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# Species comparison and pharmacological characterization of rat and human $CB_2$ cannabinoid receptors

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**Abstract**

Pharmacological effects of cannabinoid ligands are thought to be mediated through cannabinoid $CB_1$ and $CB_2$ receptor subtypes. Sequence analysis revealed that rat and human cannabinoid $CB_2$ receptors are divergent and share 81% amino acid homology. Pharmacological analysis of the possible species differences between rat and human cannabinoid $CB_2$ receptors was performed using radioligand binding and functional assays. Pronounced species selectivity at the rat cannabinoid $CB_2$ receptor (50- to 140-fold) was observed with AM-1710 (3-(1,1-Dimethyl-heptyl)-1-hydroxy-9-methoxy-benzo[c]chromen-6-one) and AM-1714 (3-(1,1-Dimethyl-heptyl)-1-9-dihydroxy-benzo[c]chromen-6-one). In contrast, JWH-015 ((2-Methyl-1-propyl-1H-indol-3-yl)-napthalen-1-yl-methanone) was 3- to 10-fold selective at the human cannabinoid $CB_2$ receptor. Endocannabinoid ligands were more human receptor selective. Cannabinoid $CB_2$ receptor antagonist, AM-630 ((6-Iodo-2-methyl-1-(2-morpholin-4-yl-ethyl)-1H-indol-3-yl)-(4-methoxy-phenyl)-methanone) was more potent at the rat receptor in radioligand binding and functional assays than that of the human receptor. The findings of the pharmacological differences between the human and rat cannabinoid $CB_2$ receptors in this study provide critical information for characterizing cannabinoid ligands in in vivo rodent models for drug discovery purpose.


*Keywords:* GPCR; Cannabinoid; Cannabinoid $CB_2$ receptor; Species selectivity; Pharmacology

## 1. Introduction

The principal constituent of cannabis ($\Delta^9$THC) exerts a wide spectrum of therapeutic effects such as analgesic, appetite-stimulating and antiemetic effects through cannabinoid receptors, $CB_1$ and $CB_2$. Cannabinoid $CB_1$ receptors are localized mainly in the central nervous system (Matsuda et al., 1993), but are also distributed in the periphery in a variety of tissues, such as cardiovascular and gastrointestinal systems (Matsuda et al., 1990; Gerard et al., 1991; Glass et al., 1997). Activation of the central $CB_1$ receptor has been associated with psychotropic effects including sedation, hypothermia and catalepsy. On the other hand, cannabinoid $CB_2$ receptors, initially identified in myeloid cells (Munro et al., 1993) are localized largely in the peripheral tissues, such as the immune system (Galigue et al., 1995; Klein et al., 1998). It was recently reported that the expression of cannabinoid $CB_2$ receptor is induced within the lumbar spinal cord in chronic pain models associated with peripheral nerve injury, and the appearance of cannabinoid $CB_2$ expression coincides with activated microglia (Zhang et al., 2003). It has been shown that cannabinoid $CB_2$ receptor agonists are efficacious in preclinical models of inflammation and pain (Hanus et al., 1999; Malan et al., 2001; Iwamura et al., 2001; Ibrahim et al., 2003). Therefore, an alternative approach to drug

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discovery may involve selectively targeting the cannabinoid $CB_2$ receptor subtype, thereby avoiding cannabinoid $CB_1$ receptor-mediated undesirable central effects.

The cannabinoid $CB_2$ receptor belongs to the G protein-coupled receptor (GPCR) superfamily containing seven transmembrane domains with the typical structural motif such as DRY at the end of the third transmembrane domain, and is negatively coupled to adenylyl cyclase through the $G_{i/o}$ proteins. The human, rat and mouse cannabinoid $CB_2$ receptor cDNAs have been cloned (Griffin et al., 2000). The human and rat cannabinoid $CB_2$ receptors share 81% amino acid identity (81% nucleic acid identity), but the rat and mouse cannabinoid $CB_2$ receptors are more homologous with 93% amino acid identity (90% nucleic acid identity). The sequences for rat, human and mouse cannabinoid $CB_2$ receptors share most identity in the putative transmembrane domains and the largest divergence in their C-termini. Distinct pharmacology has been observed in rat and human tissues with endogenous ligand palmitoylethanolamide (PEA). PEA was shown to be able to displace [$^3$H]WIN-55212-2 binding in mast cells isolated from rat (Facci et al., 1995), but had a low affinity for the cloned human cannabinoid $CB_2$ receptor (Showalter et al., 1996). Therefore, these discrepancies could be the result of species differences with the cannabinoid $CB_2$ receptors, as has been reported for other GPCRs such as bradykinin (Hess et al., 1994) and histamine $H_3$ (Yao et al., 2003) receptors. However, detailed analysis of species differences in cannabinoid $CB_2$ receptor pharmacology has not been available.

In the present study, cannabinoid agonists and antagonists were utilized to characterize and elucidate the species differences observed between the human and rat cannabinoid $CB_2$ receptors stably expressed in HEK cells in binding and functional assays. High-affinity CB ligand [$^3$H]CP-55,940 was used to characterize cannabinoid ligands in binding assays. The cannabinoid receptor agonists have been mostly characterized using assays measuring inhibition of cAMP (Howlett, 1985; Felder et al., 1992). Recently, methods using chimeric G protein technology have provided an alternative way for pharmacological characterization of GPCRs that are linked to adenylyl cyclase. Conklin et al. (1993) have shown that chimeric $G_{\alpha qi}$ or $G_{\alpha qo}$ allows alteration of $G_{\alpha i/o}$-induced inhibition of cyclase activity to activation of phospholipase C/inositol 1,4,5-triphosphate (IP3)-diacylglycerol pathway resulting in elevation of $[Ca^{2+}]_i$ level. This was achieved by replacement of the C-terminal amino acids of $G_{\alpha q}$ with those of $G_{\alpha i}$ or $G_{\alpha o}$ generating $G_{\alpha qo}$ and $G_{\alpha qi}$ chimeric proteins, respectively. Fluorescence Image Plate Reader (FLIPR) assay was developed to measure the $CB_2$-evoked change of $[Ca^{2+}]_i$ concentration in this study. The pharmacological characterization and detailed analysis of the species differences observed for cannabinoid ligands at the human and rat cannabinoid $CB_2$ receptor are summarized here.

