British Journal of Pharmacology (2006) 149, 431–440

www.brjpharmacol.org

RESEARCH PAPER

In vitro and *in vivo* pharmacology of synthetic olivetol- or resorcinol-derived cannabinoid receptor ligands

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Background and purpose: We have previously reported the development of CB-25 and CB-52, two ligands of CB_1 and CB_2 cannabinoid receptors. We assessed here their functional activity.
Experimental approach: The effect of the two compounds on forskolin-induced cAMP formation in intact cells or GTP-γ-S binding to cell membranes, and their action on nociception in vivo was determined.
Key results: CB-25 enhanced forskolin-induced cAMP formation in N18TG2 cells ($EC_{50} \sim 20$ nM, max. stimulation = 48%), behaving as an inverse CB_1 agonist, but it stimulated GTP-γ-S binding to mouse brain membranes, behaving as a partial CB_1 agonist ($EC_{50} = 100$ nM, max. stimulation = 48%). At human CB_1 receptors, CB-25 inhibited cAMP formation in hCB_1-CHO cells ($EC_{50} = 1600$ nM, max. inhibition = 68% of CP-55,940 effect). CB-52 inhibited forskolin-induced cAMP formation by N18TG2 cells ($IC_{50} = 450$ nM, max. inhibition = 40%) and hCB_1-CHO cells ($EC_{50} = 2600$ nM, max. inhibition = 62% of CP-55,940 effect), and stimulated GTP-γ-S binding to mouse brain membranes ($EC_{50} = 11$ nM, max. stimulation ~16%). Both CB-25 and CB-52 showed no activity in all assays of CB_2-coupled functional activity and antagonized CP55940-induced stimulation of GTP-γ-S binding to hCB_2-CHO cell membranes. In vivo, both compounds, administered i.p., produced dose-dependent nociception in the plantar test carried out in healthy rats, and antagonised the anti-nociceptive effect of i.p. WIN55,212-2. In the formalin test in mice, however, the compounds counteracted both phases of formalin-induced nociception.
Conclusions and implications: CB-25 and CB-52 behave in vitro mostly as CB_1 partial agonists and CB_2 neutral antagonists, whereas their activity in vivo might depend on the tonic activity of cannabinoid receptors.
British Journal of Pharmacology (2006) **149,** 431–440. doi:10.1038/sj.bjp.0706888; published online 4 September 2006

Keywords: agonist; antagonist; inverse agonist; partial agonist; receptor; endocannabinoid; pain

Abbreviations: CB_1, cannabinoid receptor subtype 1; CB_2, cannabinoid receptor subtype 2; DMSO, dimethyl sulphoxide; hCB_1-CHO cells, Chinese hamster ovary cells overexpressing the human CB_1 receptor; hCB_2-CHO cells, Chinese hamster ovary cells overexpressing the human CB_2 receptor; PAG, periaqueductal grey; RVM, rostral ventromedial medulla; THC, Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential vanilloid type 1

Introduction

Over the past few years, two receptors have gained growing importance as targets for the discovery of new potential therapeutic, and particularly analgesic and anti-inflammatory, drugs, namely the cannabinoid receptors subtype 1 and 2 (CB_1 and CB_2). These receptors were identified during studies on the molecular mode of action of a plant natural product, the psychotropic principle of *Cannabis*, Δ^9-tetrahydrocannabinol (THC). Agonists of both cannabinoid receptor subtypes (see for review Iversen and Chapman, 2002) produce strong anti-nociceptive effects in animal models of chronic, neuropathic and inflammatory pain (see for review Di Marzo *et al*., 2002). Also 'indirect' agonists of cannabinoid receptors, that is, inhibitors of endogenous cannabinoid (endocannabinoid) inactivation, have been suggested as possible leads for the development of analgesic

