Pharmacological Evaluation of a Novel Cannabinoid 2 (CB₂) Ligand, PF-03550096, In Vitro and In Vivo by Using a Rat Model of Visceral Hypersensitivity

Akira Kikuchi¹*, Katsuyo Ohashi¹, Yutaka Sugie¹, Hiromi Sugimoto¹, and Hirofumi Omura¹

¹Discovery Research, Pfizer Global Research & Development, Nagoya Laboratories, Pfizer Inc., 5-2, Taketoyo, Chita-gun, Aichi 470-2318, Japan

Received September 14, 2007; Accepted November 30, 2007

Abstract. Previous studies have shown that cannabinoid 2 (CB₂)-receptor agonists might have analgesic effects on visceral hypersensitivity. To extend these results, we have determined the pharmacological characteristics of a newly designed CB₂ ligand, N-[(1S)-1-(aminocarbonyl)-2,2-dimethylpropyl]-3-(3-hydroxy-3-methylbutyl)-2-oxo-2,3-dihydro-1H-benzimidazole-1-carboxamide (PF-03550096), in vitro and in vivo. PF-03550096 showed high affinity to human (Kᵢ = 7.9 ± 1.7 nM) and rat CB₂ receptors (Kᵢ = 47 ± 5.6 nM). In a cell-based functional assay, PF-03550096 behaved as a full agonist and showed high selectivity for human CB₂ receptors. Orally administered PF-03550096 (3, 10 mg/kg) inhibited the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced decrease in colonic pain threshold with statistical significance. The inhibitory effect of PF-03550096 (10 mg/kg) was significantly reversed by a selective CB₂ antagonist, N-(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl-5-(4-chloro-3-methylphenyl)-1(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528), while SR144528 itself did not modify colonic pain threshold. These results indicate that PF-03550096 is a potent CB₂ agonist and possesses efficacy in a rat model of visceral hypersensitivity.

Keywords: cannabinoid 2 (CB₂) agonist, colonic distension, irritable bowel syndrome (IBS), 2,4,6-trinitrobenzene sulfonic acid (TNBS), visceral hypersensitivity

Introduction

Activation of cannabinoid receptors is known to induce various pharmacological effects in the gastrointestinal tract (1, 2). Two cannabinoid receptor subtypes, cannabinoid 1 (CB₁) and cannabinoid 2 (CB₂) receptors, both of which are G protein–coupled receptors, have been identified and cloned. CB₁ receptors are mostly expressed in the central and peripheral nervous systems (3, 4), while CB₂ receptors are mostly expressed in immune cells, such as mast cells, and the peripheral nervous system (5, 6). Activation of CB₁ receptors in the central nervous system produces a number of psychoactive effects, including hypothermia, catalepsy, and hypolocomotion in rats; hence, the therapeutic use of systemically administered nonselective cannabinoid (CB) agonists or CB₁ receptor–selective agonists may be limited. In contrast, activation of CB₂ receptors has no psychoactive effects in rats (7, 8) and therefore is of particular therapeutic interest. A recent study using CB₂-receptors knock-out mice showed that the CB₂ agonist AM1241 modulates visceral afferent responses to the immunogenic agent bradykinin via activation of CB₂ receptors (9). In the colitis-induced hypersensitivity model, the CB₂ agonist JWH015 reduced the abdominal responses to rectal distension mediated by CB₂ receptors (10). These results suggest that CB₂ agonists might have analgesic effects on visceral hypersensitivity. To explore the possible use of CB₂ agonists to treat functional gut disorders associated with visceral hypersensitivity, such as irritable bowel syndrome (IBS), we have synthesized a novel CB₂ ligand, N-[(1S)-1-(aminocarbonyl)-2,2-dimethylpropyl]-3-(3-hydroxy-3-methylbutyl)-2-oxo-2,3-dihydro-1H-benzimidazole-1-carbox-
amide (PF-03550096), which is a derivative of benzimidazolone (Fig. 1). Here, we have determined the affinity of PF-03550096 for the CB₁ and CB₂ receptors and examined whether it shares the ability of cannabinoid receptor agonists to inhibit forskolin-stimulated cyclic adenosine monophosphate (cAMP) production in Chinese hamster ovary (CHO) cells expressing CB₁ or CB₂ receptors. Furthermore, we examined the effects of PF-03550096 on 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced visceral hypersensitivity in vivo.