## 2. Materials and methods

### 2.1. Materials

(−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP-55,940), (R)-(+)-[2,3-dihydro-5-methyl-3-[4-morpholinylmethyl]-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl) methanone mesylate (WIN-55212-2), (2-Methyl-1-propyl-1H-indol-3-yl)-napthalen-1-yl-methanone (JWH-015), [6-Iodo-2-methyl-1-(2-morpholin-4-yl-ethyl)-1H-indol-3-yl]-(4-methoxy-phenyl)-methanone(AM-630), anandamide and 2-arachidonyl glycerol were purchased from Tocris (Ellisville, MO). [$^3$H]CP-55,940, UniFilter-96 GF/C filter plates and Microscint-20 were purchased from Perkin-Elmer (Boston, MA). Fatty acid-free bovine serum albumin, forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO). The human embryonic kidney (HEK) cell line was obtained from the American Type Culture Collection (Rockville, MD). Lipofectamine 2000, geneticin and zeocin, Dulbecco's Modified Eagle Medium (DMEM), hygromycin and superscript II were purchased from Invitrogen (Carlsbad, CA) except that serum was purchased from Hyclone (Logan, UT). Costar tissue culture flasks, pipettes and V-bottomed 96-well assay plates were purchased from Corning (Corning, NY). Biocoat™ 96-well poly-L-lysine coated clear-bottomed black wall plates were purchased from BD Biosciences (San Jose, CA). FLIPR assay kit and tube strips were purchased from Molecular Devices (Sunnyvale, CA). Robocycler and Quickchange Mutagenesis kit were from Stratagene (La Jolla, CA); 4-(2-Aminoethyl) benzenesulfonylfluoride, HCl (AEBSF, HCl) from Calbiochem (San Diego, CA); Protease inhibitor complete tablets from Roche Diagnostics (Indianapolis, IN) and Advantage-HF2 PCR Kit from Clontech (Palo Alto, CA).

### 2.2. Cloning of the rat cannabinoid $CB_2$ receptor cDNA

Single-strand cDNA was synthesized from rat spleen poly $A^+$ RNA (Clontech) using Superscript II according to the manufacturer's instructions. Oligonucleotide primers were designed according to the sequence of the predicted translation initiation and termination site of the rat $CB_2$ gene (GenBank accession number AF176350). The forward primer contained a Kozak sequence and corresponded to base pairs 7–30 of the rat genomic sequence (CACCCGGCCACCATGGAGGGATGCCGGGAGA). The reverse primer corresponded to base pairs 1095–1076 of rat genomic sequence TCAGCAGTTGGAGCAGCCTG. The rat $CB_2$ cDNA was amplified using the Advantage-HF2 polymerase chain reaction (PCR) Kit according to the manufacturer's instructions. Briefly, the reaction mixture was incubated for 15 s at 94 °C and 4 min at 68 °C for 25 cycles with a final 3 min extension at 68 °C. A ~1.1 kb PCR product was generated, gel purified, digested and cloned into the

pcDNA3.1© directional TOPO expression vector and the rat $CB_2$ cDNA sequence was confirmed. Human $CB_2$ pcDNA3.1/GS© containing a zeocin resistance gene was obtained from Research Genetics, Huntsville, AL, accession number X74328.

### 2.3. Generation of $G_{\alpha qo}$ clone

The $G_{\alpha qi}$ clone (pCEP-$G_{qi5}$) that encodes the $G_{\alpha q}$ protein containing 5 C-terminal amino acids of $G_{\alpha i}$ in place of those of $G_{\alpha q}$ was obtained from Molecular Devices. Primers were designed to change the amino acids from the C-terminus of $G_{\alpha i}$ (DCGLF) to those of $G_{\alpha o}$ (GCGLY) to generate $G_{\alpha qo}$. The Quickchange Mutagenesis kit was used as per manufacturer's recommendations, using 0.08 μM forward primer GCTGAACCTTAAGGGCTGTGGCCTCTACT-GAATCGAGGCCG and reverse primer CGGCCTCGATT-CAGTAGAGGCCACAGCCCTTAAGGTTCAGC, as well as 25 ng of the $G_{\alpha qi}$ clone (pCEP-$G_{qi5}$) as template. PCR reaction was carried out in a Robocycler by incubation at 96 °C for 30 s followed by 18 cycles of 96 °C for 30 s and 72 °C for 12 min. Clones of the PCR products were sequenced to confirm that only the targeted changes were incorporated.

### 2.4. Cell culture and generation of cell lines

HEK cells were grown in DMEM media with high glucose, containing 10% fetal bovine serum at 37 °C with 5% $CO_2$ in a sterile humidified environment. Transfection of HEK cells with human $CB_2$ or rat $CB_2$ cDNAs was performed using Lipofectamine 2000 according to the protocol provided by the vendor. Stable transformants were selected in growth medium containing zeocin (25 μg/ml) for the human $CB_2$ or geneticin (500 μg/ml) for the rat $CB_2$. Colonies were selected (about 2 weeks post-transfection) and $CB_2$ expression screened with radioligand binding assays using [$^3$H]CP-55,940. To generate cell lines used for FLIPR assays, stable human $CB_2$ and rat $CB_2$ cell lines were transfected with $G_{\alpha qo}$ cDNA. The stable transformants were selected in growth media for respective receptors supplemented with 200 μg/ml hygromycin.