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Received 18 May 2006; revised 7 July 2006; accepted 3 August 2006; published online 4 September 2006

drugs (Lichtman *et al*., 2004a, b). Finally, although activation of CB_2 causes pain relief (Costa *et al*., 2004; Ibrahim *et al*., 2006), recent evidence also points to cannabinoid CB_2 receptor antagonists/inverse agonists as promising anti-inflammatory agents (Iwamura *et al*., 2001; Ueda *et al*., 2005; Lunn *et al*., 2006; Oka *et al*., 2006). This finding is seen by some as paradoxical, but it reflects a typical property of endocannabinoids, which can behave as mediators often playing opposing roles under pathological (and especially neurodegenerative and inflammatory) states, depending on the phase of the disorder, the cellular source of the endocannabinoids and the cellular localization and biological effect of their molecular target. For example, both the CB_1 receptor inverse agonist SR141716A and the inhibitor of endocannabinoid inactivation VDM-11 are protective against β-amyloid-induced amnesia in mice (Mazzola *et al*., 2003; van der Stelt *et al*., 2006) depending on the phase of the disorder at which the two substances are administered. It has been recently shown that also SR141716A can induce paradoxical analgesic and anti-inflammatory actions, thus behaving as an agonist (Croci *et al*., 2003; Costa *et al*., 2005), an effect that might be due to targeting of non-CB_1 receptors by this compound or to the fact that, with CB_1 receptors blocked, endocannabinoids might tonically activate other targets involved in pain and inflammation. Indeed, an agonist-like neuroprotective effect of SR141716A against ischaemia *in vivo* was recently suggested to be due to indirect activation of vanilloid transient receptor potential vanilloid type 1 (TRPV1) receptors (Pegorini *et al*., 2006).

Some contradictory results obtained with cannabinoid receptor ligands on the pharmacological control of pain and inflammation are probably also due to the fact that some of these compounds might behave as partial or full agonists or as neutral antagonists or inverse agonists also depending on the activation state of cannabinoid receptors. Thus, if the pathological condition is such to cause a strong elevation of endocannabinoid levels, a potent but inefficacious agonist might behave as an antagonist. This was recently demonstrated with THC, which, compared to the endocannabinoid 2-arachidonoylglycerol (2-AG), is a potent but partial agonist at cannabinoid receptors, and, accordingly, abolishes the cannabinoid CB_2 receptor-mediated migration of human natural killer cells (Kishimoto *et al*., 2005) and occludes the CB_1-mediated depolarization-induced inhibition of excitatory neurotransmission in the hippocampus (Straiker and Mackie, 2005), two effects mediated by 2-AG. By contrast, with little or no endogenous activation of cannabinoid receptors, a partial agonist will still behave as such, and it will be possible to distinguish between a neutral antagonist (which should be inactive in this case) and an inverse agonists (which should still be active and behave in an opposite way as compared to agonists and partial agonists). Species differences can also play a role in determining the overall activity of a certain pharmacological tool, as in the case of the CB_2 inverse agonist SR144528, which exacerbates formalin-induced pain in mice (Calignano *et al*., 1998), and inhibits it in rats (Beaulieu *et al*., 2000). These observations underscore the importance of always measuring the amounts of endocannabinoids during physiological or pathological conditions, and of developing new tools that act as neutral antagonists. To date, only neutral CB_1 receptor antagonists have been reported (see for review Pertwee, 2005), but no neutral CB_2 antagonists.

We have previously reported the development of several synthetic compounds derived from olivetol (the biosynthetic precursor of THC in *Cannabis*) or substituted resorcinol and fatty acids as ligands of cannabinoid CB_1 and CB_2 receptors (Brizzi *et al*., 2005) (Figure 1). We describe here the functional activity of two of these compounds, CB-25 and CB-52, and provide data indicating that, although they might behave as partial agonists at CB_1 receptors, they always act as neutral CB_2 antagonists *in vitro*. We also investigated the analgesic activity of the two compounds, and show that they appear to behave as CB_1 antagonists or partial agonists depending on the type of noxious stimulus used.

Materials and methods

Assays of adenylate cyclase activity

Cyclic AMP (cAMP) assays were performed on intact confluent N18TG2 cells plated in six-well dishes and stimulated for 10 min at 37°C with forskolin 1 μM in 400 μl serum-free Dulbecco's modified Eagle's medium containing 20 mM HEPES, 0.1 mg ml^{-1} bovine serum albumin and 0.1 mM 1-methyl-3-isobutylxanthine (Melck *et al*., 1999). Cells were treated with vehicle (methanol, 0.1%) or compounds, or WIN55,212-2 at various concentrations, or with SR141716A (100 nM). After incubation, 800 μl ethanol was added, cells were extracted and cAMP was determined by means of a cAMP assay kit (Amersham Biosciences, Piscataway, NJ, USA), as advised by the manufacturer.

