

In Vitro and in Vivo Pharmacological Characterization of JTE-907, a Novel Selective Ligand for Cannabinoid CB₂ Receptor

HIROYUKI IWAMURA, HIDEKAZU SUZUKI, YOSHIFUMI UEDA, TETSUDO KAYA, and TAKASHI INABA

Japan Tobacco Inc., Central Pharmaceutical Research Institute, Osaka, Japan

Received July 5, 2000; accepted October 4, 2000 This paper is available online at <http://jpet.aspetjournals.org>

ABSTRACT

JTE-907 [*N*-(benzo[1,3]dioxol-5-ylmethyl)-7-methoxy-2-oxo-8-pentyl-1,2-dihydroquinoline-3-carboxamide] was evaluated in vitro and in vivo as a novel selective ligand for cannabinoid receptor of peripheral type (CB₂). The compound binds with high affinity to human CB₂ or mouse CB₂ expressed on CHO cell membrane and to rat CB₂ on splenocytes. The *K_i* affinities for human, mouse, and rat CB₂ were 35.9, 1.55, and 0.38 nM, respectively. The selectivity ratio for the CB₂ receptors compared with central nervous type receptors (CB₁) of human (expressed on CHO cells), and mouse and rat CB₁ on cerebellum were 66, 684, and 2760, respectively. JTE-907 showed concentration-dependent increase of forskolin-stimulated

cAMP production in CHO cells expressing human and mouse CB₂ in vitro, i.e., JTE-907 behaved as an inverse agonist, which is in contrast to Win55212-2 that reduces cAMP as an agonist. JTE-907 dosed orally inhibited carrageenin-induced mouse paw edema dose dependently. The same in vivo effect was observed with other cannabinoid receptor ligands such as SR144528, Δ⁹-tetrahydrocannabinol (THC), and Win55212-2. This is the first report that a CB₂-selective inverse agonist, JTE-907, has an anti-inflammatory effect in vivo, and how the inverse agonist showed the same effect as Win55212-2 and Δ⁹-THC is discussed.

Since the discovery of peripheral type cannabinoid receptor CB₂ (Munro et al., 1993), the physiological function of the receptor has been a subject of active investigations. The CB₂ was shown to interact with G protein signaling-like central type receptor (CB₁), reducing cAMP level by coupling with Gi protein when the receptor is stimulated by ligands (Felder et al., 1995). The CB₂ is distinct from CB₁ not only in its amino acid sequence but also in the tissue distributions, i.e., abundant in splenic macrophages and B lymphocytes (Munro et al., 1993; Lynn and Herkenham, 1994), peripheral blood mononuclear cells, and tonsillar B cells (Galiègue et al., 1995). These observations have evoked a possible role of the CB₂ in immune system. In fact, many in vitro immunological effects of Δ⁹-tetrahydrocannabinol (Δ⁹-THC) as an active component of marijuana have been reported. They include inhibited production of tumor necrosis factor-α, interleukin-2, and nitric oxide from macrophages and T cells (Coffey et al., 1996; Condie et al., 1996; Zheng and Specter, 1996), and induction of arachidonic acid release from mouse macrophages (Burstein et al., 1994; Hunter and Burstein, 1997). Δ⁹-THC was also shown to have in vivo anti-inflammatory effect on mouse paw edema (Sofia et al., 1973; Burstein et al., 1989). Yet, most of these effects were not definitely confirmed to be mediated by CB₂, in contrast to the role of CB₁ in central nervous system demonstrated by using SR141716 as a CB₁-selective antagonist (Compton et al., 1996) or by receptor knockout mice (Reibaud et al., 1999).

SR144528 (Rinaldi-Carmona et al., 1998) was reported to be a CB₂-selective inverse agonist that antagonizes the binding and signal transduction of agonistic ligand in CHO cells expressing CB₂ (Portier et al., 1999). This CB₂-selective ligand is a very useful tool for studying CB₂ function induced by agonist. For example, a recent study reported that arachidonic acid release induced by Δ⁹-THC in RAW264.7 mouse macrophage cells was partially blocked by SR144528 (Pestonjamasap and Burstein, 1998), which suggests CB₂-mediated release of arachidonic acid. However, in vivo pharmacological efficacy of SR144528 by itself has not been shown yet.

Here, we report a novel selective ligand for CB₂, JTE-907 (Fig. 1), as an inverse agonist. We also show that JTE-907 has antiedema effect in vivo, and compared its effect with other cannabinoid ligands, SR144528, Win55212-2, and Δ⁹-THC.

Materials and Methods

Reagents. JTE-907 [*N*-(benzo[1,3]dioxol-5-ylmethyl)-7-methoxy-2-oxo-8-pentyl-1,2-dihydroquinoline-3-carboxamide], SR144528 [*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-chloro-3-methyl-phenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide)], and Δ⁹-THC were synthesized by Japan Tobacco Inc. (Osaka, Japan). Win55212-2 and forskolin were purchased from Research Biochemicals International (Natick, MA), Ro20-1724 from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), and prednisolone from

ABBREVIATIONS: CB, cannabinoid receptor; Δ⁹-THC, Δ⁹-tetrahydrocannabinol; CHO, Chinese hamster ovary; PCR, polymerase chain reaction.