### 2.5. Radioligand binding assays

HEK cells stably expressing human or rat cannabinoid $CB_2$ receptors were grown until a confluent monolayer was formed. The cells were harvested and homogenized in Tris–EDTA (TE) buffer (50 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mM EDTA) using a polytron for 2×10 s bursts in the presence of protease inhibitors, followed by centrifugation at 45,000×$g$ for 20 min. The final membrane pellet was re-homogenized in storage buffer (50 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mM EDTA and 10% sucrose) and frozen at −80 °C until used. Saturation binding reactions were initiated by the addition of membrane preparation (protein concentration of 5 μg/well for the human $CB_2$ and 20 μg/well for the rat $CB_2$) into wells of a deep-well plate containing ([$^3$H]CP-55,940 (120 Ci/mmol)) in assay buffer (50 mM Tris–HCl, pH 7.4, 2.5 mM EDTA, 5 mM $MgCl_2$, and 0.5 mg/ml fatty acid-free bovine albumin serum). After incubation at 30 °C for 90 min, binding reaction was terminated by the addition of 300 μl/well of cold assay buffer followed by rapid vacuum filtration through a UniFilter-96 GF/C filter plates (pre-soaked in 1 mg/ml bovine serum albumin for 2 h). The bound activity was counted in a TopCount using Microscint-20. In some experiments with endogenous ligands, experiments were performed in the presence of AEBSF (50 μM). Saturation experiments were conducted with 12 concentrations of [$^3$H]CP-55,940 ranging from 0.01 to 8 nM. Competition experiments were conducted with 0.5 nM [$^3$H]CP-55,940 and five concentrations (1 nM to 10 μM) of displacing ligands. Nonspecific binding was defined by the addition of 10 μM unlabeled CP-55,940 in both saturation and competition binding assays. $K_d$ values from saturation binding assays and $K_i$ values from the competition binding assays were determined with one site binding or one site competition curve fitting equations using Prism software (GraphPad, San Diego, CA).

### 2.6. cAMP assays

HEK cells stably expressing human and rat cannabinoid $CB_2$ receptors were maintained in DMEM, 10% fetal bovine serum and 200 μg/ml geneticin selection for rat $CB_2$ and 25 μg/ml zeocin for human $CB_2$ prior to cAMP assays. Cells were plated at 100,000 per well in 100 μl in 96 well poly-L-lysine-coated clear bottom black plates and grown overnight. Activation of human and rat cannabinoid $CB_2$ receptors were measured by reduction of forskolin-induced release of cAMP. Cells expressing the human or rat cannabinoid $CB_2$ receptors were preincubated for 10 min at room temperature with IBMX (1 mM) in PBS. Cells were treated with cannabinoid agonists in PBS plus 0.1% bovine serum albumin for 10 min at room temperature and further challenged with 10 μM forskolin for 10 min. The reaction was terminated by the addition of 0.1 N HCl and centrifuged to remove the debris. The pH was adjusted to 8 using 1 N NaOH and the cAMP level was then measured using an enzyme linked immunosorbent assay (ELISA) kit (Assay Designs, Ann Arbor, MI). For antagonist assays, cells were preincubated with AM-630 for 10 min at room temperature before the addition of CP-55,940. Results are expressed as percent inhibition of forskolin stimulated cAMP accumulation. Sigmoidal dose response curves were analyzed using Prism and $EC_{50}$ values were calculated. The cAMP levels at each compound concentration were expressed as percentage inhibition of forskolin stimulated cAMP level.

### 2.7. FLIPR assays

HEK cells stably co-expressing the $G_{\alpha qo}$ with either the human or rat cannabinoid $CB_2$ receptor were seeded the day

before the assay at 60,000 cells/well in a 96-well poly-D-lysine coated clear-bottomed black wall plates and were incubated overnight. Media was aspirated from 96-well plates gently so as not to disrupt the cells. One vial of $Ca^{2+}$ dye (Molecular Devices, product #R8041) was prepared with 10 ml of assay buffer without bovine serum albumin and used as a stock of 10× concentration. For the assay, 150 μl of 1× $Ca^{2+}$ dye in assay buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 2 mM $CaCl_2$, 0.05% bovine serum albumin) containing 250 μM probenicid (prepared in a 1:1 solution of assay buffer and 1 N NaOH) was added to wells and incubated with the cells for 1 h (Martin et al., 2002). Test compounds were prepared in tube strips in assay buffer at a 4× stock of the desired concentration. Assay buffer was used as a vehicle control and 10 μM CP-55,940 as a positive control. The fluorescence level was measured using FLIPR from 10 s prior to the addition of compound up to 2 min after the compound addition. For antagonist protocols, antagonist was added as the first addition, followed by 50 μl of buffer or 10 μM CP-55,940 as the second addition. The time interval between the two additions was 5 min. All data were corrected for background signal by subtracting vehicle controls and normalized to the response of cells to 10 μM CP-55,940. Maximum agonist responses were plotted as percent response compared to 10 μM CP-55,940 (100%) and analyzed using the sigmoidal dose response curve fitting to obtain the $EC_{50}$ values with Prism. Antagonist data were also normalized to the maximum response of CP-55,940.

## 3. Results

### 3.1. Radioligand binding assays using [$^3$H]CP-55,940 ligand for the human and rat cannabinoid $CB_2$ receptors

Stable cell lines were established in HEK cells that expressed either the rat or the human cannabinoid $CB_2$ receptor. Saturation experiments using membrane preparations expressing the human cannabinoid $CB_2$ receptor showed that the radioligand [$^3$H]CP-55,940 binds to the human receptor ($K_d$=0.58 nM and $B_{max}$=9.2 pmol/mg) (Fig. 1A). Rat $CB_2$ exhibited $K_d$ value of 0.45 nM and $B_{max}$ value of 1.0 pmol/mg (Fig. 1B). No specific binding of [$^3$H]CP-55,940 was detected in membranes from untransfected HEK cells (data not shown). Competition experiments were performed using the same membranes preparations expressing the human or the rat cannabinoid $CB_2$ receptor. The affinities of cannabinoid ligands were established based upon their abilities to displace the binding of [$^3$H]CP-55,940. The cannabinoid ligands used were from different structural classes. JWH-015 showed 3-fold higher affinity at the human cannabinoid $CB_2$ receptor ($K_i$=54 nM) compared to rat ($K_i$=150 nM). In contrast, AM-1714 and AM-1710 showed 10- to 15-fold higher

