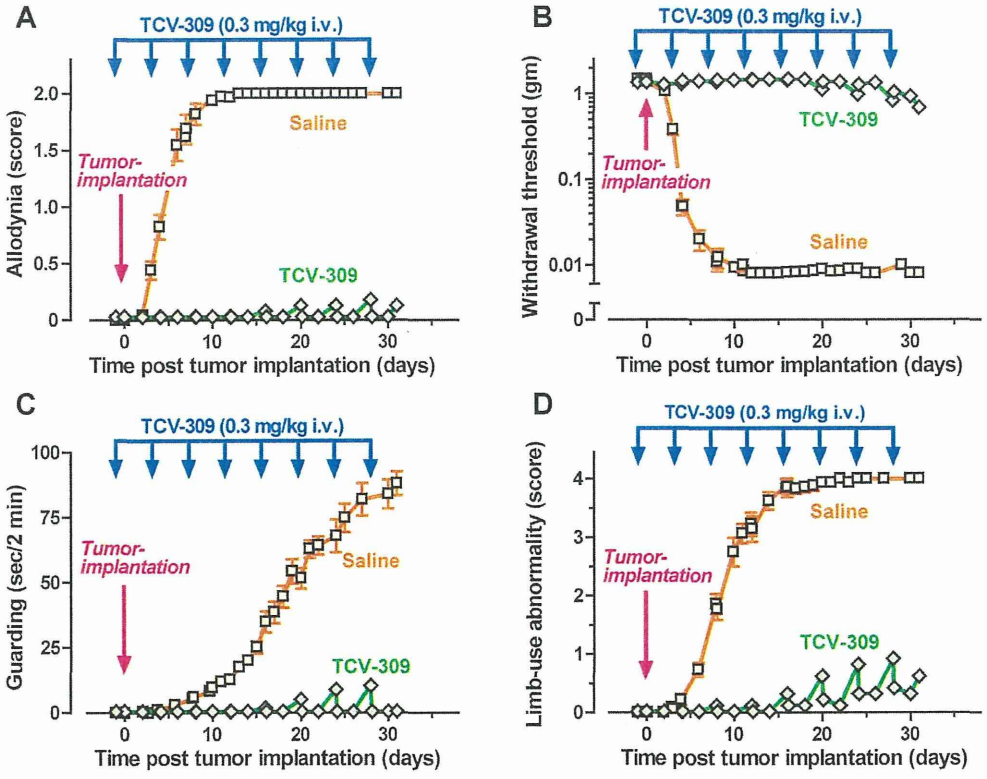


Figure 7. Effect of the repeated administration of TCV-309 on the pain-like behaviors in FBC mice. The administration of TCV-309 0.3 mg/kg i.v. was started 6 hr before the tumor implantation, given once a day and continued every 4 days up to 28 days. Allodynia (A, B), guarding behavior (C) and limb-use 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

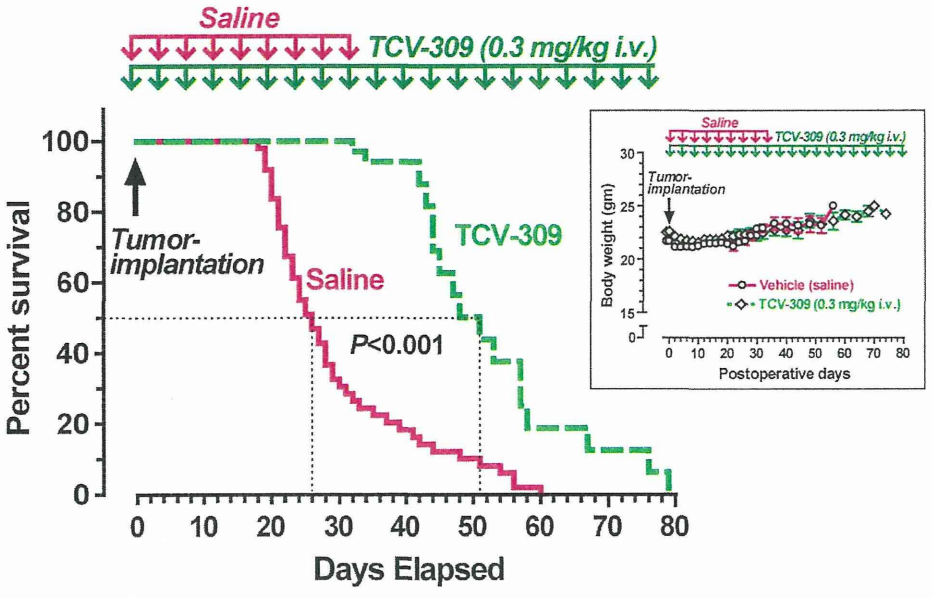


Figure 8. The Kaplan-Mayer survival curve of FBC mice and the change of body weight (insert). For the survival experiments, TCV-309 and saline were given once a day and continued every 4 days until the animals died (n = 17 and 50, respectively). Control mice received saline for 32 days. Days for 50% 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