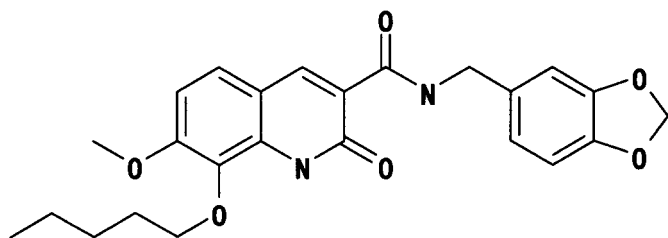


Fig. 1. Chemical structure of JTE-907.

Nakalai Tesque (Kyoto, Japan). Test compounds were dissolved in dimethyl sulfoxide, and the vehicle was kept at a final concentration of 0.1% in vitro.

Construction of Expression Vectors. With previously published sequence information (Gerard et al., 1990; Munro et al., 1993; Shire et al., 1996), the following oligonucleotides were designed to prime for PCR amplification of human CB₁: 5'-GAGGTTATGAAGTCGATCCTAG-3' (sense primer) and 5'-CATCAGGCTCACAGAGCCTC-3' (antisense primer); human CB₂: 5'-AGGGCCCACACCATGGAGGAATGCTG-3' (sense primer) and 5'-TCATCAGCAATCAGAGAGGTC-3' (antisense primer); and mouse CB₂: 5'-GCATCTAGACCATGGAGGGATGCCGG-GAGACAG-3' (sense primer) and 5'-CCATCTAGACTAGGTGGTTT-TCACATCAGCCTC-3' (antisense primer). PCR amplification of human CB₁ sequences from human brain QUICK-Clone cDNA (CLONTECH, Palo Alto, CA), human CB₂ sequences from human spleen QUICK-Clone cDNA (CLONTECH), and mouse CB₂ sequences from mouse spleen QUICK-Clone cDNA (CLONTECH) were conducted for 30 cycles (94°C for 30 s, 55°C for 30 s, and 74°C for 60 s, respectively), yielding a product that was 1.4, 1.1, and 1.0 kb in length, respectively. The human CB₁, CB₂, and mouse CB₂ PCR products were cloned into the multicloning site of the cloning vector pGEM-3Zf(+) (Promega, Madison, WI).

All cDNAs were verified by dideoxy sequencing, and then ligated into the multicloning site of the mammalian expression vector pMAM2-BSD (Funakoshi, Tokyo, Japan) or pEF-BSD, which was constructed with human EF-1 promoter derived from pEF-BOS (Mizushima and Nagata, 1990) and blasticidin S deaminase gene derived from pMAM2-BSD.

Cell Culture and Transfection. CHO cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 50 units/ml penicillin, and 50 µg/ml streptomycin in an atmosphere of 5% CO₂. CHO cells expressing CB receptor were created by transfection of pEF-BSD/human CB₁ or CB₂ into CHO cells by Trans IT LT-1 (Mirus, Madison, WI), or pEF-BSD/mouse CB₂ into CHO cells by Genetransfer (Wako, Tokyo, Japan). Stable transformants were selected in growth medium containing 10 µg/ml blasticidin S (Funakoshi). Approximately 200 colonies were picked and allowed to expand, and then tested for expression of receptor mRNA by reverse transcription-PCR and cAMP-reducing response by Win55212-2 upon forskolin stimulation. The CHO cells containing moderate-to-high levels of receptor mRNA were tested for receptor-binding assays. Transfected cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with growth medium containing 10 µg/ml blasticidin S in an atmosphere of 5% CO₂.

Ligand Binding Assays. CHO cells expressing human CB₁ or human CB₂, or mouse CB₂ were cultured and harvested for the study. Male Sprague-Dawley rats (7 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and male C57BL/6J mice (6 weeks old) from JAPAN CLEA (Shizuoka, Japan). Rat and mouse cerebellum and rat spleen were collected from decapitated animals under ether anesthesia. The harvested cells or the tissues were homogenized in ice-cold buffer C [2 mM EDTA·4Na, 5 mM MgCl₂, 50 mM Tris (Sigma Chemical, St. Louis, MO) and 320 mM sucrose at pH 7.4] followed by centrifugation three times at 1900g for 10 min at 4°C. The supernatant was centrifuged at 39,000g for 10 min at 4°C. The pellet was suspended in buffer A (2 mM EDTA·4Na, 5 mM MgCl₂, and 50 mM Tris at pH 7.4), and incubated for 10 min at 37°C. The suspension

was centrifuged at 23,000g for 10 min at 4°C and then the pellet was resuspended and incubated for 40 min at 30°C. The suspension was centrifuged again at 11,000g for 15 min at 4°C to collect pellet as P2 membrane. The membrane was suspended in buffer B (2 mM EDTA·4Na, 3 mM MgCl₂, and 50 mM Tris at pH 7.4). Rat splenocytes as CB₂ preparation were collected from spleen dispersed through filter mesh and suspended in buffer C. Contaminating red blood cells were lysed and washed with Dulbecco's phosphate-buffered saline (–), Nissui Pharmaceuticals Co., LTD., Tokyo, Japan). The splenocytes were suspended in buffer B at a density of 1 × 10⁸ cells/ml.