**Materials and Methods**

**Materials**

PF-03550096 and N-(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) were synthesized at Pfizer Global Research Development (PGRD) Nagoya Laboratories (Aichi). [³H]CP55,940 (specific activity: 160.6 Ci/mmol) and [³H]SR141716A (specific activity: 49.0 Ci/mmol) were purchased from Perkin Elmer (Waltham, MA, USA) and Amersham Biosciences (Buckinghamshire, UK), respectively. In this study, the following reagents were used: Ham’s F-12 medium, HEPES, phosphate-buffered saline (PBS), geneticin, and penicillin/streptomycin (Gibco, Paisley, UK); CP55,940, WIN55,212-2, bovine serum albumin (BSA), IBMX, protease inhibitor cocktail, Ro-20-1724, forskolin, dialyzed FBS, Tris-HCl, and ketamine/xylazine (Sigma-Aldrich, St. Louis, MO, USA); AM251 (Tocris, Avonmouth, Bristol, UK); EDTA (Dojindo, Gaithersburg, MD, USA); dimethyl sulfoxide (DMSO), polyethylene glycol 400 (PEG400), MgCl₂, and ethyl alcohol (Wako Pure Chemical Industries Ltd., Osaka); TNBS (Fluka, Buchs, Switzerland). For the in vivo experiments, PF-03550096 was suspended in 0.5% methylcellulose solution. SR144528 was dissolved in DMSO/PEG400 (1:19). The solvent alone had no effect on the responses in the in vivo studies.

**Membrane preparation and binding**

HEK293 cells expressing human CB₁ (hCB₁) receptors and CHO-K1 cells expressing human CB₂ (hCB₂) receptors were obtained from PGRD Groton laboratories (Groton, CT, USA). CHO-K1 cells stably expressing rat CB₂ (rCB₂) receptors were obtained from PGRD Nagoya Laboratories. Cells transfected with hCB₁, hCB₂, or rCB₂ were grown in flasks containing F-12 medium supplemented with 10% FBS, 20 mM HEPES, 100 units/ml penicillin, 100 µl/ml streptomycin, and 500 µg/ml geneticin. Harvested cells were homogenized in ice-cold 50 mM Tris-HCl containing protease inhibitor cocktail, followed by centrifugation at 10000 x g for 10 min at 4°C. The supernatant was centrifuged at 40,000 x g for 10 min at 4°C, and the pellets were suspended in 50 mM Tris-HCl (pH 7.4). The suspension was centrifuged once more in the same manner, and the pellet was resuspended in 50 mM Tris-HCl (pH 7.4). For the rat CB₁ (rCB₁) binding assay, whole brains were collected from euthanized male Sprague-Dawley rats purchased from Charles River, Inc. (Shiga). Tissues were homogenized in 5 mM Tris-HCl containing 2 mM EDTA at 4°C for 15 min. Following centrifugation as described above, pellets were resuspended in 25 mM Tris-HCl containing 5 mM MgCl₂, 1 mM EDTA, and 0.5% BSA. Competition binding experiments were performed in 96-well plates with 1 nM [³H]SR141716A for membranes from cells transfected with hCB₂ (40 µg/ml) or rCB₂ (40 µg/ml) or with 1 nM [³H]SR141716A for membranes from cells transfected with hCB₁ (40 µg/ml) or whole rat brain (20 µg/ml). Competitors were present at varying concentrations, and the nonspecific binding of the radioligands was determined in the presence of 10 µM CP55,940 for CB₂ binding assays or 10 µM AM251 for CB₁ binding assays. Reactions were incubated for 60 min at 25°C, and then 100 µl stop buffer (TME buffer containing 5% BSA) was added. The assays were terminated by filtration using a Unifilter cell harvester (Perkin Elmer) followed by three washes of 25 mM Tris-HCl (pH 7.4 at 25°C) onto Unifilter 96 well GF/C. Radioactivity was measured by scintillation counting using the Packard Top-Count Microplate Scintillation Counter. Binding isotherms were analyzed by non-linear regression using GraphPad Prism ver.2.01 (San Diego, CA, USA).
Cell-based cAMP functional assay