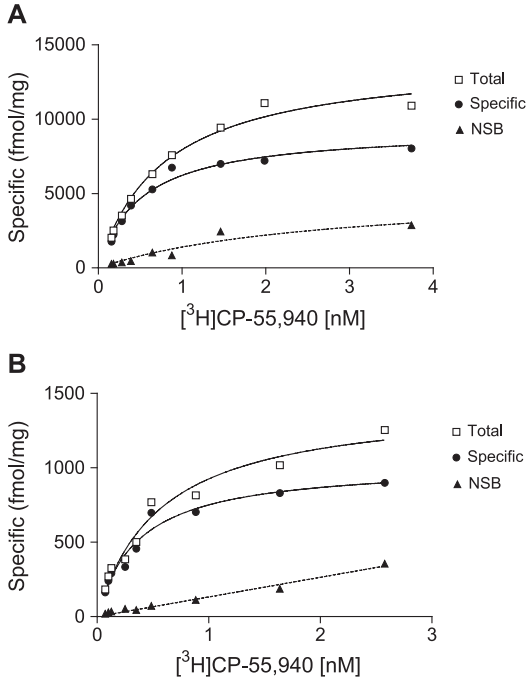


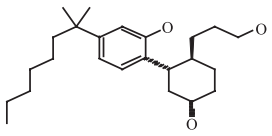
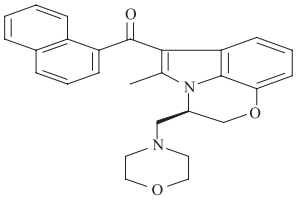
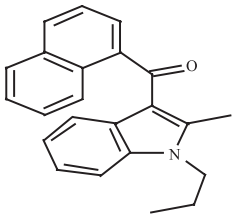
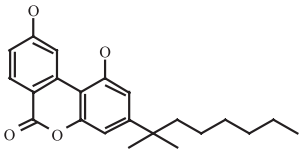
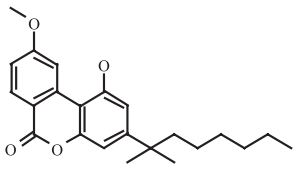
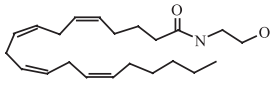
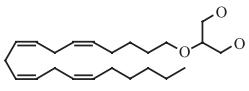
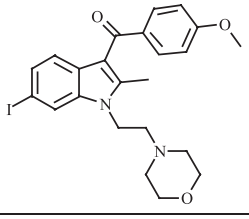

Fig. 1. Saturation binding assays of [$^3$H]CP-55,940 at human cannabinoid $CB_2$ (A) and rat cannabinoid $CB_2$ (B) receptors. Total (□), NSB (▲) and specific (●) represent total, nonspecific and specific bindings, respectively. Data are representative of three experiments performed in triplicate.

affinities at the rat cannabinoid $CB_2$ receptor. AM-1714 exhibited $K_i$ values of 2 nM at the rat receptor and 29 nM at the human receptor, and AM-1710 showed $K_i$ of 3 nM at the rat compared to 28 nM at human cannabinoid $CB_2$ receptor, respectively. Also, AM-630, a $CB_2$-selective ligand was more potent at the rat receptor compared to human with binding affinities of 2.3 and 26 nM at the rat and human cannabinoid $CB_2$ receptors, respectively. CP-55,940 and WIN-55212-2 showed comparable affinities at the rat and human cannabinoid $CB_2$ receptors. The endocannabinoids showed 2- to 3-fold higher affinity at the human cannabinoid $CB_2$ receptor compared to rat receptor. Table 1 summarizes the comparison of affinities at the human and the rat receptors for the cannabinoid ligands in binding experiments.

### 3.2. Functional characterization of human and rat cannabinoid $CB_2$ receptors using cAMP assays

The human or rat cannabinoid $CB_2$ receptors are negatively coupled to adenylyl cyclase and the activation of cannabinoid $CB_2$ receptors inhibit forskolin-stimulated cAMP production. In cAMP assays, CP-55,940 exhibited a potency of 9 nM with 74% efficacy at the human cannabinoid $CB_2$ receptor (Fig. 2A), comparable to that seen at the rat receptor (19 nM with 74% efficacy). AM-1714 and AM-1710 showed 2- to 5-fold higher potencies at the rat cannabinoid $CB_2$ receptor compared to human (Fig. 2B). However, WIN-55212-2 was selective at the human receptor by 30-fold and, 2-arachidonyl glycerol and

Table 1
Comparison of ligand $K_i$ (nM) values of competition binding assays

| Ligand | Structure | Human $K_i$ (nM) (95% confidence interval) | Rat $K_i$ (nM) (95% confidence interval) | Ratio $K_i$ $rCB_2/hCB_2$ |
|---|---|---|---|---|
| CP-55,940 |  | 0.68[a] (0.42–1.1) | 0.52 (0.42–0.73) | 0.76[b] |
| WIN-55212-2 |  | 1.2 (1.0–3.1) | 2.1 (1.7–5.1) | 1.7 |
| JWH-015 |  | 54 (41–71) | 150 (90 –250) | 2.7[c] |
| AM-1714 |  | 29 (21–41) | 1.9 (1.1–4.0) | 0.065 |
| AM-1710 |  | 28 (24–30) | 2.0 (1.7–6.0) | 0.071 |
| Anandamide |  | 160 (140–390) | 240 (180–310) | 1.5 |
| 2-arachidonyl glycerol |  | 1100 (920–1200) | 3700 (1200–11000) | 3.3 |
| AM-630 |  | 26 (20–29) | 2.3 (1.7–4.2) | 0.088 |

[a] $n \geq 4$ experiments.
[b] Ratio<1 means more potent at the rat receptor.
[c] Ratio>1 means more potent at the human receptor.