Competition binding assays with membrane preparations [20 µg/ml except for human CB₂ (5 µg/ml)] were performed by 24-well plate filtration with 1 nM [³H]CP-55,940 (129.95 Ci/mmol; NEN Life Sciences, Boston, MA) in assay buffer D (1 mM EDTA·4Na, 3 mM MgCl₂, 50 mM Tris, 0.2% bovine serum albumin, and 0.2% ethanol at pH 7.4) at 30°C for 90 min. For the competitive binding assays with splenocytes (1 × 10⁷ cells/ml) we used assay buffer E (1 mM EDTA·4Na, 4 mM NaHCO₃, 50 mM Tris, 0.2% bovine serum albumin, and 0.95% Hank's balanced salt solution at pH 7.4) at 4°C for 3 h. Nonspecific binding was determined in the presence of 10 µM Δ⁹-THC.

cAMP Assays. CHO cells expressing CB receptors were harvested and cultured at a density of 1 × 10⁴ cells/well in 96-well culture plate. After 24-h culture at 37°C, cells were washed with phosphate-buffered saline (–) and incubated at 37°C for 10 min in HEPES buffer [137 mM NaCl, 4.5 mM KCl, 1.2 mM MgCl₂, 1 mM CaCl₂·2H₂O, 20 mM HEPES, and 10 mM D-(+)-glucose at pH 7.4] containing 0.25 mM Ro20-1724 in the presence or absence of test compounds. Cells were then incubated with 5 µM forskolin at 37°C for 15 min, followed by the addition of ice-cold 2.5% dodecyltrimethylammonium bromide (Amersham Pharmacia Biotech, Piscataway, NJ) to stop the reaction. The wells were agitated for 60 min at room temperature. cAMP concentration in the medium of each well was measured by enzyme immunoassay kit (Amersham Pharmacia Biotech).

Carrageenin-Induced Paw Edema. Male C57BL/6J mice (8 weeks old; JAPAN CLEA) were randomized by their weight. Right hind paw was measured by plethysmometer (TK-101; Unicom Inc., Chiba, Japan) 2 h before the compound dosing. Test compounds were suspended in 0.5% methyl cellulose and dosed orally at a volume of 10 ml/kg. Carrageenin (Picin A; Zushikagaku Laboratory, Kanagawa, Japan) was dissolved in saline by incubation for 24 h at 4°C to make up 1% solution and inoculated subcutaneously at a volume of 50 µl at foot pad of the right hind paw 1 h after test compound dosing. Three hours after carrageenin injection, paw volume was measured and compared with predosing value, and the edema formation of each mouse was determined. All the measurements were performed in a blind manner. Data were calculated as percentage of increase of the paw volume by comparing pre- and postcarrageenin injection. The data were further corrected for percentage of control swelling by comparison between saline- and carrageenin-treated animals. ED₅₀ values for JTE-907 and SR144528 to inhibit paw swelling were calculated from linear regression of dose-response curve.

Statistical Analysis. Values are expressed as mean ± S.E.M. The differences between control and compound were analyzed by ANOVA with Dunnett's test or Student's *t* test.

Results

Ligand Binding Assays. To assess binding activity of JTE-907 to cannabinoid receptors from several animal species, we performed ligand displacement experiments using [³H]CP-55,940, which is known as a ligand for CB₁ and CB₂. The *K_d* values of [³H]CP-55,940 were as follows: 0.57 and 0.23 nM for human CB₁ and CB₂, 0.23 and 0.70 nM for rat CB₁ and CB₂, and 0.16 and 0.16 nM for mouse CB₁ and CB₂. Corresponding *B_{max}* values of [³H]CP-55,940 were 0.73 and

20.0 pmol/mg, 1.9 and 3.8 fmol/ 10^6 cells, and 0.87 and 6.3 pmol/mg. The binding affinities expressed as K_i value for JTE-907, SR144528, Win55212-2, and Δ^9 -THC on CB receptors of human, mouse, and rat are shown in Table 1. JTE-907 displaced [3 H]CP-55,940 binding to human, mouse, and rat CB₂ with high affinities, whereas it showed lower affinities to human, mouse, and rat CB₁. The selectivity ratio of JTE-907 for CB₂ was higher than those of SR144528, Win55212-2, and Δ^9 -THC in three animal species. Figure 2 illustrates a competitive displacement of specific [3 H]CP-55,940 binding by cannabinoid ligands in mouse CB₁ and CB₂. JTE-907 and SR144528 showed good selectivities for mouse CB₂, whereas Win55212-2 and Δ^9 -THC were equipotent with mouse CB₁ and CB₂.

cAMP Assays. We examined whether JTE-907 affects cAMP production in forskolin-stimulated CHO cells expressing human CB₁ and CB₂ and mouse CB₂. As shown in Fig. 3, B and C, JTE-907 showed a concentration-dependent increase of forskolin-stimulated cAMP production in human CB₂ and mouse CB₂. This increase by JTE-907 was apparent above 0.01 μ M in a way similar to that of SR144528 and reached nearly maximum at 1 and 0.01 μ M in human CB₂ and mouse CB₂, respectively. In contrast, Win55212-2 decreased the cAMP production in a concentration-dependent manner in all the receptors tested. Δ^9 -THC showed a weak effect in decreasing cAMP compared with Win55212-2 in human CB₁, CB₂, and mouse CB₂, and the decrease in cAMP by Δ^9 -THC was minimum in human CB₂ (Fig. 3, A–C). JTE-907 and SR144528 did not affect human CB₁ at concentrations up to 10 μ M (Fig. 3A). All the compounds tested had little effect on the cAMP level in untransfected CHO cells stimulated with forskolin under the same condition (data not shown).

