC80[9] (Figure 1) exerted convulsive behaviors.[3] However, some DOR agonists such as ADL5747[10] and KNT-127[11,12] (Figure 1) have recently been reported to induce no convulsion. Although SNC80 has been reported to induce the internalization of the DORs and to develop tolerance toward the analgesic, locomotor, and anxiolytic effects, ARM390[13] (Figure 1) induced hardly any internalization of the DORs and showed tolerance to analgesia but not to locomotor or anxiolytic responses.[14,15] Thus, a distinct DOR agonist interacting with the same DOR sometimes exerted different pharmacological responses. Recently, SNC80, a well-known representative selective DOR agonist, was reported to activate the MOR/DOR heteromer more selectively than the DOR homomer.[16] It is not yet clear why the various DOR agonists mentioned above elicit different pharmacological responses, but the structure of the DOR agonist may account, in part, for their distinct activities. For example, a structural feature of DOR agonists may influence the induction of convulsive behaviors: the DOR agonists that do not cause convulsion had a structure distinct from diarylmethylpiperazine and its related structures such as BW373U86 and SNC80.[3] However, diarylmethylpiperazine derivative AZD2327 (Figure 1) reportedly produced no convulsion.[17] The synthesis and pharmacological characterization of DOR agonists with different chemotypes will help to better understand the different pharmacological profiles of distinct DOR agonists. We have recently reported the synthesis and binding affinities for the MOR, DOR, and KOR of an oxazatricyclodecane derivative **2a**, which was obtained from endoethanotetrahydrothebaine derivative **1** by a novel rearrangement reaction[18] (Scheme 1). This new compound exhibited moderate affinities for the opioid receptors (K_i (MOR) = 47.7 nM, K_i (DOR) = 174.6 nM, and K_i (KOR)

Received: November 29, 2013
Accepted: January 27, 2014
Published: January 27, 2014

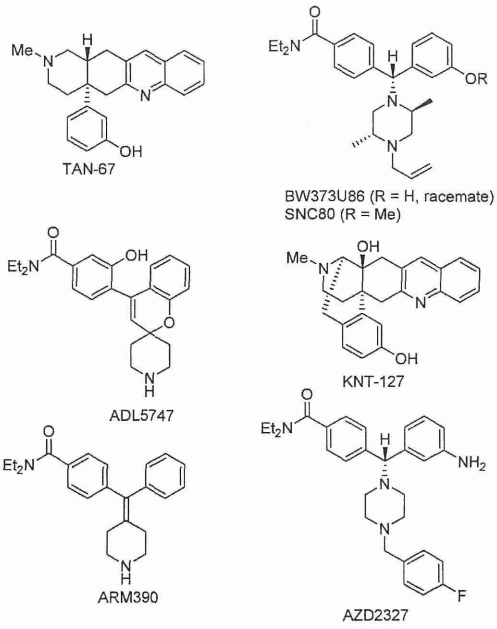
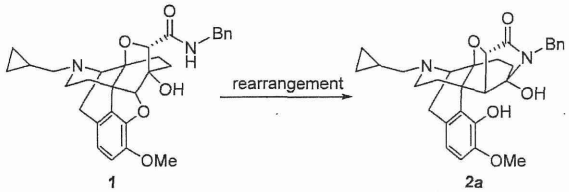
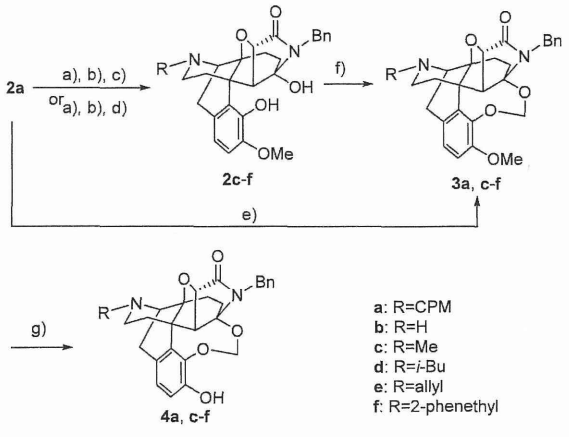


Figure 1. Structures of DOR agonists, TAN-67, BW373U86, SNC80, ADL5747, KNT-127, ARM390, and AZD2327.