Cellular cAMP was measured using HTRF cAMP dynamic reagent purchased from CISBIO (Cedex, France). CHO-K1 cells expressing hCB1 or hCB2 receptors were obtained from PGRD Groton laboratories and were grown in flasks containing F-12 medium supplemented with 10% FBS, 20 mM HEPES, 50 units/ml penicillin, 50 µl/ml streptomycin, and 500 µg/ml geneticin. For the hCB1 functional assay, cells expressing hCB1 were harvested, resuspended in incubation buffer (F-12 medium, 20 mM HEPES, 1 mM IBMX, and 1 mg/ml acid-free BSA), and dispensed into 384-well plates at a density of 1.0×10^3 cells/well in the presence or absence of test compounds. Cells were incubated with 10 µM forskolin at 37°C for 30 min, and the cAMP-XL665–conjugated antibody and the anti-cAMP-cryptase–conjugated antibody were added to the plate. After incubation for 60 min at room temperature, measurements were made using a Wallac 1420 ARVOsx multilabel counter (Perkin Elmer). Data analysis was performed based on the ratio of fluorescence intensity of each well at 620 and 665 nm. The sigmoidal dose-response equation was used to determine EC50 and Emax values. These values represent the relative efficacy that was defined as the ratio of the response of test compound to the maximum response of 2-arachidonoylglycerol. For the hCB2 functional assay, cells expressing hCB2 were harvested, re-suspended in incubation buffer (F-12 medium, 20 mM HEPES, 1 mM IBMX, and 0.1 mM Ro-20-1724), and dispensed into 96-well plates at a density of 2.5×10^3 cells/well in the presence or absence of test compounds. Reactions were initiated by adding 5 µM forskolin at 37°C, and the assay was carried out as described above.

Animals

Male Sprague-Dawley rats (240 – 270 g) were purchased from Charles River, Inc. and were housed in pairs in polypropylene cages with free access to food and water. The animals were kept under conditions of constant temperature (23 ± 2°C) and humidity (55 ± 15%) with a 12-h light/dark cycle (lights on 7:00 a.m.). All experiments employed in this study were approved by the Animal Ethics Committee of Nagoya Laboratories, PGRD, according to the Laboratory Animal Welfare guidelines.

Visceral hypersensitivity model

Rats were fasted for 16 – 18 h before surgery and were anesthetized by intramuscular injection of ketamine (20 mg/kg) and xylazine (3 mg/kg). After abdominal laparotomy, TNBS was injected into the proximal colon (1 cm from the cecum) at a dosage of 50 mg/kg in 0.5 ml 30% ethanol. Sham animals underwent the same surgery but were not administered TNBS. Seven days after surgery, colonic pain threshold was measured as previously reported (11, 12). Animals were fasted for 16 – 18 h and then placed individually into polypropylene cages. After acclimation, a latex balloon (5-cm in length; Okamoto, Tokyo) was placed in the distal colon 5 cm from the anus, and it was fixed at the tail with tape. The balloon was progressively inflated from 0 to 70 mmHg, by 5-mmHg increments every 30 s, using an electronic barostat purchased from G&J Electronics, Inc. (Willowdale, Ontario, Canada). The colonic pain threshold was determined as the pressure required to elicit behavioral signs of pain (13, 14).

Pharmacokinetic study

The blood samples were drawn from the jugular vein at specified time intervals after oral or subcutaneous administration of PF-03550096 or SR144528, respectively. The blood samples (100 µl) were precipitated by adding 1 ml acetonitrile and then centrifuged at 10,000 × g for 10 min. The supernatant was analyzed for drug concentration by using a mass spectrometer (API-300; MDS Sciex, Ontario, Canada) equipped with an HPLC system (HP1100; Agilent Technologies, Palo Alto, CA, USA).

Data analyses

The data obtained from colonic distension studies are represented as the median and first and third quartiles that indicate the range of median values calculated by GraphPad Prism Software ver. 2.01. The data were subjected to the individual Mann-Whitney U-test. Statistical significance was determined at P<0.05.