Carrageenin-Induced Mouse Paw Edema. Various ligands for cannabinoid receptors were tested in carrageenin-induced mouse paw edema model. As shown in Fig. 4A, treatment of mice with Win55212-2 and Δ^9 -THC at 1 mg/kg orally showed potent antiedema effects, which are comparable to that with 3 mg/kg prednisolone. JTE-907 inhibited the edema in a dose-dependent manner. A small dose of 0.01 mg/kg showed a significant effect, and at 1 mg/kg it was almost equivalent to those of Win55212-2, Δ^9 -THC, and prednisolone. A similar effect was seen in SR144528-treated mice (Fig. 4B). Antiedema effects of JTE-907 and SR144528 expressed as ED₅₀ were 0.05 and 0.12 mg/kg, respectively.

Discussion

The data in this article clearly show that JTE-907 is a highly selective CB₂ ligand, which behaves as an inverse

agonist in vitro. The affinity of JTE-907 for CB₂ receptors is high, although the potency (K_i value) is different in three animal species; rat (0.38 nM) > mouse (1.55 nM) > human (35.9 nM). The homology of amino acid sequence between human and mouse CB₂ receptor is 82% (Shire et al., 1996), and the structure of JTE-907 (Fig. 1) is very different from known cannabinoid ligands. Therefore, the difference in binding affinity of JTE-907 in three species can be attributed to both the receptor homologies between the species and the novel structure of JTE-907. Notable observation was that JTE-907 is the weakest CB₁ ligand among the compounds tested, and is 240- to 7500-fold and 38- to 50-fold less active with CB₁ than Win55212-2 and SR144528 as studied in three animal species, respectively. The advantage of such a higher CB₂ selectivity ratio is a lower possibility to affect central nervous system and to cause clinically unfavorable effects. SR144528 is the first reported selective ligand for CB₂, the selectivity of which was approximately 728 with human CB₂ and CB₁ expressed in CHO cells (Rinaldi-Carmona et al., 1998). However, the selectivity ratio was only 25 in our study. The selectivity ratio between CB₁ and CB₂ is actually variable depending on the assay conditions. For example, the human CB₂/CB₁ ratio of Δ^9 -THC and that of Win55212-2 were reported to be 0.99 to 2.49 (Bayewitch et al., 1996; Showalter et al., 1996; Rhee et al., 1997) and 6.75 to 30.00 (Felder et al., 1995; Shire et al., 1996), respectively. Our results with these ligands were 1.6 and 34, respectively, which are almost in the range of the previously reported values. In the present study, there is a tendency that SR144528 has a relatively higher affinity to CB₁ receptor than previous report (Rinaldi-Carmona et al., 1998). Similar results were also shown in a recent article that K_i values of SR144528 in mouse and rat CB₁ were 33.0 and 54.6 nM, respectively (Griffin et al., 1999). Thus, the binding affinities of SR144528 for CB₁ vary depending on the assay condition, and care must be taken in discussing the effect of the compound with high concentrations.

Both CB₁ and CB₂ coupled with Gi proteins are known to reduce cAMP upon the receptor stimulation (Felder et al., 1995). Generally, a ligand that reduces cAMP through the receptor has been defined as agonist. We, therefore, tested JTE-907 as well as other ligands for CB₁ or CB₂ expressed in CHO cells to see whether these compounds are agonists or antagonists. JTE-907 showed a concentration-dependent increase in cAMP production in forskolin-stimulated CHO cells expressing human CB₂ and more potently in mouse CB₂, whereas it did not affect CHO cells expressing human CB₁ or CHO without receptor expression. These results clearly reflect our finding that JTE-907 is 23 times more potent in

TABLE 1

Binding affinities and selectivities of various cannabinoid ligands to membranes of CHO cells expressing human CB₁ and CB₂, mouse cerebellum as CB₁ preparation and CHO cells expressing mouse CB₂, and membrane of rat cerebellum as CB₁ preparation and rat splenocyte as CB₂ preparation

Each value represents mean \pm S.E.M. of three experiments.

Test Compounds	K_i (nM)								
	Human CB ₁	Human CB ₂	Selectivity for CB ₂	Mouse CB ₁	Mouse CB ₂	Selectivity for CB ₂	Rat CB ₁	Rat CB ₂	Selectivity for CB ₂
JTE-907	2370 \pm 297	35.9 \pm 7.32	66	1060 \pm 90.0	1.55 \pm 0.09	684	1050 \pm 35.4	0.38 \pm 0.1	2760
SR144528	50.3 \pm 8.37	1.99 \pm 0.94	25	20.1 \pm 4.20	0.04 \pm 0.00	503	27.6 \pm 2.89	0.24 \pm 0.02	115
Win55212-2	9.87 \pm 1.52	0.29 \pm 0.12	34	0.41 \pm 0.16	0.56 \pm 0.02	0.7	0.14 \pm 0.07	1.30 \pm 0.33	0.1
Δ^9 -THC	5.05 \pm 0.65	3.13 \pm 0.34	1.6	8.33 \pm 3.45	1.73 \pm 0.09	4.8	13.5 \pm 3.81	6.80 \pm 2.28	2