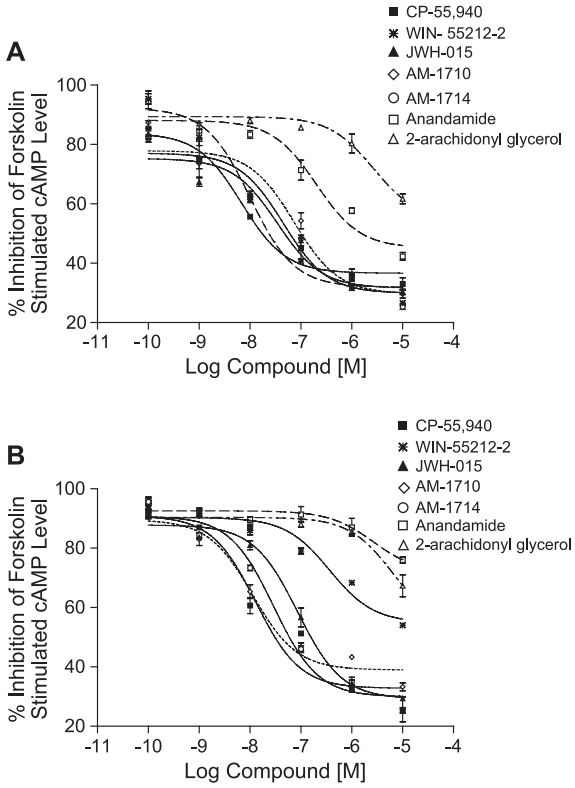



Fig 2. Effects of cannabinoid receptor ligands on forskolin-stimulated cAMP production in HEK cells expressing human (A) and rat (B) cannabinoid $CB_2$ receptors, respectively. The concentration of forskolin used in these experiments was 10 μM. Points indicate mean±S.E.M. percentage of forskolin stimulated cAMP calculated from three to four experiments, each performed in triplicate. The ligands used were CP-55,940, WIN-55212-2, JWH-015, AM-1710, AM-1714, Anandamide and 2-arachidonyl glycerol.

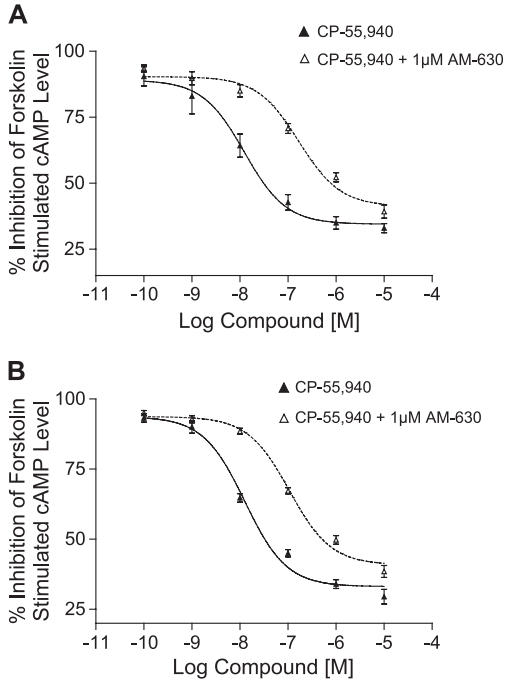



Fig 3. The effect of 1 μM AM-630 (dotted line) on the CP-55,940 induced inhibition of forskolin stimulated cAMP production in HEK cells expressing the human (A) and rat (B) cannabinoid $CB_2$ receptors, respectively. Cells were preincubated with 1 μM AM-630 before the addition of increasing concentration of CP-55,940. Each point represents mean±S.E.M. ($n \geq 3$) percentage change in forskolin stimulated cAMP production.

anandamide exhibited weak potencies at the human receptor but were essentially inactive at the rat receptor (Table 2). WIN-55212-2 showed $EC_{50}$ of 11 nM at human cannabinoid $CB_2$ receptor comparable to that observed for CP-55,940. However, at the rat cannabinoid $CB_2$ receptor, WIN-55212-2 showed much weaker potency ($EC_{50}$=330 nM) and efficacy (29%) (Fig. 2B). The endocannabinoid, anandamide-inhibited forskolin stimulated cAMP production at the human cannabinoid $CB_2$ receptor ($EC_{50}$=230 nM), whereas at the rat receptor the $EC_{50}$ was >1 μM. 2-arachidonyl glycerol was not active at the rat cannabinoid $CB_2$ receptor ($EC_{50}$>10 μM) and weak at the human $CB_2$

Table 2
Comparison of ligand $EC_{50}$ (nM) values of cAMP and FLIPR assays

| Ligand | cAMP ($hCB_2$) | | cAMP ($rCB_2$) | | Preferred species | FLIPR ($hCB_2$) | | FLIPR ($hCB_2$) | | Preferred species |
|---|---|---|---|---|---|---|---|---|---|---|
| | $EC_{50}$ (nM) | Efficacy (%) | $EC_{50}$ (nM) | Efficacy (%) | | $EC_{50}$ (nM) | Efficacy (%) | $EC_{50}$ (nM) | Efficacy (%) | |
| CP-55,940 | 9.1[a] (7.3–12)[b] | 78 | 19 (18–35) | 74 | Human | 40[a] (18–89)[b] | 97 | 17 (11–27) | 91 | Rat |
| WIN-55212-2 | 11 (7.5–20) | 76 | 330 (160–490) | 29 | Human | 82 (55–160) | 79 | >10 μM | 29 | Human |
| JWH-015 | 61 (38–99) | 72 | 99 (60–160) | 70 | Human | 540 (260–690) | 81 | >1 μM | 67 | Human |
| AM-1714 | 70 (58–84) | 75 | 13 (15–20) | 78 | Rat | >10 μM | 48 | 56 (44–70) | 72 | Rat |
| AM-1710 | 49 (25–62) | 72 | 25 (17–31) | 79 | Rat | >3 μM | 58 | 66 (47–85) | 78 | Rat |
| Anandamide | 230 (180–280) | 55 | >1 μM | 35 | Human | >10 μM | 59 | >10 μM | 35 | NA |
| 2-arachidonyl glycerol | 2 μM | 32 | >10 μM | 28 | Human | >8 μM | 54 | >10 μM | 34 | NA |

[a] $n \geq 4$ experiments.
[b] 95% confidence interval.

receptor ($EC_{50}$>2 μM) (Fig. 2B). AM-630, a cannabinoid $CB_2$ receptor antagonist was utilized to block the effect of CP-55,940 at the human and rat receptors. Indeed, addition of 1 μM of AM-630 blocked the CP-55,940 dose response with $EC_{50}$=170 nM at human and $EC_{50}$=110 nM at rat cannabinoid $CB_2$ receptor (Fig. 3A and B).