Results

Ligand binding assays

To assess the binding of PF-03550096 to human and rat CB receptors, we performed ligand displacement experiments using [3H]SR141716A and [3H]CP55,940 for CB1 and CB2 binding assays, respectively. The Kd and Bmax values for each experiment were as follows: Kd (Bmax) of hCB1: 1.7 nM (2.8 pmol/mg), hCB2: 3.3 nM (15 pmol/mg), rCB1: 0.74 nM (4.2 pmol/mg), and rCB2: 0.81 nM (1800 pmol/mg). The binding affinities expressed as Ki for PF-03550096 and CP55,940 on CB receptors are summarized in Table 1. PF-03550096 displaced [3H]CP55,940 bound to human and rat CB2 receptors with high affinities (hCB2: 7.9 ± 1.7 nM, rCB2: 47 ± 5.6 nM), whereas it showed lower affinities to human and rat CB1 receptors (hCB1: 1500 ± 180 nM, rCB1: 1300 ± 210 nM). The selectivity ratios of PF-
The ability of PF-03550096 to activate CB receptors was assessed in a functional cAMP accumulation assay using cells expressing human CB$_1$ or CB$_2$ receptors (Table 2). Similar to WIN55,212-2, PF-03550096 potently inhibited forskolin-mediated cAMP production by human CB$_2$ receptors, with an EC$_{50}$ value of 2.1 $\pm$ 0.50 nM. In contrast, the EC$_{50}$ value of PF-03550096 on human CB$_1$ receptors was 450 $\pm$ 250 nM; this indicates a 213-fold selectivity ratio for human CB$_2$ receptors. In addition, both PF-03550096 and WIN-55,212-2 showed maximum inhibition of forskolin-mediated cAMP production by human CB$_2$ receptors, with E$_{max}$ values of 120 $\pm$ 5.7% and 110 $\pm$ 4.7%, respectively.

**PF-03550096 inhibited TNBS-induced decreased colonic pain threshold in rats**

Seven days after surgery, the colonic pain threshold in response to colonic distension was measured in sham and TNBS-treated rats. We observed a decreased pain threshold in animals treated with TNBS in comparison to sham animals (Fig. 2). Oral administration of PF-03550096 (3, 10 mg/kg) inhibited the TNBS-induced decrease in pain threshold with statistical significance. No abnormal behaviors were observed during the experimental period.

**SR144528 reversed the inhibitory effects of PF-03550096 in TNBS-treated rats**

We examined whether the inhibitory effects of PF-03550096 could be reversed by the CB$_2$-selective antagonist SR144528. Inhibitory effects of PF-03550096 (10 mg/kg) were reproducibly observed with statistical significance; these effects were reversed by subcutaneous pretreatment with SR144528 (10 mg/kg). SR144528 itself did not modulate the pain threshold (Fig. 3).

**Plasma concentrations of PF-03550096 and SR144528**

Pharmacokinetics studies of PF-03550096 (1 mg/kg) and SR144528 (10 mg/kg) were assessed in the rat. After the oral administration of PF-03550096, the plasma concentration reached its maximum (21 $\pm$ 4.9 ng/ml) at 30 min after dosing and gradually decreased up to 8 h. The plasma concentration of SR144528 gradually increased post dosing and saturated at 4 h after subcutaneous administration (33 $\pm$ 11 ng/ml).

### Table 1. Binding affinities and selectivities of PF-03550096 at human and rat cannabinoid receptors

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Human CB$_1$ (nM)</th>
<th>Human CB$_2$ (nM)</th>
<th>Selectivity (Rat CB$_1$/Rat CB$_2$)</th>
<th>Selectivity (Human CB$_1$/Human CB$_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-03550096</td>
<td>1500 $\pm$ 180</td>
<td>7.9 $\pm$ 1.7</td>
<td>190</td>
<td>28</td>
</tr>
<tr>
<td>CP55,940</td>
<td>13 $\pm$ 0.95</td>
<td>2.1 $\pm$ 0.82</td>
<td>6.2</td>
<td>16</td>
</tr>
</tbody>
</table>

Data represent the mean $\pm$ S.E.M. from at least three separate experiments each performed in dupulicate.

### Table 2. Effects of PF-03550096 on forskolin-stimulated cAMP production by CHO cells expressing human CB$_1$ and CB$_2$ receptors

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Human CB$<em>1$ EC$</em>{50}$ (nM)</th>
<th>Human CB$<em>1$ E$</em>{max}$ (%)</th>
<th>Human CB$<em>2$ EC$</em>{50}$ (nM)</th>
<th>Human CB$<em>2$ E$</em>{max}$ (%)</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-03550096</td>
<td>450 $\pm$ 250</td>
<td>100 $\pm$ 2.7</td>
<td>2.1 $\pm$ 0.50</td>
<td>120 $\pm$ 5.7</td>
<td>210</td>
</tr>
<tr>
<td>WIN-55212.2</td>
<td>12.0 $\pm$ 2.5</td>
<td>120 $\pm$ 5.5</td>
<td>0.50 $\pm$ 0.11</td>
<td>110 $\pm$ 4.7</td>
<td>24</td>
</tr>
</tbody>
</table>