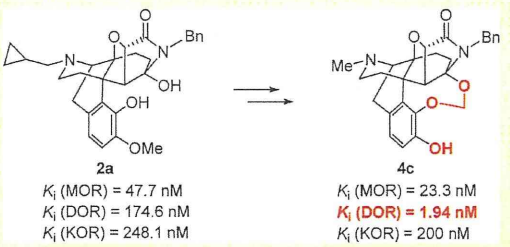
[§]Division of Cancer Pathophysiology, National Cancer Center Research Institute, 5-1-1 Tsukiji, Tokyo 104-0045, Japan

[∥]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

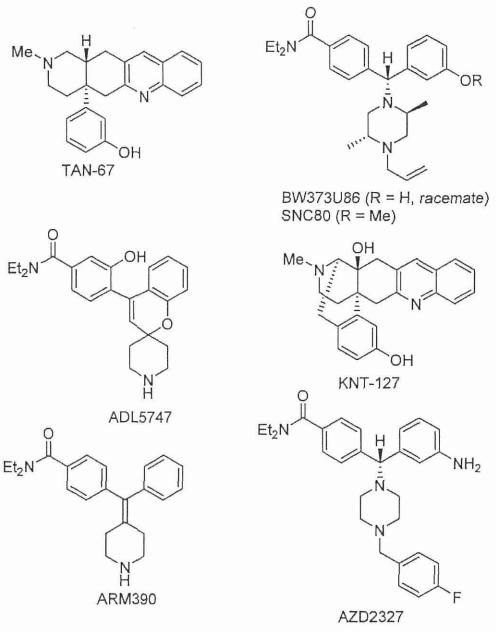
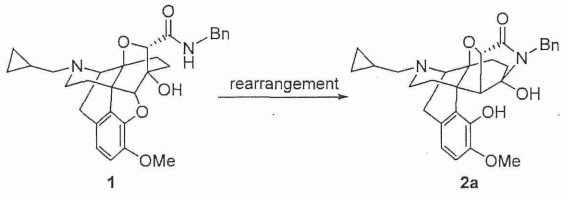
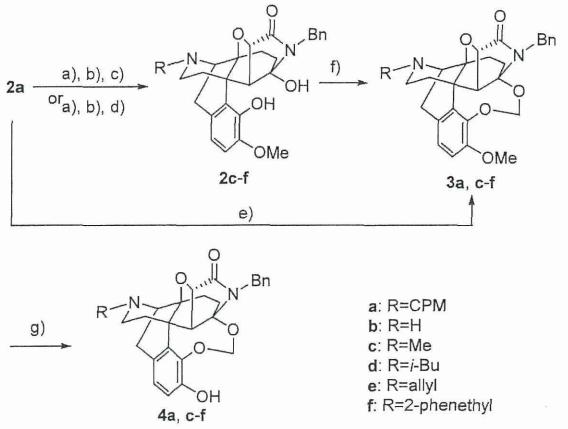


Figure 1. Structures of DOR agonists, TAN-67, BW373U86, SNC80, ADL5747, KNT-127, ARM390, and AZD2327.

Scheme 1. Potential Opioid Ligand 2a

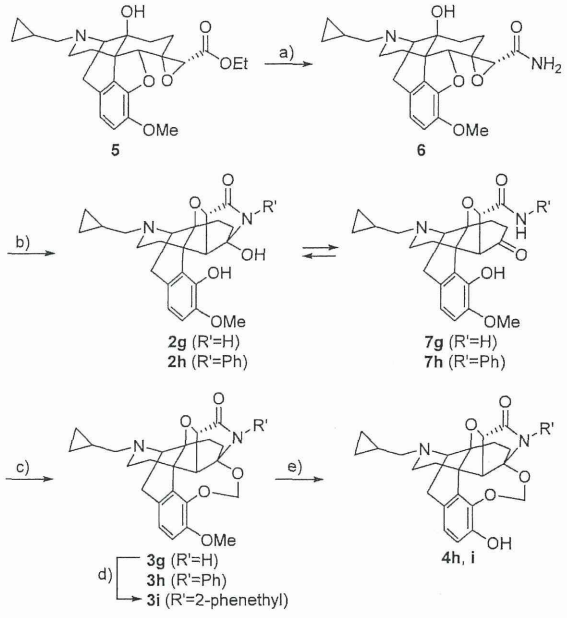


Scheme 2. Synthesis of 4a,c–f[a]



[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.