### 3.3. FLIPR assays

FLIPR assays measuring rapid changes in $[Ca^{2+}]_i$ level was performed with HEK cells co-expressing the chimeric $G_{\alpha qo}$ protein with either rat or the human cannabinoid $CB_2$ receptor. Significant differences in potencies of cannabinoid ligands were observed at the rat cannabinoid $CB_2$ receptor compared to the human receptor in FLIPR assays. JWH-015 and WIN-55212-2 were human selective with $EC_{50}$ values of 542 and 82 nM, respectively, but were weak and inactive at the rat receptor (Figs. 4A and B). In contrast, AM-1714 and AM-1710 were significantly more potent and efficacious at the rat cannabinoid $CB_2$ receptor with $EC_{50}$ values and efficacies determined as 66 and 56 nM, and 72% to 78%, respectively, but were not active at the human cannabinoid $CB_2$ (Table 2 and Fig. 4B). CP-55,940 exhibited a potency of 40 nM at the human receptor and 17 nM at the rat receptor. Endocannabinoid anandamide and 2-arachidonyl glycerol were not active in the FLIPR assays at the human and rat receptors. AM-630 behaved as an antagonist and blocked the ability of CP-55,940 to increase $[Ca^{2+}]_i$ at human (Fig. 5A) and rat receptors (Fig. 5B).

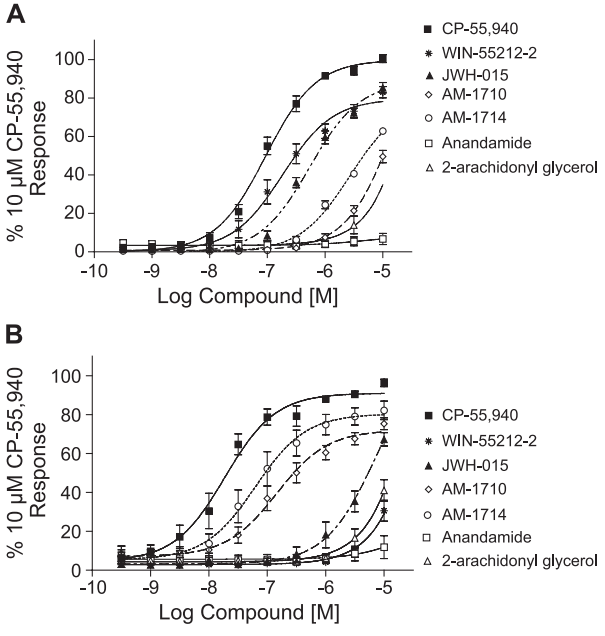



Fig 4. Ability of cannabinoid ligands to show increases in $[Ca^{2+}]_i$ in a concentration-dependent manner in HEK cells co-expressing the $G_{\alpha qo}$ protein and either the human (A) or rat (B) cannabinoid $CB_2$ receptor. $EC_{50}$ values were derived using nonlinear regression curve fitting by Prism and are represented by mean±S.E.M. of three to four concentration–response curves performed in duplicate.

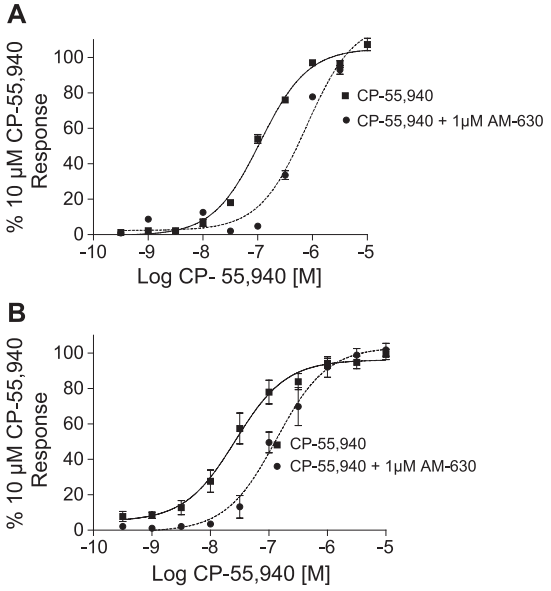



Fig 5. Inhibition of CP-55,940 evoked $[Ca^{2+}]_i$ response by of 1 μM AM-630 (dotted line) in HEK cells co-expressing $G_{\alpha qo}$ protein and either the human (A) or rat (B) cannabinoid $CB_2$ receptor. Results represent the mean±S.E.M. of two to three concentration–response curves performed in duplicate.

AM-630 (1 μM) was more potent at blocking the rat ($EC_{50}$=130 nM) cannabinoid $CB_2$ receptor activation by CP-55,940 compared to the human ($EC_{50}$=470 nM).

## 4. Discussion

Species differences are important aspects of compound characterization in drug discovery, since animal models are commonly used for the preclinical evaluation of chemical leads. The different pharmacological profiles of receptor proteins of various species are presumably the result of the sequence heterogeneity of the receptors. Unlike the $CB_1$ receptor, which is conserved across diverse species like mammals, amphibians, birds and fish (Yamaguchi et al., 1996; Soderstrom and Johnson, 2000; Soderstrom et al., 2000; Soderstrom et al., 2000), the cannabinoid $CB_2$ receptors are more divergent (Griffin et al., 2000; Brown et al., 2002). The human and rat cannabinoid $CB_2$ receptors share only 81% sequence identity in their amino acid sequence and the divergent nature of rat and human cannabinoid $CB_2$ receptors could possibly contribute to the species differences that are reflected in pharmacological profiles of receptor ligands.