Adenylate cyclase assays were also carried out using CHO cells stably overexpressing the human recombinant CB_1 or CB_2 receptor (obtained from Euroscreen and denoted as hCB_1-CHO cells and hCB_2-CHO cells, respectively). Compounds and reference (CP55,940) were diluted in serum-free medium containing 10 μM rolipram and 2 μM forskolin; dispensing equipment (Beckman BioMek FX) was used to transfer 10 μl to an assay plate (384-well white culture plate, Perkin-Elmer, Monza, Milano, Italy). Cell suspensions con-

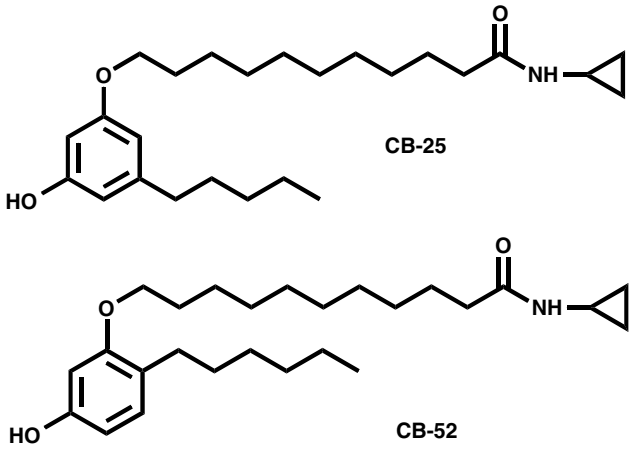

Figure 1 Chemical structures of CB-25 and CB-52 (Brizzi *et al*., 2005).

taining 10^6 cells ml^{-1} were prepared from hCB1-CHO and hCB2-CHO cells and 10 μl (10 000 cells well^{-1}) was transferred to the assay plate; cells were incubated for 45 min at 37°C. Homogeneous time-resolved fluorescence (CisBio) was used as a read-out by sequentially adding 10 μl cAMP-XL665 and 10 μl anti-cAMP(Eu) cryptate; after 1 h incubation at room temperature, fluorescence at 615 and 665 nm was measured on Envision (Perkin-Elmer). Results were calculated from the 665 nm/615 nm ratios obtained for individual compounds and were compared to values obtained for the reference compound.

Assays of [^{32}S]GTP-γ-S binding

These assays were performed as described previously (Thomas *et al*., 2005), using (1) membranes from CHO cells stably overexpressing the human recombinant CB_2 receptor (10 μg ml protein^{-1}), 0.7 nM [^{35}S]GTP-γ-S and 320 μM GDP, and a final assay volume of 250 μl, and (2) membranes from mouse brain (10 μg ml protein^{-1}), 0.1 nM [^{35}S]GTP-γ-S and 30 μM GDP, and a final assay volume of 500 μl.

Test of anti-nociceptive activity in normal rats

Animals. Male Wistar rats (250–300 g) and Swiss-Webster mice (40–45 g) were housed three per cage under controlled illumination (12 h light/12 h dark cycle; light on 0600 hours) and standard environmental conditions (ambient temperature 20–22°C, humidity 55–60%) for at least 1 week before the commencement of experiments. Rat chow and tap water were available *ad libitum*. All surgery and experimental procedures were performed during the light cycle and were approved by the Animal Ethics Committee of The Second University of Naples. Animal care was in compliance with European regulations on the protection of laboratory animals (OJ of EC L358/1 18/12/86). In agreement with the Ethical Guidelines of the IASP, all efforts were made to reduce both animal numbers and suffering during the experiments.