Scheme 1. Potential Opioid Ligand 2a

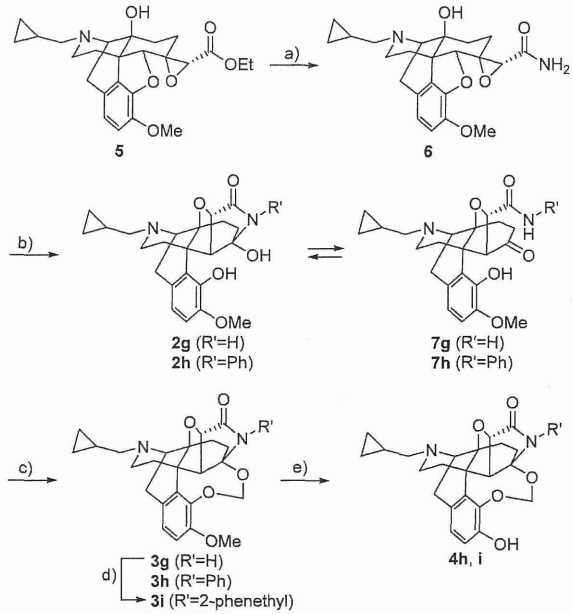


Scheme 2. Synthesis of 4a,c–f[a]



[a]Reagents and conditions: (a) Troc-Cl, K_2CO_3, 1,1,2,2-tetrachloroethane, 150 °C; (b) Zn, AcOH, rt, 80% from **2a**; (c) aldehyde, AcOH, $NaB(OAc)_3H$, 1,2-dichloroethane, rt, 74%-quant. (for R = Me, 2-phenethyl); (d) alkyl bromide, $NaHCO_3$, DMF, rt, 48–92% (for R = allyl, *i*-Bu); (e) CH_2Br_2, K_2CO_3, DMF (0.0005 M), rt, 66%; (f) CH_2ClBr, K_2CO_3, DMF (0.0004 M), rt, a solution of **2c–f** in DMF was added portion-wise. 69–98%; (g) BBr_3, CH_2Cl_2, 0 °C, 76–95%.

Scheme 3. Synthesis of 4h and 4i[a]



[a]Reagents and conditions: (a) 12 M NH_3aq, EtOH, rt, 73%; (b) *t*-BuOK, *t*-BuOH, reflux, quant.; (c) CH_2ClBr, K_2CO_3, DMF (0.0004 M), rt, a solution of **2g** and **7g** or **2h** and **7h** in DMF was added portion-wise. 73–93%; (d) 60% NaH, $PhCH_2CH_2Br$, DMF, rt, 83%; (e) BBr_3, CH_2Cl_2, 0 °C, 61–89%.

Table 1. Binding Affinities of 4a,c–f,h,i for the Opioid Receptors[a]

	K_i (nM)			selectivity	
compd	MOR[b]	DOR[c]	KOR[d]	MOR/DOR	KOR/DOR
SNC80	695	1.04	>1000	668	962
2a[e]	47.7	175	248	0.27	1.4
4a	3.14	0.313	5.14	10.0	16.4
4c	23.3	1.94	200	12.0	103
4d	186	7.00	119	26.6	17.0
4e	68.4	1.23	56.6	55.8	46.2
4f	45.9	2.59	588	17.7	227
4h	4.61	0.534	1.69	8.6	3.2
4i	1.75	1.16	1.94	1.5	1.7

[a]Binding assays were carried out in duplicate using mouse whole brain without cerebellum membranes for MOR and DOR or guinea pig cerebellum membranes for KOR. [b][^{3}H] DAMGO was used. [c][^{3}H] DPDPE was used. [d][^{3}H] U-69,593 was used. [e]Ref 18.