To compare the rat and human cannabinoid $CB_2$ receptors, radioligand binding assays were carried out in HEK cells stably expressing human and rat cannabinoid $CB_2$ receptors. Both the human and the rat receptors exhibited similar affinities to the radioligand [$^3$H]CP-55,940 with $K_d$ values of 0.58 and 0.36 nM at the human and rat cannabinoid $CB_2$ receptors, respectively. In the

competition experiments, CP-55,940 and WIN-55212-2 showed comparable affinities at the rat and human cannabinoid $CB_2$ receptors. However, AM-1710 and AM-1714 were 10- to 15-fold more selective at the rat cannabinoid $CB_2$ receptor compared to the human receptor. On the contrary, JWH-015 showed higher affinity at the human cannabinoid $CB_2$ receptor compared to rat in binding assays. Also, anandamide and 2-arachidonyl glycerol were 2- to 3-fold weaker at the rat receptor. In our studies, WIN-55212-2 exhibited similar binding affinity at the human and rat cannabinoid $CB_2$ receptor, distinct from the report by Griffin et al. (2000), where WIN-55212-2 was shown to be 10-fold more selective at the human cannabinoid $CB_2$ receptor. This discrepancy is not expected to arise from the receptor expression level, although the $B_{max}$ for the rat receptor in the current study was 9-fold lower than that of the human receptor, whereas in the report by Griffin et al. (2000), the human receptor $B_{max}$ was 4-fold less than that of the rat. First, the affinity of [$^3$H] CP-55,940 was comparable at the rat and human receptors in our study as well as comparable to that reported by Griffin et al. (2000). Second, the majority of the cannabinoid ligands showed comparable binding affinities in the present study compared to Griffin et al. (2000) study. In addition, we have performed competition experiments (data not shown) utilizing membranes from a cell passage where the human cannabinoid $CB_2$ receptor was expressed at a lower level, comparable to that of the rat receptor, and the binding affinities of cannabinoid ligands were similar to those performed with membrane preparation from a higher expressing passage.

With the exception of WIN-55212-2, the differences in binding potencies of the ligands AM-1710, AM-1714, JWH-015, anadamide and 2-arachidonyl glycerol correlated well with the differences in the functional potencies. Both AM-1710 and AM-1714 were highly potent and efficacious at the rat cannabinoid $CB_2$ receptor compared to the human receptor in FLIPR and cAMP assays. In contrast, JWH-015 exhibited a higher potency at the human cannabinoid $CB_2$ receptor compared to the rat in both FLIPR and cAMP assays. Moreover, the endocannabinoids anandamide and 2-arachidonyl glycerol were more potent at the rat receptor compared to human in cAMP assays, similar to findings by Berglund et al. (1998). However, they were not active in FLIPR assays. On the other hand, WIN-55212-2 showed similar binding affinities at the human and rat cannabinoid $CB_2$ receptors, but exhibited higher potencies at the human receptor in both FLIPR and cyclase assays. Although the concentration responses for cannabinoid ligands at the cell lines coexpressing the human or rat cannabinoid $CB_2$ and $G_{\alpha qo}$ showed comparable rank order of potencies with respect to binding ($K_i$ values) and cAMP ($EC_{50}$ values) assays, the ligands were about 10-fold weaker in FLIPR assays. It is possible that slow receptor binding kinetics may contribute to the differences of potencies in these two assays. The cAMP and binding assays are performed at equilibrium whereas the FLIPR assays measures transient increases in $[Ca^{2+}]_i$ levels. A similar shift in potencies was observed in FLIPR assays using chimeric $G_{\alpha qi}$ proteins for opioid receptors when compared to other functional assays (Coward et al., 1999).

WIN55212-2 was significantly more potent at the human cannabinoid $CB_2$ receptor compared to the rat in both cAMP and FLIPR assays. JWH-015 showed a similar trend like WIN-55212-2 and was more potent at the human receptor, although the effect was more pronounced in FLIPR assays. In contrast, AM-1714 and AM-1710 were more potent at the rat receptor than human and the species preference column (Table 2) depicts that observation. Endocannabinoids were much weaker at the rat cannabinoid $CB_2$ receptor compared to human in cAMP assays and are inactive in FLIPR assays. CP-55,940 was more active at the human receptor in the cAMP assays compared to FLIPR. However, the 2-fold difference observed between the two assays was not significant.

AM-630, a cannabinoid $CB_2$ receptor-specific antagonist (Ross et al., 1999), exhibited 13-fold higher affinity at the rat cannabinoid $CB_2$ receptor. The addition of AM-630 at 1 μM resulted in a dextral shift of CP-55,940 dose–response curve in both cyclase and FLIPR assays, with more pronounced shifts at the rat receptor than the human receptor, consistent with the higher binding affinity of AM-630 at the rat receptor. In cyclase assays, AM630 significantly blocked (87% to 91%) the effects of CP-55,940 on adenylyl cyclase activity. The higher concentrations of CP-55940 could not be tested in the blocking experiments due to possible DMSO effects on the assay at high ligand concentration.

In summary, we report the detailed analysis of the species differences observed between the rat and human cannabinoid $CB_2$ receptors using radioligand binding assays and cyclase and FLIPR functional assays. The observed differences between human and rat species for the cannabinoid $CB_2$ receptor are consistent with the findings reported in earlier publications (Griffin et al., 2000; Brown et al., 2002). However, we have extended the finding using functional assays and have shown that the pharmacological differences in ligand binding for human and rat cannabinoid $CB_2$ receptors are largely correlated with those in functional assays. Future studies with mutant receptors altering specific amino acid residues in the human and rat cannabinoid $CB_2$ receptors, as well as using molecular modeling tools, will assist in elucidating the key amino acid residues in the cannabinoid $CB_2$ receptor that are responsible for the species specificities. Understanding species differences of human and rat cannabinoid $CB_2$ receptors will provide valuable information on the action of cannabinoid compounds in in vivo rodent models and therefore help in extrapolating the effects observed in rodent models to humans.

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## References

Berglund, B.A., Boring, D.L., Wilken, G.H., Makriyannis, A., Howlett, A.C., 1998. Structural requirements for the archidonylethanolamide interaction with $CB_1$ and $CB_2$ cannabinoid receptors: Pharmacology of the carbonyl and ethanolamide groups. Prostaglandins. Leukot. Essent. Fatty. Acids. 59 (2), 111–118.