Surgical preparation. For intra-periaqueductal grey (PAG) microinjections, rats were anaesthetized with pentobarbital (60 mg kg^{-1}, intraperitoneally (i.p.)) and a 23-gauge, 12-mm-long stainless-steel guide cannula was stereotaxically lowered until its tip was 1.4 mm above the ventrolateral PAG by applying coordinates from the Atlas of Paxinos and Watson (1986) (A: −7.8 mm from bregma, L: 0.5 mm, V: 4.3 mm below the dura). The cannula was anchored with dental cement to a stainless-steel screw in the skull. We used a David Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) with the animal positioned on a homeothermic temperature control blanket (Harvard Apparatus Limited, Edenbridge, Kent, UK). Ventrolateral PAG was considered in this study because previous studies have shown in that area the presence of excitatory output neurons projecting monosynaptically to OFF neurons in the rostral ventromedial medulla (RVM) (Tortorici and Morgan, 2002). Direct intra-PAG administration of drugs or their vehicle (10% dimethyl sulphoxide (DMSO) in artificial cerebrospinal fluid (ACSF) of composition (in mM) KCl 2.5, NaCl 125, $MgCl_2$ 1.18 and $CaCl_2$ 1.26) was achieved with a stainless-steel cannula connected by a polyethylene tube to a Hamilton 1-microlitre syringe, inserted through the guide cannula and extended 1.4 mm beyond the tip of the guide cannula to reach the ventrolateral PAG. Volumes of 200 nl drug solutions, or vehicle, were injected into the ventrolateral PAG over a period of 60 s and the injection cannula gently removed 5 min later. At the end of the experiment, a volume of 200 nl of neutral red (0.1%) was also injected in the ventrolateral PAG 40–50 min before killing the rat. Rats were then perfused intracardially with 20 ml phosphate-buffered solution (PBS) followed by 20 ml of 10% formalin solution in PBS. The brains were removed and immersed in a saturated formalin solution for 2 days. The injection sites were ascertained by using two consecutive sections (40 μm), one stained with cresyl violet to identify nuclei and the other one unstained to determine dye spreading. Only those rats whose microinjection site and diffusion were located within ventrolateral PAG were included in the results.

Thermoceptive responses. Changes in thermoceptive responses were evaluated according to Hargreaves *et al*. (1988) using a Plantar Test Apparatus (Ugo Basile, Varese, Italy). On the day of the experiment, rats were placed in a plastic cage (22 cm × 17 cm × 14 cm; length × width × height) with a glass floor. After 1 h habituation period, the plantar surface of the hind paw was exposed to a beam of radiant heat through the glass floor. The radiant heat source consisted of an infrared bulb (Osram halogen-bellaphot bulb; 8 V, 50 W). A photoelectric cell detected light reflected from the paw and turned off the lamp when paw movement interrupted the reflected light. The paw withdrawal latency was automatically displayed to the nearest 0.1 s; the cutoff time was 25 s in order to prevent tissue damage. The latency of nociceptive reaction was measured in seconds under basal condition and after intra-PAG treatment with drugs. Each rat served as its own control, the latency to nociceptive response being measured both before and after treatments. At time 0, five baseline responses were already obtained for each animal at 15 min intervals and averaged. Rats received CB-25 (1 or 2.5 mg kg^{-1}, i.p.), CB-52 (1 and 5 mg kg^{-1}, i.p.) or WIN55,212-2 (2.5 mg kg^{-1}, i.p.). CB-25 and CB-52 (1 mg kg^{-1}, i.p.) were also given in combination with WIN55,212-2 (2.5 mg kg^{-1}, i.p.). We chose these doses of CB-25 and CB-52 on the basis of previous *in vitro* and *in vivo* studies of their potencies and receptor affinities relative to the reference compound CP55,940 (Brizzi *et al*., 2005; Vigano *et al*., 2005).

Groups of 10 animals per treatment were used, with each animal used for one treatment only. The results are expressed as a percentage of the maximum possible effect (%MPE), using the following formula:

$$\%\text{MPE} = \frac{(\text{test latency}) - (\text{control latency})}{(\text{cutoff time}) - (\text{control latency})} \times 100$$

Extracellular electrophysiological recording in normal rats

After implantation of the guide cannula into the ventrolateral PAG matter, a tungsten microelectrode was stereotaxically lowered, through a small craniotomy into the RVM

using the following stereotaxic coordinates: 2.8–3.3 mm caudal to lambda, 0.4–0.9 mm lateral and 8.9–10.7 mm depth from the surface of the brain (Paxinos and Watson, 1986) to record the activity of ON and OFF cells. These neurons were identified by the characteristic OFF cell pause, and ON cell burst of activity just before tail flick responses (Fields *et al.*, 1983). Anaesthesia was maintained with a constant, continuous infusion of sodium methohexital (40–60 mg kg^{-1} h^{-1}, i.v.). The jugular vein was cannulated to allow intravenous anaesthetic administration. Anaesthesia was adjusted so that tail flicks were elicited with a constant latency of 4–5 s. From 35°C, the temperature increased linearly to 53°C and it was adjusted at the beginning of each experiment in order to elicit a constant tail flick latency of 4–5 s. The thermal stimulus was elicited by a radiant heat source of a tail flick unit (Ugo Basile, Varese, Italy) focused on the rat tail approximately 3–5 cm from the tip. Tail flicks were elicited every 3 min for at least 15–20 min before microinjecting drugs, or respective vehicles, into the PAG.