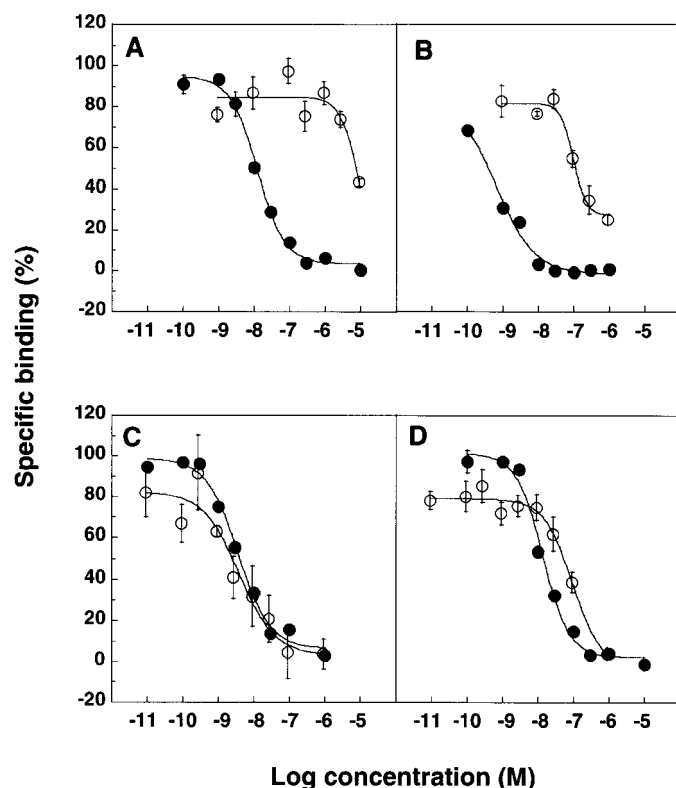


Fig. 2. Competitive displacement of specific [³H]CP-55,940 binding by JTE-907 (A), SR144528 (B), Win55212-2 (C), and Δ⁹-THC (D) to membranes from mouse cerebellum as CB₁ preparation (○) and CHO cells expressing CB₂ (●). Experiments were carried out at 30°C for 90 min using 1 nM [³H]CP-55,940 under the experimental procedures described under *Materials and Methods*. The 100% binding and the nonspecific binding levels (dpm) were 9301 and 4068 in mouse CB₁, and 46996 and 5215 in mouse CB₂, respectively. Each value represents mean ± S.E.M of three series of experiments, each of which was run by triplicate measurements. Data are expressed as the percentage of specific binding of [³H]CP-55,940.

binding affinity to mouse CB₂ than human CB₂, and has a very low binding affinity for human CB₁. These results also suggest that JTE-907 is a CB₂-selective inverse agonist like SR144528 (Portier et al., 1999). Interestingly, SR144528 up to 10 μM did not significantly affect cAMP production in CHO cells expressing human CB₁, despite its relatively high affinity to the CB₁. This may suggest that SR144528 is an antagonist in CB₁ receptor. A synthetic cannabinoid receptor ligand, Win55212-2, showed a concentration-dependent inhibition of cAMP production, and behaved as a full agonist in CHO cells expressing human CB₁ and CB₂ and mouse CB₂. On the other hand, the activity of Δ⁹-THC on cAMP production was different from receptor to receptor. Δ⁹-THC behaved as a full agonist in CHO cells expressing human CB₁ and mouse CB₂, and a partial agonist in CHO cells expressing human CB₂. This result is consistent with previous reports that Δ⁹-THC antagonizes human CB₂-mediated cAMP reduction (Bayewitch et al., 1996) and krox24 pathway (Portier et al., 1999), whereas another report describes that Δ⁹-THC inhibits CB₂-mediated cAMP production both in human and mouse (Shire et al., 1996). Δ⁹-THC may affect the receptor signaling depending on animal species, unlike stably acting agonists such as Win55212-2, CP55940, and HU-210 (Bayewitch et al., 1996).