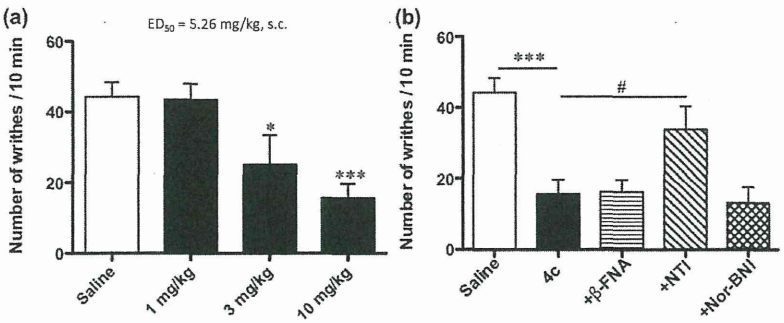


Figure 2. (a) Antinociceptive effect of **4c** administered subcutaneously in the mice acetic acid writhing tests. The statistical significance of differences between the groups was assessed with one-way ANOVA followed by Bonferroni's test. *$p < 0.05$ and ***$p < 0.001$ versus saline treated mice. (b) 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, (4a*S**,12a*R**)-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-[(α*R*)-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.

Neurosteroids Allopregnanolone Sulfate and Pregnanolone Sulfate Have Diverse Effect on the α Subunit of the Neuronal Voltage-gated Sodium Channels $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ Expressed in *Xenopus* Oocytes

Takafumi Horishita, M.D., Ph.D., Nobuyuki Yanagihara, Ph.D., Susumu Ueno, M.D., Ph.D., Yuka Sudo, Ph.D., Yasuhito Uezono, M.D., Ph.D., Dan Okura, M.D., Tomoko Minami, M.D., Takashi Kawasaki, M.D., Ph.D., Takeyoshi Sata, M.D., Ph.D.

ABSTRACT

Background: The neurosteroids allopregnanolone and pregnanolone are potent positive modulators of γ-aminobutyric acid type A receptors. Antinociceptive effects of allopregnanolone have attracted much attention because recent reports have indicated the potential of allopregnanolone as a therapeutic agent for refractory pain. However, the analgesic mechanisms of allopregnanolone are still unclear. Voltage-gated sodium channels (Na_v) are thought to play important roles in inflammatory and neuropathic pain, but there have been few investigations on the effects of allopregnanolone on sodium channels.

Methods: Using voltage-clamp techniques, the effects of allopregnanolone sulfate (APAS) and pregnanolone sulfate (PAS) on sodium current were examined in *Xenopus* oocytes expressing $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ α subunits.

Results: APAS suppressed sodium currents of $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ at a holding potential causing half-maximal current in a concentration-dependent manner, whereas it markedly enhanced sodium current of $Na_v1.8$ at a holding potential causing maximal current. Half-maximal inhibitory concentration values for $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ were 12 ± 4 (n = 6), 41 ± 2 (n = 7), and 131 ± 15 (n = 5) μmol/l (mean ± SEM), respectively. The effects of PAS were lower than those of APAS. From gating analysis, two compounds increased inactivation of all α subunits, while they showed different actions on activation of each α subunit. Moreover, two compounds showed a use-dependent block on $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$.

Conclusion: APAS and PAS have diverse effects on sodium currents in oocytes expressing four α subunits. APAS inhibited the sodium currents of $Na_v1.2$ most strongly. **(ANESTHESIOLOGY 2014; XXX:XX-XX)**

NEUROSTEROIDS are neuroactive steroids synthesized from cholesterol in both central and peripheral nervous systems, and they accumulate in the nervous system.[1] They rapidly alter neuronal excitability by mediating actions through ion-gated neurotransmitter receptors, but not through classic steroid hormone nuclear receptors.[2] Many of them are converted to sulfated metabolites by hydroxysteroid sulfotransferases, and neurosteroid sulfates are also known to regulate physiological processes. They are thought to be potentially therapeutic because of their many pharmacological properties.[3,4]