Extracellular single-unit recordings were made in the RVM with glass insulated tungsten filament electrodes (3–5 MΩ) (FHC Frederick Haer & Co., Bowdoin, ME, USA). The recorded signals were amplified and displayed on analog and digital storage oscilloscopes to ensure that the unit under study was unambiguously discriminated throughout the experiment. Signals were also fed into a window discriminator, whose output was processed by an interface (CED 1401) (Cambridge Electronic Design Ltd, Cambridge, UK) connected to a Pentium III PC. Spike2 software (CED, version 4) was used to create peristimulus rate histograms on-line and to store and analyse digital records of single-unit activity off-line. Configuration, shape and height of the recorded action potentials were monitored and recorded continuously, using a window discriminator and Spike2 software for on-line and off-line analysis. Once an ON or OFF cell was identified from its background activity, we optimized spike size before all treatments. This study only included neurons whose spike configuration remained constant and could clearly be discriminated from activity in the background throughout the experiment, indicating that the activity from only one and the same neuron was measured. In each rat, only one neuron was recorded.

Direct intra-PAG administration of drugs or respective vehicle (10% DMSO in ACSF) was conducted as follows: 2 or 4 nmol per rat of CB-25 or CB-52, or WIN55,212-2 (4 nmol per rat) or CB-25 (2 nmol per rat) + WIN55,212-2 (4 nmol per rat) or CB-52 (2 nmol per rat) + WIN55,212-2 (4 nmol per rat). At the end of the experiment, each animal was killed with a lethal dose of urethane and the recording site was marked with a 20 μA DC current for 20 s. After fixation by immersion in 10% formalin, the recording sites were identified.

Test of anti-nociceptive activity in mice treated with formalin

Formalin injection induces a biphasic stereotypical nocifensive behaviour. Nociceptive responses are divided into an early, short-lasting first phase (0–7 min) caused by a primary afferent discharge produced by the stimulus, followed by a quiescent period and then a second, prolonged phase (15–60 min) of tonic pain. Mice received formalin (1.25% in 0.9% NaCl, 50 μl) in the dorsal surface of one side of the hind paw. Each mouse was randomly assigned to one of the experimental groups and placed in a Plexiglas cage and allowed to move freely for 15–20 min. A mirror was placed at a 45° angle under the cage to allow full view of the hind paws. Lifting, favouring, licking, shaking and flinching of the injected paw were recorded as nociceptive responses. Mice received CB-25 (2.5 or 5 mg kg^{-1}, i.p.) alone or in combination with AM630 (2 mg kg^{-1}, i.p.). CB-52 was given (1 or 5 mg kg^{-1}, i.p.) either alone or in combination with WIN55,212-2 (2.5 mg kg^{-1}, i.p.), SR141716A (0.5 mg kg^{-1}, i.p.), AM630 (2 mg kg^{-1}, i.p.) or capsazepine (5 mg kg^{-1}, i.p.). CB-25 or CB-52 was administered 15 min before formalin alone or in combination with WIN55,212-2, SR141716A, AM630 or capsazepine. These last drugs were administered 5 min before the CB-25 or CB-52. The total time of the nociceptive response was measured every 5 min and expressed as the total time of the nociceptive responses in minutes (mean ± s.e.m.). Recording of nociceptive behaviour commenced immediately after the formalin injection and was continued for 60 min.

Data analysis

Results from the *in vitro* studies of adenylate cyclase activity and [^{32}S]GTP-γ-S binding were analysed by analysis of variance (ANOVA) followed by the Bonferroni's *post hoc* test. Behavioural data are shown as means ± s.e. and were analysed by ANOVA, followed by Student–Newman–Keuls *post hoc* test. Extracellular recording single-unit activity (action potentials) was analysed off-line from peristimulus rate histograms using Spike2 software (CED, version 4) and expressed as spikes per second (Hz). Baseline activities of neurons were obtained by averaging the activities recorded 50–60 s before the application of four thermal stimulations (tail flick). Data are presented as mean ± standard error (s.e.) of changes in neuronal responses (extracellular recordings). Statistical comparisons of values under control conditions or following drug applications were made using the Wilcoxon signed rank test. Throughout, differences between means were deemed to be significant when $P<0.05$.