=248.1 nM). The potential opioid ligand **2a** was expected to lead to other ligands selective for an opioid receptor type with a unique core structure. Herein, we report the synthesis of novel DOR agonists **4a,c–f,h,i** with oxazatricyclodecane structure derived from **2a** and their pharmacological properties.

The synthesis of the objective compounds **4a,c–f** commenced with compound **2a**[18] (Scheme 2). The treatment of **2a** with 2,2,2-trichloroethyl chloroformate (Troc-Cl) in the presence of K_2CO_3 and the subsequent zinc/AcOH treatment gave norcompound **2b**.[19] Various *N*-substituents were introduced by reductive alkylation of **2b** or the alkylation of **2b** with an alkyl bromide to provide **2c–f**. Compound **2a** reacted with CH_2Br_2 in the presence of K_2CO_3 under high dilution

dx.doi.org/10.1021/ml400491k | ACS Med. Chem. Lett. 2014, 5, 368–372

Table 2. Functional Activities of 4a,c–f,h,i for the Opioid Receptors Assessed by [^{35}S]GTPγS Binding Assays[a]

	MOR		DOR		KOR	
compd	EC_{50} (nM)	E_{max} (%)[b]	EC_{50} (nM)	E_{max} (%)[c]	EC_{50} (nM)	E_{max} (%)[d]
SNC80	NT[e]	NT[e]	1.9	100	NT[e]	NT[e]
4a	2.8	13.7	1.1	92.8	80.5	69.1
4c	113	110	11	112	478	83.6
4d	223	8.4	15.6	96.4	760	65.6
4e	2.7	5.4	6.5	94.6	231	74.0
4f	2.3	83.0	9.2	115	ND[f]	ND[f]
4h	9.0	25.6	0.98	118	6.5	42.9
4i	2.1	19.7	0.41	103	3.9	51.2

[a][^{35}S]GTPγS binding assays were carried out in duplicate using human MOR, DOR, or KOR expressed CHO cells. [b]E_{max} was calculated as the % of the response obtained with DAMDO. [c]E_{max} was calculated as the % of the response obtained with SNC80. [d]E_{max} was calculated as the % of the response obtained with U-69,593. [e]Not tested. [f]Not determined.

Table 3. Functional Activities of 4a,c–f,h,i for the Opioid Receptors Assessed by CellKey Assays[a]

	MOR		DOR		KOR	
compd	EC_{50} (nM)	E_{max} (%)[b]	EC_{50} (nM)	E_{max} (%)[c]	EC_{50} (nM)	E_{max} (%)[d]
SNC80	0.14	6.8	1.7	100	5264	5.9
4a	1.8	9.6	1.54	88.7	39.8	50.5
4c	1350	36.1	141	138	307	79.2
4d	400	12.2	140	130	333	57.9
4e	5.1	11.3	2.0	77.2	ND[e]	ND[e]
4f	639	40.2	20.5	108	12530	22.6
4h	1.2	8.8	0.39	123	1.2	80.4
4i	5.2	4.8	0.62	90.6	2.4	75.8

[a]CellKey assays were carried out in duplicate using human MOR, DOR, or KOR expressed HEK293 cells. [b]E_{max} was calculated as the % of the response obtained with DAMGO. [c]E_{max} was calculated as the % of the response obtained with SNC80. [d]E_{max} was calculated as the % of the response obtained with (−)-U-50,488H. [e]Not determined.