Brown, S.M., Wagner-Miller, J., Mackie, K., 2002. Cloning and molecular characterization of the rat $CB_2$ cannabinoid receptor. Biochim. Biophys. Acta 1576, 255–264.

Conklin, B.R., Farfel, Z., Lustig, K.D., Julius, D., Bourne, H.R., 1993. Substitution of three amino acids switches receptor specificity of $G_{q\alpha}$ to that of $G_{i\alpha}$. Nature 363, 274–276.

Coward, P., Chan, S.D., Wada, H.G., Humphries, G.M., Conklin, B.R., 1999. Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors. Anal. Biochem. 270, 242–248.

Facci, L., Toso, R.D., Romanello, S., Buriani, A., Skaper, S.D., Leon, A., 1995. Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanlamide. Proc. Natl. Acad. Sci. U. S. A. 92, 3376–3380.

Felder, C.C., Veluz, J.S., Williams, H.L., 1992. Cannabinoid agonists stimulate both receptor and non receptor mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. Mol. Pharmacol. 42, 838–845.

Galigue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., LeFur, G., Casella, P., 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulation. Eur. J. Biochem. 232, 54–61.

Gerard, C.M., Mollereau, C., Vassart, G., Parmentier, M., 1991. Molecular cloning of human cannabinoid receptor which is also expressed in testis. Biochem. J. 279, 129–134.

Glass, M., Dragunow, M., Faull, R.L., 1997. Cannabinoid receptors in the human brain: a detailed anatomical and quantitative autoradiographic study in the fetal, neonatal and adult human brain. Neuroscience 77, 299–318.

Griffin, G., Tao, Q., Abood, M.E., 2000. Cloning and pharmacological characterization of rat $CB_2$ cannabinoid receptor. J. Pharmacol. Exp. Ther. 292, 886–894.

Hanus, L., Breuer, A., Tchilibon, S., Shiloah, S., Goldenberg, D., Horowitz, M., Pertwee, R.G., Ross, R.A., Mechoulam, R., Fride, E., 1999. HU-308: a specific agonist for $CB_2$, a peripheral cannabinoid receptor. Proc. Natl. Acad. Sci. U. S. A. 96, 14228–14233.

Hess, J.F., Borkowski, J.A., Macneil, T., Stonesifer, G.Y., Fraher, J., Strader, C.D., Ransom, R.W., 1994. Differential pharmacology of cloned human and mouse B2 bradykinin receptors. Mol. Pharmacol. 45, 1–8.

Howlett, A.C., 1985. Cannabinoid inhibition of adenylate cyclase: biochemistry and the response in neuroblastoma cell membranes. Mol. Pharmacol. 27, 429–436.

Ibrahim, M.M., Deng, H., Zvonok, A., Cockayne, D.A., Kwan, J., Mata, H.P., Vanderah, T.W., Lai, J., Porreca, F., Makriyannis, A., Malan Jr., T.P., 2003. Activation of $CB_2$ cannabinoid receptors by AM-1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. Proc. Natl. Acad. Sci. U. S. A. 100, 10529–10533.

Iwamura, H., Suzuki, H., Ueda, Y., Kaya, T., Inaba, T., 2001. In vitro and in vivo pharmacological characterization of JTE-907, a novel selective ligand for cannabinoid $CB_2$ receptor. J. Pharmacol. Exp.Ther. 296, 420–425.

Klein, T.W., Newton, C., Friedman, H., 1998. Cannabinoid receptors and immunity. Immunol. Today 19, 373–381.

Malan Jr., T.P., Ibrahim, M.M., Deng, H., Liu, Q., Mata, H.P., Vanderah, T., Porreca, F., Makriyannis, A., 2001. $CB_2$ cannabinoid receptor-mediated peripheral antinociception. Pain 93, 239–245.

Martin, R.S., Reynen, P.H., Calixto, J.J., Reyes, C.L., Chang, T.K., Dietrich, P.S., Bonhaus, D.W., MacLennan, S.J., 2002. Pharmacological comparison of a recombinant $CB_1$ cannabinoid receptor with its $G_{\alpha 16}$ fusion product. J. Biomol. Screen 7, 281–289.

Matsuda, L.A., Bonner, T.I., Lolait, S.J., 1993. Localization of cannabinoid receptor mRNA in rat brain. J. Comp. Neurol. 327 (4), 535–550.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of peripheral receptor for cannabinoids. Nature 365, 61–65.

Ross, R.A., Brockie, H.C., Stevenson, L.A., Murphy, V.L., Templeton, F., Makriyannis, A., Pertwee, R.G., 1999. Agonist-inverse agonist characterization at $CB_1$ and $CB_2$ cannabinoid receptors of L759633, L75656 and AM-630. British. J. Pharmacol. 126, 665–672.

Showalter, V.M., Compton, D.R., Martin, B.R., Abood, M.E., 1996. Evaluation of binding in transfected cell line expressing a peripheral cannabinoid receptor ($CB_2$): identification of cannabinoid receptor subtype selective ligands. J. Pharmacol. Exp. Ther. 278, 989–999.

Soderstrom, K., Johnson, F., 2000. $CB_1$ cannabinoid receptor expression in brain regions associated with zebra finch song control. Brain Res. 857, 151–157.

Soderstrom, K., Leid, M., Moore, F.L., Murray, T.F., 2000. Behavioral, pharmacological, and molecular characterization of an amphibian cannabinoid receptor. J. Neurochem. 75, 413–423.

Yamaguchi, F., Macrae, A.D., Brenner, S., 1996. Molecular cloning of two cannabinoid type-1 like receptor genes from the puffer fish *Fugu rubripes*. Genomics 35, 603–605.

Yao, B., Hutchins, C.W., Carr, T.L., Cassar, S., Masters, J.N., Bennani, Y.L., Esbenshade, T.L., Hancock, A.A., 2003. Molecular modeling and pharmacological analysis of species-related histamine H3 receptor heterogeneity. Neuropharmacology 44, 773–786.

Zhang, J., Hoffert, C., Vu, H.K., Groblewski, T., Ahmad, S., O'Donnell, D., 2003. Induction of $CB_2$ receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain. Eur. J. Neurosci. 17, 2750–2754.