Drugs

CB-25 (11-(3-hydroxy-5-pentyl-phenoxy)-undecanoic acid cyclopropylamide) and CB-52 (11-(5-hydroxy-2-pentyl-phenoxy)-undecanoic acid cyclopropylamide) (Figure 1) were synthesized in our laboratory as described previously (Brizzi *et al.*, 2005). AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide), capsazepine (*N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide, CP-55,940 ((−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol), WIN55,212-2 ((*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) and AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1-*H*-indol-3-yl](4-methoxyphenyl)methanone) were purchased from Tocris Cookson Ltd (Bristol, UK). SR141716A (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-

methyl-3-pyrazolearboxamide) was generously provided by Sanofi-Synthelabo Recherche (France).

Results

Functional activity of CB-25 and CB-52 at the mouse CB_1 receptor

Adenylate cyclase assays. As reported previously (Brizzi *et al*., 2005), CB-25 behaved as an inverse agonist by enhancing forskolin-induced cAMP formation by mouse neuroblastoma N18TG2 cells ($EC_{50} = 20 \pm 3$ nM, max. stimulation $= 48 \pm 5\%$, means $\pm$ s.e., $N = 4$), which constitutively express CB_1, but not CB_2, receptors (see also Brizzi *et al*., 2005). By contrast, CB-52 behaved as a partial agonist in this assay by inhibiting forskolin-induced cAMP formation with lower efficacy as compared to WIN55,212-2 ($IC_{50} = 450 \pm 35$ and 20 ± 2 nM, max. inhibition 40 ± 3 and $63 \pm 5\%$, respectively, means $\pm$ s.e., $N = 4$; see also Brizzi *et al*., 2005). The effect of CB-25 (100 nM) was blocked by WIN55,212-2 (5 nM, a concentration inactive by itself) ($8.2 \pm 4.5\%$ stimulation, $P > 0.05$), whereas the effect of CB-52 (500 nM) was blocked by the CB_1 antagonist SR141716A (10 nM) ($3.1 \pm 1.0\%$ inhibition, $P > 0.05$).

GTP-γ-S binding assays. Both CB-25 and CB-52 stimulated [^{32}S]GTP-γ-S binding to membranes from mouse brain ($EC_{50} = 100$ and 11 nM, maximum Stimulation was 48 and 16%, respectively), thus behaving as rather potent agonists, albeit inefficacious as compared to the potent full CB_1 receptor agonist CP55,940 (Figure 2).

Functional activity of CB-25 and CB-52 at the human CB_1 receptor

Both CB-25 and CB-52 inhibited forskolin-induced cAMP formation by hCB_1-CHO cells ($EC_{50} = 1600$ and 2600 nM, max. inhibition $= 68$ and 62%, respectively), thus behaving as partial agonists in this assay as compared to the potent CB_1 receptor ligand CP55,940 (Figure 3a and b). No effect on forskolin-induced cAMP formation was observed in non-transfected CHO cells.

Functional activity of CB-25 and CB-52 at the human CB_2 receptor

Unlike CP55,940, neither CB-25 nor CB-52 significantly inhibited forskolin-induced cAMP formation by hCB_2-CHO cells at concentrations up to 10 μM. Furthermore, neither compound stimulated [^{32}S]GTP-γ-S binding to membranes from hCB_2-CHO cells over the same range of concentrations (Figure 4a). However, CB-25 (100 nM) and CB-52 (1 μM) antagonized the effect of CP55,940 on [^{32}S]GTP-γ-S binding to these membranes (K_b 5.4 and 9.8 nM, respectively), thus behaving as neutral antagonists at the human CB_2 receptor (Figure 4b and c).

Effect of CB-25 and CB-52 on nociception in normal rats

The systemic administration of CB-25 (1 and 2.5 mg kg^{-1}, i.p.) and CB-52 (1 and 5 mg kg^{-1}, i.p.) caused a long-lasting and dose-dependent decrease of the pain threshold to thermal stimulation as assessed in the plantar test in rats at the highest doses used (Figure 5a and b). By contrast, the potent cannabinoid receptor agonist WIN55,212-2 (2.5 mg kg^{-1}, i.p.) caused a strong anti-nociceptive effect, which was blocked by pretreatment with low doses (1 mg kg^{-1}, i.p.) of CB-25 and CB-52 , which were inactive when given alone (Figure 5a and b).