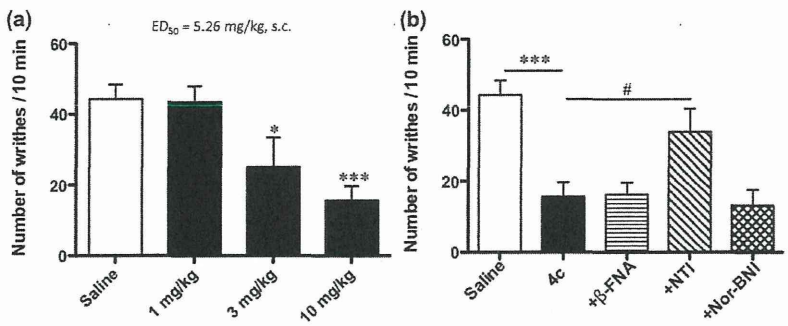


Figure 2. (a) Antinociceptive effect of **4c** administered subcutaneously in the mice acetic acid writhing tests. The statistical significance of differences between the groups was assessed with one-way ANOVA followed by Bonferroni's test. *$p < 0.05$ and ***$p < 0.001$ versus saline treated mice. (b) Effects of opioid receptor antagonists on the antinociception induced by subcutaneous treatment of **4c** in the mice acetic acid writhing tests. The statistical significance of differences between the groups was assessed with one-way ANOVA followed by Bonferroni's test. ***$p < 0.001$ versus saline treated mice. #$p < 0.05$ versus **4c** treated mice.

conditions (0.0005 M) to provide dioxymethylene compound **3a** in 66% yield concomitantly with a dimer in 30% yield in which two **2a** units were tethered with a methylene group (see the Supporting Information for details). A portion-wise addition of a solution of **2c–f** markedly improved the yields of **3c–f** and prevented formation of the dimer. Finally, the *O*-methyl group in **3a,c–f** was removed by a treatment with BBr_3 to give **4a,c–f**. Compounds **4h** and **4i** with respective phenyl and 2-phenethyl groups as the lactam nitrogen substituents were prepared as shown in Scheme 3. After a conversion of ester **5** into **6**, the treatment of **6** with *t*-BuOK in *t*-BuOH provided an equilibrium mixture of **2g** and **7g**. An equilibrium mixture of **2h** and **7h** was prepared from **5** by a previously reported method.[18] The mixture of **2g** and **7g** or **2h** and **7h** was reacted with CH_2ClBr in the same manner shown in Scheme 2 to afford dioxymethylene compounds **3g,h**. The 2-phenethyl group was introduced on the lactam nitrogen in **3g** by alkylation to give **3i**.

The affinities of the prepared compounds **4a,c–f,h,i** were evaluated by competitive binding assays (Table 1). All the compounds **4a,c–f,h,i** bound to the opioid receptors. The phenolic hydroxy group at the 3-position appeared to play an important role in improving the binding affinities for the opioid receptors compared to the parent compound **2a**.[20] Except for *N*-(2-phenethyl)lactam **4i**, compounds **4a,c–f,h** showed selectivities for the DOR, suggesting that the phenyl group of