Δ⁹-THC was reported to be anti-inflammatory in vivo (So-

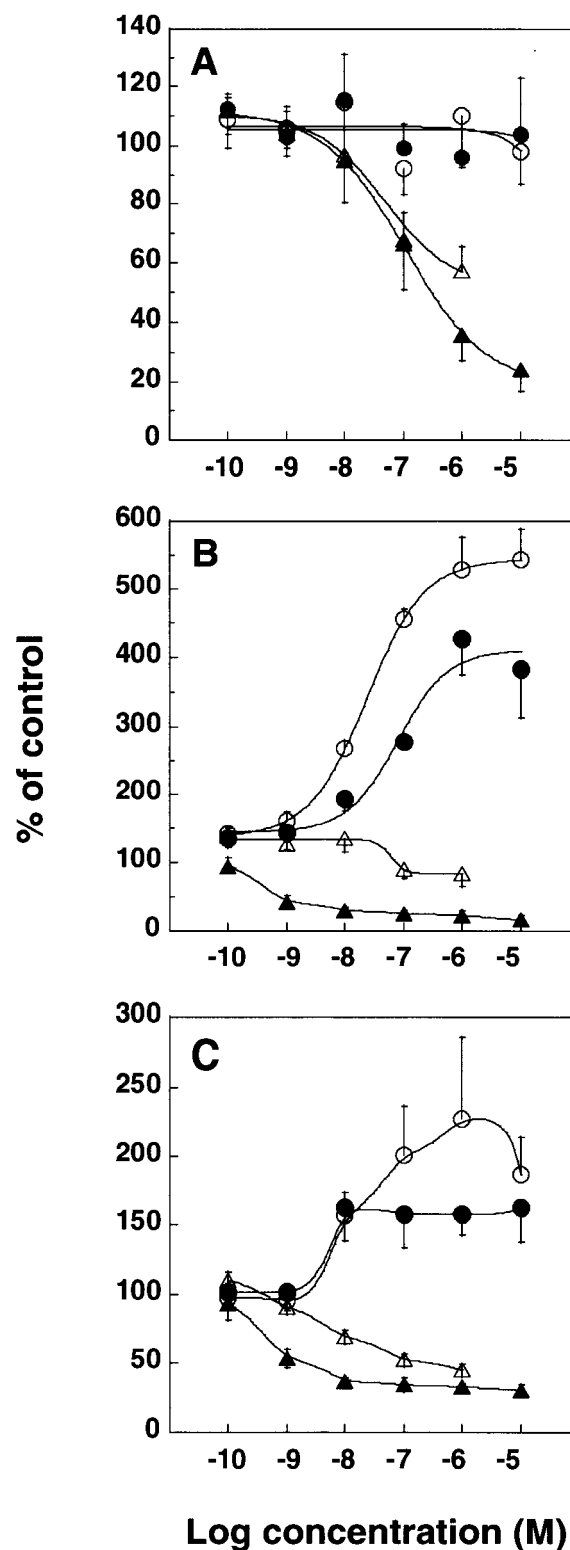


Fig. 3. Effects of JTE-907 (●), SR144528 (○), Win55212-2 (▲), and Δ⁹-THC (△) on cAMP production in CHO cells expressing human CB₁ (A), human CB₂ (B), and mouse CB₂ (C). Cells were preincubated with each test compound at 37°C for 10 min, and then were stimulated with 5 μM forskolin at 37°C for 15 min. Supernatant of the reaction mixture was collected and measured for cAMP by enzyme immunoassay. The cAMP production (pmol/ml) in the absence or presence of 5 μM forskolin was 0.62 ± 0.03, 29.0 ± 3.33 in human CB₁; 0.46 ± 0.06, 6.87 ± 1.48 in human CB₂; and 0.79 ± 0.06, 41.5 ± 2.12 in mouse CB₂, respectively (*n* = 3). Each value represents mean ± S.E.M of three series of experiments, each of which was run by duplicate measurements.

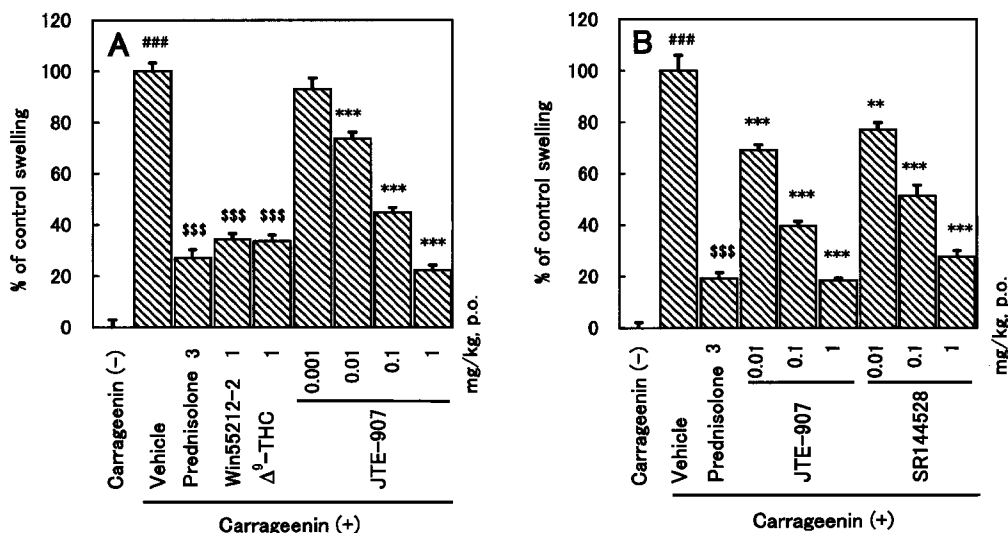


Fig. 4. Effects of various ligands for cannabinoid receptors on carrageenin-induced paw edema in mice. Mice were treated with oral treatment of test compounds as indicated 1 h before the carrageenin injection to mouse foot pad. After 3 h, the increased paw volume was measured in a blind manner. Data are calculated as percentage of increase of paw volume comparing pre- and postcarrageenin injection, and expressed as percentage of control calculated from vehicle and carrageenin treatment group. Paw volumes of pre- and postcarrageenin injections were 205 ± 5 and $310 \pm 8 \mu\text{l}$ in Fig. 4A (prednisolone, Win55212-2, Δ^9 -THC, and JTE-907, $n = 6$), and 197 ± 7 and $302 \pm 5 \mu\text{l}$ in Fig. 4B (prednisolone, JTE-907, and SR144528, $n = 6$). Each value represents mean \pm S.E.M. of six animals. ###, *** $p < 0.001$ compared with vehicle group by Student's t test; ** $p < 0.01$, *** $p < 0.001$ compared with vehicle group by Dunnett's test.