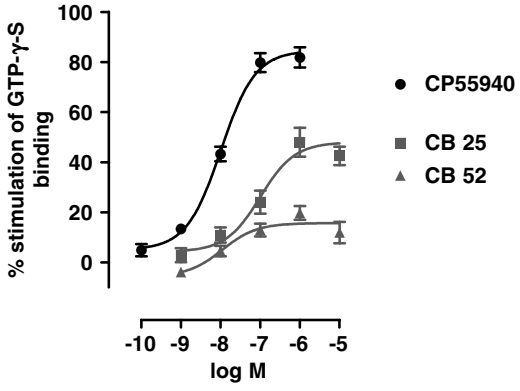

Figure 2 The effect of CP55940 ($n = 7$–9), CB-25 ($n = 5$–7) and CB-52 ($n = 8$) on the level of [^{35}S]GTP-γ-S binding to mouse whole brain membranes. Each symbol represents the mean percentage increase in [^{35}S]GTP-γ-S binding $\pm$s.e.

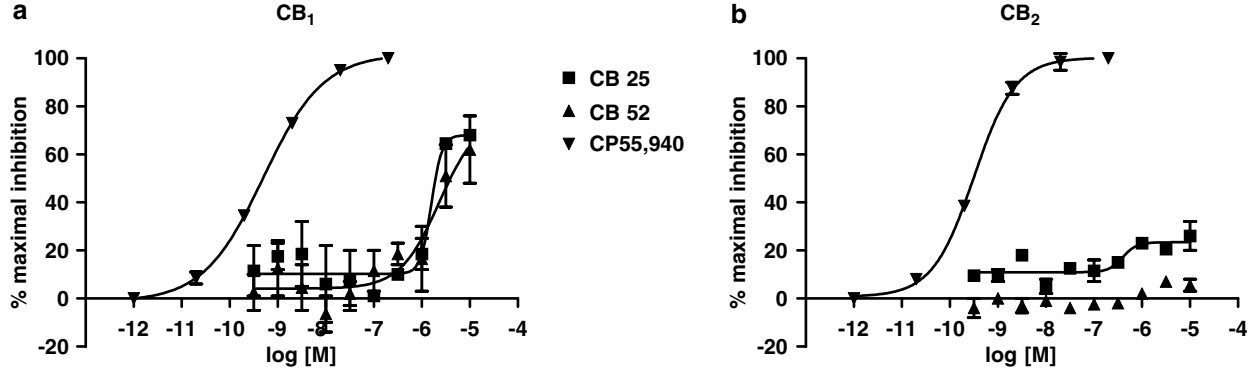

Figure 3 The effect of CP55940, CB-25 and CB-52 on forskolin-stimulated cAMP levels in CHO cells overexpressing the human recombinant CB_1 (**a**) or CB_2 (**b**) receptors. Data are expressed as percent inhibition and are means $\pm$ s.e. of four experiments.

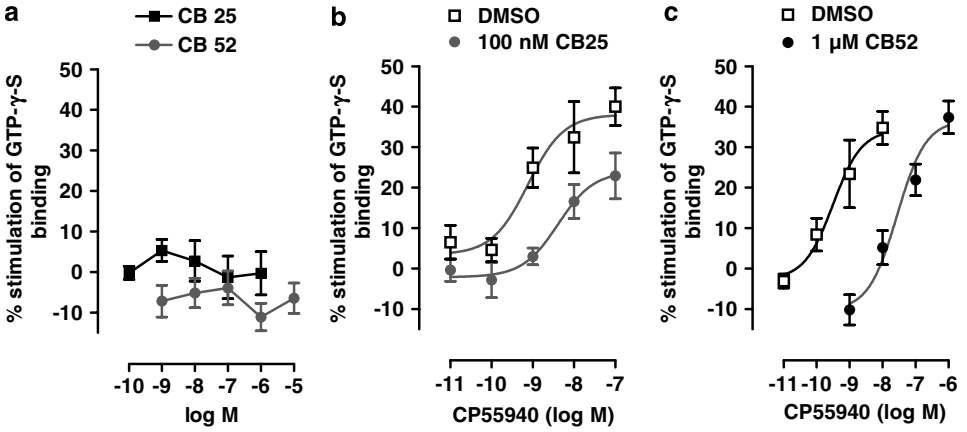

Figure 4 (**a**) The effect of CB-25 ($n=6$) or CB-52 ($n=6$) on the level of [^{35}S]GTP-γ-S binding to CB_2-transfected CHO cell membranes and the effect of (**b**) 100 nM CB-25 ($n=3$–4) or (**c**) 1 μM CB-52 ($n=3$–4) on the mean log concentration–response curve of CP55940 for stimulation of [^{35}S]GTP-γ-S binding to CB_2-transfected CHO cell membranes. Each symbol represents the mean percentage increase in [^{35}S]GTP-γ-S binding $\pm$s.e.