Two 3α-hydroxylated metabolites of progesterone, allopregnanolone (3α-hydroxy-5α-pregnane-20-one) and pregnanolone (3α-hydroxy-5β-pregnane-20-one), are known

What We Already Know about This Topic

- Sodium channels are important targets for analgesic actions in the spinal cord, but their role in neurosteroid analgesia is unclear
- The effects of two sulfated neurosteroids with analgesic and anesthetic properties were tested on heterologously expressed rat voltage-gated sodium channel function

What This Article Tells Us That Is New

- The neurosteroids tested produced voltage and use-dependent block of all the subtypes tested, with more potent effects on $Na_v1.2$
- Inhibition of $Na_v1.2$ in the spinal cord by allopregnanolone is a plausible mechanism for its analgesic effects if confirmed in neuronal preparations and pain models

Submitted for publication October 25, 2013. Accepted for publication April 9, 2014. From the Department of Anesthesiology (T.H., D.O., T.M., T.K., T.S.) and Department of Pharmacology (N.Y.), School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan; Department of Occupational Toxicology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Kitakyushu, Japan (S.U.); Department of Molecular Pathology and Metabolic Disease, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba, Japan (Y.S.); and Cancer Pathophysiology Division, National Cancer Center Research Institute, Tokyo, Japan (Y.U.).

 Anesthesiology 2014; XXX:00-00

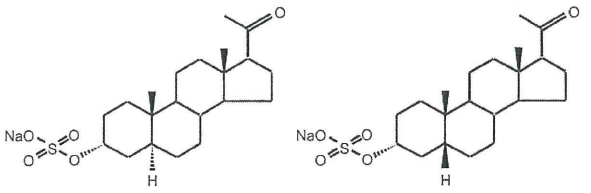
<zdoi;10.1097/ALN.0000000000000296>

to be positive modulators at γ-aminobutyric acid type A ($GABA_A$) receptors with high potency.[5] These neurosteroids have been shown to have greater anesthetic potencies than those of other intravenous anesthetics that are clinically used, and not to cause acute tolerance that are observed in other anesthetics, suggesting usefulness of these neurosteroids as general anesthetics.[6,7] On the contrary, allopregnanolone was shown to have the most potent analgesic effects among all neurosteroids in pain models.[8] Recent studies demonstrated its analgesic effects in neuropathic pain models. Allopregnanolone alleviates thermal and mechanical hyperalgesia by ligation of the sciatic nerve in rats,[9] produces analgesic effects on formalin-induced pain in rats,[10] and prevents anticancer drug oxaliplatin-induced cold and mechanical allodynia and hyperalgesia.[11] In addition, it was suggested that stimulation of allopregnanolone synthesis might be involved in the antinociceptive effects of several analgesic drugs in neuropathic pain models.[12–14] Its effect on $GABA_A$ receptors may be important for its antinociceptive properties because GABA is involved in pain pathways in the nervous systems, and drugs targeting subtypes of GABA receptors have analgesic effects in chronic pain.[15] However, these two neurosteroids, allopregnanolone and pregnanolone, also act on other ion channels in pain signaling pathways, including T-type calcium channels[16] and *N*-methyl-D-aspartate receptors.[17]

Voltage-gated sodium channels (Na_v) have an important role in action potential initiation and propagation in excitable nerve and muscle cells. Nine α subunits ($Na_v1.1$ to $Na_v1.9$) and four auxiliary β subunits have been identified in mammals.[18,19] Each pore-forming α subunit has a different pattern of development and localization and has distinct physiological and pathophysiological roles. Sodium channel α subunits expressed in the dorsal root ganglion are considered possible targets for analgesics for inflammatory and neuropathic pain.[20–22] However, there has been little investigation on the effects of allopregnanolone on sodium channel function. It is important to examine these effects because they may be useful in clarifying the mechanisms of the analgesic effects of allopregnanolone and developing natural and safe neurosteroid-based analgesics for refractory pain. In addition, our recent report demonstrated the importance of neurosteroid sulfonation for regulation of ion channels because of more potent effects of sulfated steroid than those of nonsulfated steroids.[23] Here, we investigate the effects of two sulfated neurosteroids, allopregnanolone sulfate (APAS) and pregnanolone sulfate (PAS) (fig. 1), on several sodium channel α subunits, including $Na_v1.2$, which is expressed in the central nervous system; $Na_v1.6$, which is expressed in the central nervous system and dorsal root ganglion neurons; and $Na_v1.7$ and $Na_v1.8$, which are expressed in dorsal root ganglion neurons.