fia et al., 1973; Burstein et al., 1989). Thus, we next tested the anti-inflammatory effects of various cannabinoid ligands in carrageenin-induced mouse paw edema as an inflammation model. Two inverse agonists, JTE-907 and SR144528, dosed orally showed anti-inflammatory effects as well as Δ^9 -THC and Win55212-2. These effects were not parallel with the findings of cAMP production. The anti-inflammatory effects of JTE-907 and SR144528 were dose-dependent, and the efficacy of 1 mg/kg of JTE-907 or SR144528 was equivalent to that of 3 mg/kg prednisolone. We also studied the specificity of JTE-907 by investigating possible influences on other inflammatory mediators in vitro. JTE-907 at 10 μM did not show any significant effect on receptor bindings or enzyme activities of known inflammatory mediators such as adenosine, bradykinin, histamine, leukotrienes, platelet-activating factor, and serotonin, or nitric-oxide synthases, phosphodiesterases, and protein kinase C (data not shown). This suggests that JTE-907 functions as a specific ligand for CB_2 without affecting other inflammatory mediators. The exact mechanism of JTE-907 and SR144528 is not clear yet, however, it is reported that the drugs that raise cAMP show anti-inflammatory effect in carrageenin hind paw edema in vivo (Mohd and Lewis, 1984; Naik, 1984). It is speculated, therefore, that JTE-907 and SR144528 showed antiedema effect by increasing cAMP level as inverse agonist through CB_2 receptor expressed on inflammatory cells. On the other hand, it has already been demonstrated that Δ^9 -THC inhibits carrageenin-induced paw edema through central nervous system because the effect is markedly attenuated by hypophysectomy (Sofia et al., 1973). The pituitary gland is abundant in CB_1 receptor (Wenger et al., 1999), and both Δ^9 -THC and Win55212-2 were proved to possess inhibitory effect on neuronal transmission, which was blocked by CB_1 antagonist SR141716A (Gessa et al., 1998; Shen and Thayer, 1999). Thus, although the effect of Δ^9 -THC and Win55212-2 on cAMP in mouse CB_2 receptor-expressing cells was in contrast to that of JTE-907 and SR144528, CB_1 -mediated effect of

Δ^9 -THC and Win55212-2 in the central nervous system may overwhelm the effect through the CB_2 receptor in vivo.

Another explanation is that endogenous cannabinoid ligands such as anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (Mechoulam et al., 1995; Sugiura et al., 2000) could be produced upon stimulation by carrageenin, and the inverse agonist antagonizes the ligands. Endogenous cannabinoid ligands can be released from neuroblastoma (Hunter and Burstein, 1997) and macrophages (Varga et al., 1998), and may be involved in inflammatory process. For example, anandamide was reported to release arachidonic acid, which would be then converted to proinflammatory prostaglandins (Wartmann et al., 1995), and 2-arachidonoylglycerol was reported to induce splenocyte proliferation (Lee et al., 1995). It was indicated in a recent report that although inverse agonistic activities of histamine "antagonists" cimetidine, ranitidine, and famotidine were observed in cells overexpressing histamine receptor, those ligands act as antagonists in vivo (de Ligt et al., 2000). Therefore, it is also speculated that JTE-907 and SR144528 acted as antagonists of endogenous cannabinoid ligands. These possible mechanisms of anti-inflammatory effect of JTE-907 are now under investigation.

In conclusion, our study demonstrates that JTE-907 is a novel selective inverse agonist of CB_2 , which has antiedema effect in vivo. Although the mechanism underlying the effect of JTE-907 has not yet been entirely explained, our results suggest an involvement of CB_2 in inflammatory process and the pharmacological efficacy of CB_2 inverse agonist by itself. Future studies are necessary to prove the mechanism, which may lead to a new approach to therapeutic use of CB_2 inverse agonist for diseases.

Acknowledgments

We thank T. Yoshida, T. Matsui, K. Takagi, and J. Nishiu for technical assistance; Dr. J. Mizushima for technical advice; and Professor S. Yamamoto for critical reading of this manuscript.