dx.doi.org/10.1021/ml400491k | ACS Med. Chem. Lett. 2014, 5, 368–372

the substituent on the lactam nitrogen would function as a DOR address such as the phenyl moiety in NTI.[21,22] The binding affinities of **4a** and **4h** for the DOR were better than that of SNC80. Compounds **4c** and **4f** with respective *N*-methyl and *N*-(2-phenethyl) substituents were over 100-fold more selective for the DOR as compared to the KOR. The functional activities of **4a,c−f,h,i** were determined by [^{35}S]GTPγS binding and CellKey assays (Tables 2 and 3).[23] The CellKey system utilizes impedance biosensors for detection of cell behaviors and is a functional cell-based assay technology enabling label-free analysis of cell surface receptor activity.[24,25] It is noteworthy that the [^{35}S]GTPγS and CellKey assays differed in the observed output, even though giving similar results. All the compounds **4a,c−f,h,i** were full agonists for the DOR. The agonistic activities for the DOR of **4c,f,h** were more efficacious than that of SNC80 in both of the functional assays. Compounds **4h** and **4i** were also potent KOR agonists, whereas compounds **4c** and **4f** exhibited agonistic activities for the MOR. Although *N*-methyl derivative **4c** had moderate to high efficacy for the MOR and KOR, the potencies for these receptors were poor, which suggested that **4c** was highly selective and the most efficacious DOR agonist among the tested compounds. Derivatives **4a,e,f** with respective cyclopropylmethyl (CPM), allyl, and 2-phenethyl substituents on the basic nitrogen were more potent agonists for the DOR than *N*-methyl derivative **4c** in both functional assays; however, their functional selectivities for the DOR were lower than that of **4c** in [^{35}S]GTPγS binding assays and lower or comparable to that of **4c** in CellKey assays. Therefore, the *N*-methyl substituent on the basic nitrogen appeared to be the optimal group among the tested compounds.

We next assessed the antinociceptive effects of **4c** in mice by acetic acid writhing tests. Subcutaneously administered **4c** significantly exhibited antinociception in a dose-dependent manner and its EC_{50} value was 5.26 mg/kg (Figure 2a). No convulsive behaviors were observed. The antinociceptive effects induced by **4c** were attenuated by the selective DOR antagonist NTI but not by the selective MOR antagonist β-FNA or the selective KOR antagonist nor-BNI (Figure 2b). Taken together, these results indicate that compound **4c** could be a promising lead compound for selective DOR agonists with a novel chemotype, the oxazatricyclodecane structure

In conclusion, we synthesized novel DOR agonists **4a,c−f,h,i** with oxazatricyclodecane structure. Among the synthesized compounds, *N*-methyl derivative **4c** was highly selective and the most effective DOR agonist. Subcutaneous administration of **4c** produced dose-dependent and NTI-reversible antinociception without any convulsive behaviors. *N*-Methyl derivative **4c** is expected to be a promising lead compound for selective DOR agonists containing the novel oxazatricyclodecane structure.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures for the synthesis and characterization of the compounds, the in vitro activity assay, the in vivo mice acetic acid writhing assay, and the spectral data of the reported compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(H.F.) E-mail: fujiih@pharm.kitasato-u.ac.jp.

Present Address

#(H.N.) International Institute for Integrative Sleep Medicine, University of Tsukuba, 1−1−1 Tennodai, Tsukuba, Ibaraki 305−8577, Japan.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This work was partly supported by JSPS KAKENHI (23590133) [Grant-in-Aid for Scientific Research (C)] (H.F) and Adaptable and Seamless Technology Transfer Program through target-driven R&D, JST (AS2315040G) (to H.F, K.H, S.H, T.I, E.N., T.N., and H.N.).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Bn, benzyl; CHO, chinese hamster ovary; CPM, cyclopropylmethyl; DAMGO, [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-enkephalin; DOR, δ opioid receptor; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin; β-FNA, β-funaltrexamine; HEK, human embryonic kidney; KOR, κ opioid receptor; MOR, μ opioid receptor; nor-BNI, nor-binaltorphimine; NTI, naltrindole; Troc, 2,2,2-trichloroethoxycarbonyl

■ REFERENCES

(1) Dondio, G.; Ronzoni, S.; Petrillo, S. Non-peptide δ opioid agonists and antagonists. *Expert Opin. Ther. Pat.* **1997**, *7*, 1075−1997.

(2) Dondio, G.; Ronzoni, S.; Petrillo, S. Non-peptide δ opioid agonists and antagonists (Part II). *Expert Opin. Ther. Pat.* **1999**, *9*, 353−374.

(3) Fujii, H.; Takahashi, T.; Nagase, H. Non-peptidic δ opioid receptor agonists and antagonists (2000−2012). *Expert Opin. Ther. Pat.* **2013**, *23*, 1181−1208.