Materials and Methods

This study was approved by the Animal Research Committee of the University of Occupational and Environmental Health, Kitakyushu, Japan.



Allopregnanolone sulfate (APAS) Pregnanolone sulfate (PAS)

Fig. 1. Structures of allopregnanolone sulfate (APAS) and pregnanolone sulfate (PAS).

Drugs

Allopregnanolone sulfate and PAS were purchased from Steraloids, Inc. (Newport, RI).

Plasmids

Rat $Na_v1.2$ α subunit complementary DNA (cDNA) was a gift from Dr. William A. Catterall, Ph.D. (Professor, Department of Pharmacology, University of Washington, Seattle, Washington). Rat $Na_v1.6$ α subunit cDNA was a gift from Dr. Alan L. Goldin, M.D., Ph.D. (Professor, Department of Anatomy and Neurobiology, University of California, Irvine, California). Rat $Na_v1.7$ α subunit cDNA was a gift from Gail Mandel, Ph.D. (Professor, Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon). Rat $Na_v1.8$ α subunit cDNA was a gift from Dr. Armen N. Akopian, Ph.D. (Assistant Professor, University of Texas Health Science Center, San Antonio, Texas), and human β_1 subunit cDNA was a gift from Dr. Alfred L. George, Jr., M.D. (Professor, Department of Pharmacology, Vanderbilt University, Nashville, Tennessee). The percentages of homology between rat and human protein of $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ are 98, 99, 93, and 83%, respectively, suggesting the possible limitations imposed by using rat α subunit for only $Na_v1.8$ to make conclusions in humans.

cRNA Preparation and Oocyte Injection

After linearization of cDNA with *Cla*I ($Na_v1.2$ α subunit), *Not*I ($Na_v1.6$, 1.7 α subunits), *Xba*I ($Na_v1.8$ α subunit), and *Eco*RI (β_1 subunit), cRNAs were transcribed using SP6 (Na_v 1.8 α, β_1 subunits) or T7 ($Na_v1.2$, 1.6, and 1.7 α subunits) RNA polymerase from the mMESSAGE mMACHINE kit (Ambion, Austin, TX). Adult female *Xenopus laevis* frogs were obtained from Kyudo Co., Ltd. (Saga, Japan). *X. laevis* oocytes and cRNA microinjection were prepared as described previously.[24] Na_v α subunit cRNAs were coinjected with β_1 subunit cRNA at a ratio of 1:10 (total volume was 20 to 40 ng/50 nl) into *Xenopus* oocytes (all α subunits were coinjected with the β_1 subunit) that were randomly assigned to four α subunit groups for injection. Injected oocytes were incubated at 19°C in incubation medium, and 2 to 6 days after injection, the cells were used for electrophysiological recordings.

Electrophysiological Recordings

All electrical recordings were performed at room temperature (23°C). Oocytes were placed in a 100-μl recording chamber and perfused at 2 ml/min with Frog Ringer's solution containing 115 mmol/l NaCl, 2.5 mmol/l KCl, 10 mmol/l HEPES, 1.8 mmol/l $CaCl_2$, pH 7.2, using a peristaltic pump (World Precision Instruments Inc., Sarasota, FL). Recording electrodes were prepared, and the whole-cell voltage clamp and recordings were achieved as described previously.[24] Transients and leak currents were subtracted using the P/N procedure, in which N subsweeps each 1/Nth of the amplitude of the main stimulus waveform (P) are applied. APAS and PAS stocks were prepared in dimethylsulfoxide and diluted in Frog Ringer's solution to a final dimethylsulfoxide concentration not exceeding 0.05%. APAS and PAS were perfused for 3 min to reach equilibrium. All recordings were performed by the experimenters who were blind to the type of compound.