Each value represents the mean $\pm$ S.E.M. of three series of experiments, each of which was run by duplicate measurement.
Discussion

The purpose of this study was to elucidate the in vitro characteristics of PF-03550096, a newly synthesized CB$_2$ ligand, and to determine the effects of PF-03550096 in the TNBS-induced visceral hypersensitivity model. Binding data showed that the selectivity of PF-03550096 for hCB$_2$ and rCB$_2$ receptors were higher than those of CP55,940. Notably, PF-03550096 had 190-fold selectivity for hCB$_2$ over hCB$_1$. Both CB$_1$ and CB$_2$ coupled to Gi reduce cAMP upon receptor stimulation (15). Generally, a ligand that reduces cAMP via receptor binding is defined as an agonist. We therefore tested PF-03550096 in a cAMP accumulation assay to determine whether this compound is an agonist or an antagonist. In this assay, PF-03550096 behaved as a full agonist upon binding human CB$_2$ receptors and displayed 210-fold selectivity for hCB$_2$ over hCB$_1$ receptors. An advantage of this CB$_2$ selectivity ratio is a reduced possibility of affecting the central nervous system and causing clinically adverse effects mediated by CB$_1$ receptors. To evaluate the utility of PF-03550096 for studying the role of CB$_2$ receptors in vivo, we characterized its pharmacokinetic properties in rats. PF-03550096 was rapidly absorbed following oral administration with peak plasma concentrations achieved 30 min after dosing at 1 mg/kg. Thus, we evaluated the efficacy of PF-03550096 30 min after oral administration. To measure the pain threshold in response to colonic distension, we used behavioral signs of pain as an endpoint in the TNBS-induced visceral hypersensitivity model. This endpoint has been previously used and defined as a pain response in other studies because morphine showed potent efficacy on TNBS-induced visceral hypersensitivity (11). Using this endpoint, we demonstrated that PF-03550096 significantly inhibited TNBS-induced visceral hypersensitivity; this effect was reversed by the CB$_2$-selective antagonist SR144528. Agonist activity of PF-03550096 on rCB$_2$ receptors was not determined in vitro; however, reversal by SR144528 indicates that PF-03550096 behaved as a CB$_2$ agonist in rats. In addition, none of the rats showed abnormal behaviors at any dose. These results indicate that the pharmacological effects of PF-03550096 via CB$_2$ receptors are visceral analgesic ones. In a previous study using this model, Ohashi et al. observed infiltration of mast cells in the distal colon in the absence of inflammation by both the myeloperoxidase assay and histological examination (14). It seemed possible that the accumulation of mast cells would be involved in visceral hypersensitivity to colonic distension. Given that activation of mast cells is inhibited via activation of CB$_2$ receptors in vitro (16, 17), a possible mechanism of PF-03550096 action is inhibiting the release of pro-inflammatory mediators from mast cells in response to colonic distension. Patients suffering from IBS demonstrate lowered visceral sensory threshold to colorectal balloon distension, indicating visceral hypersensitivity (18, 19). It has also been shown that there are increased numbers of mast cells in close proximity to nerve fibers in the colonic mucosa of some patients with IBS (20). Barbara et al. demonstrated that mucosal mast cell mediators from IBS patients excite rat nociceptive visceral sensory nerves (21). These data suggest that the infiltration and degranulation of intestinal mast cells are involved in altered excitability of primary afferent and enteric neurons, which are related to visceral hypersensitivity in IBS patients. Based on these findings, CB$_2$-receptor agonists may represent therapeutic agents for treatment of visceral hypersensitivity via activation of CB$_2$ receptors expressed on mast cells. In conclusion, we have generated a novel CB$_2$ agonist, PF-03550096, that possesses efficacy in the TNBS-induced visceral hypersensitivity model in rats. Although the mechanism underlying the effects of PF-03550096 has not been
fully elucidated, the higher selectivity for hCB₂ receptors suggests a possible use in treatment of functional gastrointestinal disorders characterized by visceral hypersensitivity, including irritable bowel syndromes.

Acknowledgments

Our special thanks to Dr. H. Iwamura for helpful suggestion on this manuscript. We thank Drs. H. Iwata and J. Lin for technical advice and assistance for the in vitro studies. We appreciate Drs. K. Inuzuka and M. Masuda for their technical assistance in the pharmacokinetic studies.

References