References

- Bayewitch M, Rhee M, Avidor-Reiss T, Breuer A, Mechoulam R and Vogel Z (1996) (-)- Δ^9 -Tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. *J Biol Chem* **271**:9902–9905.
- Burstein SH, Audette CA, Doyle SA, Hull K, Hunter SA and Latham V (1989) Antagonism to the action of platelet activating factor by a nonpsychoactive cannabinoid. *J Pharmacol Exp Ther* **251**:531–535.
- Burstein S, Budrow J, Debatis M, Hunter SA and Subramanian (1994) Phospholipase participation in cannabinoid-induced release of free arachidonic acid. *Biochem Pharmacol* **48**:1253–1264.
- Coffey RG, Yamamoto Y, Snella E and Pross S (1996) Tetrahydrocannabinol inhibition of macrophage nitric oxide production. *Biochem Pharmacol* **52**:743–751.
- Compton DR, Aceto MD, Lowe J and Martin BR (1996) *In vivo* characterization of a specific cannabinoid receptor antagonist (SR141716A): Inhibition of Δ^9 -tetrahydrocannabinol-induced responses and apparent agonistic activity. *J Pharmacol Exp Ther* **277**:586–594.
- Condie R, Herring A, Koh WS, Lee M and Kaminski NE (1996) Cannabinoid inhibition of adenylyl cyclase-mediated signal transduction and interleukin 2 (IL-2) expression in the murine T-cell line, EL-4. IL-2. *J Biol Chem* **271**:13175–13183.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Wash DC)* **258**:1946–1949.
- Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB₁ and CB₂ receptors. *Mol Pharmacol* **48**:443–450.
- Galiègue S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G and Casellas P (1995) Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* **232**:54–61.
- Gerard C, Mollereau C, Vassart G and Parmentier M (1990) Nucleotide sequence of a human cannabinoid receptor cDNA. *Nucleic Acids Res* **18**:7142.
- Gessa GL, Casu MA, Carta G and Mascia MS (1998) Cannabinoid decreases acetylcholine release in the medial-prefrontal cortex and hippocampus, reversed by SR141716A. *Eur J Pharmacol* **355**:119–124.
- Griffin G, Wray EJ, Tao Q, McAllister SD, Rorrer WK, Aung M, Martin BR and Abood ME (1999) Evaluation of the cannabinoid CB₂ receptor-selective antagonist, SR144528: Further evidence for cannabinoid CB₂ receptor absence in the rat central nervous system. *Eur J Pharmacol* **377**:117–125.
- Hunter SA and Burstein SH (1997) Receptor mediation in cannabinoid stimulated arachidonic acid mobilization and anandamide synthesis. *Life Sci* **60**:1563–1573.
- Lee M, Yang KH and Kaminski NE (1995) Effect of putative cannabinoid receptor ligands, anandamide and 2-arachidonol-glycerol, on immune function in B6C3F1 mouse splenocytes. *J Pharmacol Exp Ther* **275**:529–536.
- de Ligt RA, Kourounakis AP, IJzerman AP (2000) Inverse agonism at G protein-coupled receptors: (Patho)physiological relevance and implications for drug discovery. *Br J Pharmacol* **130**:1–12.
- Lynn AB and Herkenham M (1994) Localization of cannabinoid receptors and non-saturable high-density cannabinoid binding sites in peripheral tissues of the rat: Implications for receptor-mediated immune modulation by cannabinoids. *J Pharmacol Exp Ther* **268**:1612–1623.
- Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, Pertwee RG, Griffin G, Bayewitch M, Barg J and Vogel Z (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* **50**:83–90.
- Mizushima S and Nagata S (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* **18**:5322.
- Mohd HS and Lewis DA (1984) Anti-inflammatory action of drugs that raise adenosine-3',5'-cyclic monophosphate and putrescine levels in-vivo. *J Pharm Pharmacol* **36**:760–762.
- Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (Lond)* **365**:61–65.
- Naik SR (1984) Increased cyclic AMP-phosphodiesterase activity during inflammation and its inhibition by anti-inflammatory drugs. *Eur J Pharmacol* **104**:253–259.
- Pestonjamasap VK and Burstein SH (1998) Anandamide synthesis is induced by arachidonate mobilizing agonists in cells of the immune systems. *Biochim Biophys Acta* **1394**:249–260.
- Portier M, Rinaldi-Carmona M, Pecceu Florence, Combes T, Poinot-Chazel C, Calandra B, Barth F, Le Fur G and Casellas P (1999) SR144528, an antagonist for the peripheral cannabinoid receptor that behaves as an inverse agonist. *J Pharmacol Exp Ther* **288**:582–589.
- Reibaud M, Obinu MC, Ledent C, Parmentier M, Bohme GA and Imperato A (1999) Enhancement of memory in cannabinoid CB1 receptor knock-out mice. *Eur J Pharmacol* **379**:R1–R2.
- Rinaldi-Carmona M, Barth F, Millan J, Derocq J, Casellas P, Congy C, Oustric D, Sarran M, Bouaboula M, Calandra B, Portier M, Shier D, Breliere J and Le Fur G (1998) SR144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* **284**:644–650.
- Rhee M, Vogel Z, Barg J, Bayewitch M, Levy R, Hanus L, Breuer A and Mechoulam R (1997) Cannabinoid derivatives: Binding to cannabinoid receptors and inhibition of adenylyl cyclase. *J Med Chem* **40**:3228–3233.
- Shen M and Thayer SA (1999) Δ^9 -Tetrahydrocannabinol acts as a partial agonist to modulate glutamatergic synaptic transmission between rat hippocampal neurons in culture. *Mol Pharmacol* **55**:8–13.
- Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessègue B, Bonnin-Cabanne O, Le Fur G, Caput D and Ferrara P (1996) Molecular cloning, expression and function of murine CB2 peripheral cannabinoid receptor. *Biochim Biophys Acta* **1307**:132–136.
- Showalter VM, Compton DR, Martin BR and Abood ME (1996) Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): Identification of cannabinoid receptor subtype selective ligands. *J Pharmacol Exp Ther* **278**:989–999.
- Sofia RD, Nalepa SD, Harakal JJ and Vassar HB (1973) Anti-edema and analgesic properties of delta9-tetrahydrocannabinol (THC). *J Pharmacol Exp Ther* **186**:646–655.
- Sugiura T, Kondo S, Kishimoto S, Miyashita T, Nakane S, Kodaka T, Suhara Y, Takayama H and Waku K (2000) Evidence that 2-arachidonoylglycerol but not N-palmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. *J Biol Chem* **275**:605–612.
- Varga K, Wagner JA, Bridgen T and Kunos G (1998) Platelet and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension. *FASEB J* **12**:1035–1044.
- Wartmann M, Campbell D, Subramanian A, Burstein SH and Davis RJ (1995) The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Lett* **359**:133–136.
- Wenger T, Fernandez-Ruiz JJ and Ramos JA (1999) Immunocytochemical demonstration of CB1 cannabinoid receptors in the anterior lobe of the pituitary gland. *J Neuroendocrinol* **11**:873–878.
- Zheng Z and Specter SC (1996) Delta-9-tetrahydrocannabinol suppresses tumor necrosis factor α maturation and secretion but not its transcription in mouse macrophages. *Int J Immunopharmacol* **18**:53–68.

Send reprint requests to: Hiroyuki Iwamura, Japan Tobacco Inc., Central Pharmaceutical Research Institute, 1-1 Murasaki-Cho, Takatsuki, Osaka 569-1125, Japan. E-mail: hiroyuki.iwamura@ims.jti.co.jp