The voltage dependence of activation was determined using 50-ms depolarizing pulses from a holding potential causing maximal current (V_{max}) (−90 mV for $Na_v1.2$ and $Na_v1.6$, −100 mV for $Na_v1.7$ and $Na_v1.8$) and from a holding potential causing half-maximal current ($V_{1/2}$) (from approximately −40 mV to −70 mV) to 60 mV in 10-mV increments. V_{max} and $V_{1/2}$ holding potentials induce resting and inactivated states of sodium channels. Because the effects of many analgesics in the inactivated state are known to be important for analgesic action,[25] we used these two different holding potentials to compare the effects of compounds in the resting and inactivated states. Normalized activation curves were fitted to the Boltzmann equation as described previously[24]: briefly, $G/G_{max} = 1/(1 + \exp(V_{1/2} - V)/k)$, where G is the voltage-dependent sodium conductance, G_{max} is the maximal sodium conductance, G/G_{max} is the normalized fractional conductance, $V_{1/2}$ is the potential at which activation is half maximal, and k is the slope factor. To measure steady-state inactivation, currents were elicited by a 50-ms test pulse to −20 mV for $Na_v1.2$ and $Na_v1.6$, −10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ after 200 ms (500 ms for only $Na_v1.8$) prepulses ranging from −140 to 0 mV in 10-mV increments from a holding potential of V_{max}. Steady-state inactivation curves were fitted to the Boltzmann equation: $I/I_{max} = 1/(1 + \exp(V_{1/2} - V)/k)$, where I_{max} is the maximal sodium current, I/I_{max} is the normalized current, $V_{1/2}$ is the voltage of half-maximal inactivation, and k is the slope factor. To investigate a use-dependent sodium channel block, currents were elicited at 10 Hz by a 20-ms depolarizing pulse of −20 mV for $Na_v1.2$ and $Na_v1.6$, −10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ from a $V_{1/2}$ holding potential in both the absence and presence of 100 μmol/l APAS and PAS. Peak currents were measured and normalized to the first pulse and plotted against the pulse number. Data were fitted to the monoexponential equation $I_{Na} = \exp(-\tau_{use} \cdot n) + C$, where n is pulse number, C is the plateau I_{Na}, and τ_{use} is the time constant of use-dependent decay.

Statistical Analysis

The GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) was used to perform the statistical analysis, and a statistical power analysis was performed using G*Power software. All values are presented as means ± SEM. The n values refer to the number of oocytes examined. Each experiment was performed with oocytes taken from at least two frogs. Data were statistically evaluated by paired t test (two-tailed). We assessed the inhibitory effects at different APAS concentrations in the concentration–response curve, using one-way ANOVA followed by Dunnet *post hoc* test for

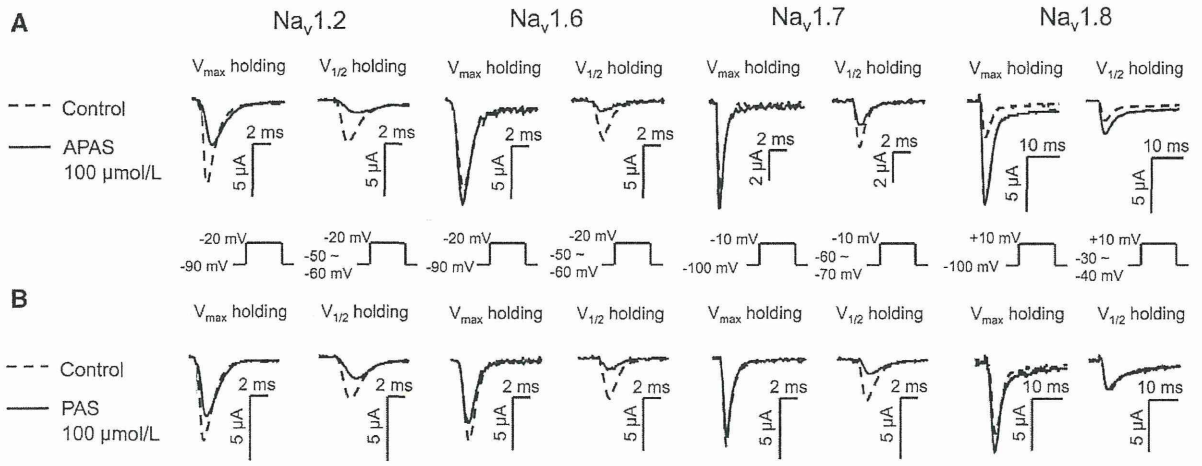


Fig. 2. Effects of allopregnanolone sulfate (APAS) (*A*) and pregnanolone sulfate (PAS) (*B*) on peak sodium inward currents in *Xenopus* oocytes expressing $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, or $Na_v1.8$ α subunits with β_1 subunits at two holding potentials. Representative traces are shown. Sodium currents were evoked by 50-ms depolarizing pulses to −20 mV for $Na_v1.2$ and $Na_v1.6$, −10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ from V_{max} or $V_{1/2}$ in both the absence and presence of 100 μmol/l of the compounds. Na_v = voltage-gated sodium channel; V_{max} holding = holding potential causing maximal current; $V_{1/2}$ holding = holding potential causing half-maximal current.