(4) Mucha, R. F.; Herz, A. Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. *Psychopharmacology* **1985**, *86*, 274−280.

(5) Millan, M. J. κ-Opioid receptors and analgesia. *Trends Pharmacol. Sci.* **1990**, *11*, 70−76.

(6) Nagase, H.; Kawai, K.; Hayakawa, J.; Wakita, H.; Mizusuna, A.; Matsuura, H.; Tajima, C.; Takezawa, Y.; Endoh, T. Rational drug design and synthesis of a highly selective nonpeptide δ-opioid agonist, (4aS*,12aR*)-4a-(3-hydroxyphenyl)-2-methyl-1,2, 3,4,4a,5,12,12a-octahydropyrido[3,4-*b*]acridine (TAN-67). *Chem. Pharm. Bull.* **1998**, *46*, 1695−1702.

(7) Nagase, H.; Yajima, Y.; Fujii, H.; Kawamura, K.; Narita, M.; Kamei, J.; Suzuki, T. The pharmacological profile of δ opioid receptor ligands, (+) and (-) TAN-67 on pain modulation. *Life Sci.* **2001**, *46*, 2227−2231.

(8) Chang, K. J.; Rigdon, G. C.; Howard, J. L.; McNutt, R. W. A novel, potent and selective nonpeptidic delta opioid receptor agonist BW373U86. *J. Pharmacol. Exp. Ther.* **1993**, *267*, 852−857.

(9) Calderon, S. N.; Rothman, R. B.; Porreca, F.; Flippen-Anderson, J. L.; McNutt, R. W.; Xu, H.; Smith, L. E.; Bilsky, E. J.; Davis, P.; Rice, K. C. Probes for narcotic receptor mediated phenomena. 19. Synthesis of (+)-4-[(αR)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC 80): A highly selective, nonpeptide δ opioid receptor agonist. *J. Med. Chem.* **1994**, *37*, 2125−2128.

(10) Bourdonnec, B. L.; Windh, R. T.; Ajello, C. W.; Leister, L. K.; Gu, M.; Chu, G. H.; Tuthill, P. A.; Barker, W. M.; Koblish, M.; Wiant, D. D.; Graczyk, T. M.; Belanger, S.; Cassel, J. A.; Feschenko, M. S.; Brogdon, B. L.; Smith, S. A.; Christ, D. D.; Derelanko, M. J.; Kutz, S.; Little, P. J.; DeHaven, R. N.; DeHaven-Hudkins, D. L.; Dolle, R. E.

Potent, orally bioavailable delta opioid receptor agonists for the treatment of pain: Discovery of *N,N*-diethyl-4-(5-hydroxyspiro-[chromene-2,4′-piperidine]-4-yl)benzamide (ADL5859). *J. Med. Chem.* **2008**, *51*, 5893−5896.

(11) Nagase, H.; Nemoto, T.; Matsubara, A.; Saito, M.; Yamamoto, N.; Osa, Y.; Hirayama, S.; Nakajima, M.; Nakao, K.; Mochizuki, H.; Fujii, H. Design and synthesis of KNT-127, a δ-opioid receptor agonist effective by systemic administration. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6302−6305.

(12) Saitoh, A.; Sugiyama, A.; Nemoto, T.; Fujii, H.; Wada, K.; Oka, J.; Nagase, H.; Mitsuhiko Yamada, M. The novel δ opioid receptor agonist KNT-127 produces antidepressant-like and antinociceptive effects in mice without producing convulsions. *Behav. Brain Res.* **2011**, *223*, 271−279.

(13) Wei, Z. Y.; Brown, W.; Takasaki, B.; Plobeck, N.; Delorme, D.; Zhou, F.; Yang, H.; Jones, P.; Gawell, L.; Gagnon, H.; Schmidt, R.; Yue, S. Y.; Walpole, C.; Payza, K.; St-Onge, S.; Labarre, M.; Godbout, C.; Jakob, A.; Butterworth, J.; Kamassah, A.; Morin, P. E.; Projean, D.; Ducharme, J.; Roberts, E. *N,N*-Diethyl-4-(phenylpiperidin-4-ylidenemethyl)benzamide: A novel, exceptionally selective, potent δ opioid receptor agonist with oral bioavailability and its analogues. *J. Med. Chem.* **2000**, *43*, 3895−3905.

(14) Pradhan, A. A. A.; Becker, J. A. L.; Scherrer, G.; Tryoen-Toth, P.; Filliol, D.; Matifas, A.; Massotte, D.; Gavériaux-Ruff, C.; Kieffer, B. L. In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One* **2009**, *4*, e5425.

(15) Pradhan, A. A. A.; Walwyn, W.; Nozaki, C.; Filliol, D.; Erbs, E.; Matifas, A.; Evans, C.; Kieffer, B. L. Ligand-directed trafficking of the δ-opioid receptor in vivo: Two paths toward analgesic tolerance. *J. Neurosci.* **2010**, *30*, 16459−16459.

(16) Metcalf, M. D.; Yekkirala, A. S.; Powers, M. D.; Kitto, K. F.; Fairbanks, C. A.; Wilcox, G. L.; Portoghese, P. S. The δ opioid receptor agonist SNC80 selectively activates heteromeric μ-δ opioid receptors. *ACS Chem. Neurosci.* **2012**, *3*, 505−509.

(17) Hudzik, T. J.; Maciag, C.; Smith, M. A.; Caccese, R.; Pietras, M. R.; Bui, K. H.; Coupal, M.; Adam, L.; Payza, K.; Griffin, A.; Smagin, G.; Song, D.; Swedberg, M. D. B.; Brown, W. Preclinical pharmacology of AZD2327: A highly selective agonist of the δ-opioid receptor. *J. Pharmacol. Exp. Ther.* **2011**, *338*, 195−204.

(18) Hayashida, K.; Fujii, H.; Hirayama, S.; Nemoto, T.; Nagase, H. Rearrangement of 4,5α-epoxymorphinan derivatives with carbamoyle-poxy rings provide novel oxazatricyclodecane structures. *Tetrahedron* **2011**, *67*, 6682−6688.

(19) Montzka, T. A.; Mtiskella, J. D.; Partyka, R. A. 2,2,2-Trichloroethyl chloroformate: A general reagent for demethylation of tertiary methylamines. *Tetrahedron Lett.* **1974**, 1325−1327.

(20) Compound **3a** with 3-OMe group showed lower binding affinities for the opioid receptors than the corresponding compound **4a** with 3-OH group (see the Supporting Information).

(21) Portoghese, P. S.; Sultana, M.; Nagase, H.; Takernori, A. E. Application of the message-address concept in the design of highly potent and selective non-peptide δ opioid receptor antagonists. *J. Med. Chem.* **1988**, *31*, 281−282.

(22) Portoghese, P. S.; Sultana, M.; Takernori, A. E. Design of peptidomimetic δ opioid receptor antagonists using the message-address concept. *J. Med. Chem.* **1990**, *33*, 1714−1720.

(23) The functional activities of **4a**,**c**−**f**,**h**,**i** were also evaluated by cAMP assays (see the Supporting Information).

(24) Peters, M. F.; Knappenberger, K. S.; Wilkins, D.; Sygowski, L. A.; Lazor, L. A.; Liu, J.; Scott, C. W. Evaluation of cellular dielectric spectroscopy, a whole-cell, label-free technology for drug discovery on G_i-coupled GPCRs. *J. Biomol. Screening* **2007**, *12*, 312−319.

(25) Scott, C. W.; Peters, M. F. Label-free whole-cell assays: expanding the scope of GPCR screening. *Drug Discovery Today* **2010**, *15*, 704−716.