Effect of CB-25 and CB-52 on basal activity of RVM cells in normal rats

We next studied the effects of CB-25 (2 and 4 nmol per rat), CB-52 (2 and 4 nmol per rat) and WIN55,212-2 (4 nmol per rat) locally injected into the ventrolateral PAG on the spontaneous firing activity of 'ON' and 'OFF' neurons of the RVM, which receive input innervation from the PAG. Moreover, CB-25 (2 nmol per rat) and CB-52 (2 nmol per rat) were also evaluated in combination with WIN55,212-2 (4 nmol per rat) in the same test. We found that, in agreement with their pro-nociceptive effects observed in the plantar test following i.p. administration, both compounds caused dose-dependent and prolonged inhibition of 'OFF' anti-nociceptive RVM neurons (Figure 6b and d) and stimulation of 'ON' pro-nociceptive RVM neurons (Figure 6a and c). Also in this case, WIN55,212-2 caused effects that were opposite to those induced by CB-25 and CB-52, and were attenuated by co-injection with the low, ineffective, doses of the two compounds.

Effects of CB-25 and CB-52 on the nocifensive behaviour induced by formalin in mice

Unlike their effects in normal rats, in mice treated with formalin, which respond with a typical biphasic nocifensive behaviour (paw flinching and licking) to the injection of the noxious agent, both CB-25 (2.5 or 5 mg kg^{-1}, i.p.) and CB-52 (1 or 5 mg kg^{-1}, i.p.) behaved like WIN55,212-2 (2.5 mg kg^{-1}, i.p.), that is, they dose-dependently inhibited both the first and second hyperalgesic phases (Figure 7a and b). CB-52 appeared to be more potent than CB-25. The effect of submaximal doses of CB-25 or CB-52 and WIN55,212-2 appeared to be additive (Figure 7c), thus suggesting a possible similar mechanism of action. Finally, the effect of both compounds was attenuated by pretreatment with selective antagonists of CB_1 and CB_2 receptors, SR141716A (0.5 mg kg^{-1}, i.p.) and AM630 (2 mg kg^{-1}, i.p.), respectively (Figure 7a and b), doses that were inactive by themselves.

Discussion

We have described here the results of a series of *in vitro* and *in vivo* pharmacological experiments aimed at (1) assessing for the first time the functional activity at mouse and human cannabinoid CB_1 and CB_2 receptors of two previously developed cannabinoid receptor ligands, CB-25 and CB-52 (Figure 1) (Brizzi *et al*., 2005), (2) establishing whether different functional activities can be observed for these compounds when using different *in vitro* bioassays carried out in different animal species and (3) investigating whether the same compound can exert agonist-like or antagonist-like analgesic activities *in vivo* depending on the type of experimental pain model used.

Our first set of data indicates that the compound with highest affinity for human CB_1 receptors, CB-25 ($K_i=5.2$ nM), behaves as a partial agonist at these receptors when using inhibition of forskolin-stimulated cAMP formation in intact hCB_1-CHO cells as a means to determine functional activity. Interestingly, in this assay, not only the efficacy of this compound was significantly lower than that of CP55,940, but also its potency ($IC_{50}=1.6$ μM) did not reflect its affinity for human CB_1 receptors. The same applies to CB-52, which also inefficiently inhibited forskolin-stimulated cAMP formation in intact hCB_1-CHO cells, although in this case the difference between affinity ($K_i=210$ nM) and potency ($IC_{50}=1.4$ μM) was less striking. Interestingly, when the functional activity of CB-25 and CB-52 was studied using mouse CB_1 receptors, that is, by examining their effect on either forskolin-stimulated cAMP formation in intact N18TG2 cells or GTP-γ-S binding to mouse brain membranes, the former compound behaved as an inverse agonist or as a partial agonist, respectively, whereas CB-52 always acted as a partial agonist. Also in the case of this latter compound, however, differences between the two assays were found, as its potency in the cAMP assay was significantly lower, and its efficacy higher, than in the GTP-γ-S binding assay. By contrast, the potency ($EC_{50}=$ 20–100 nM) and efficacy (48% stimulation) of CB-25 in the

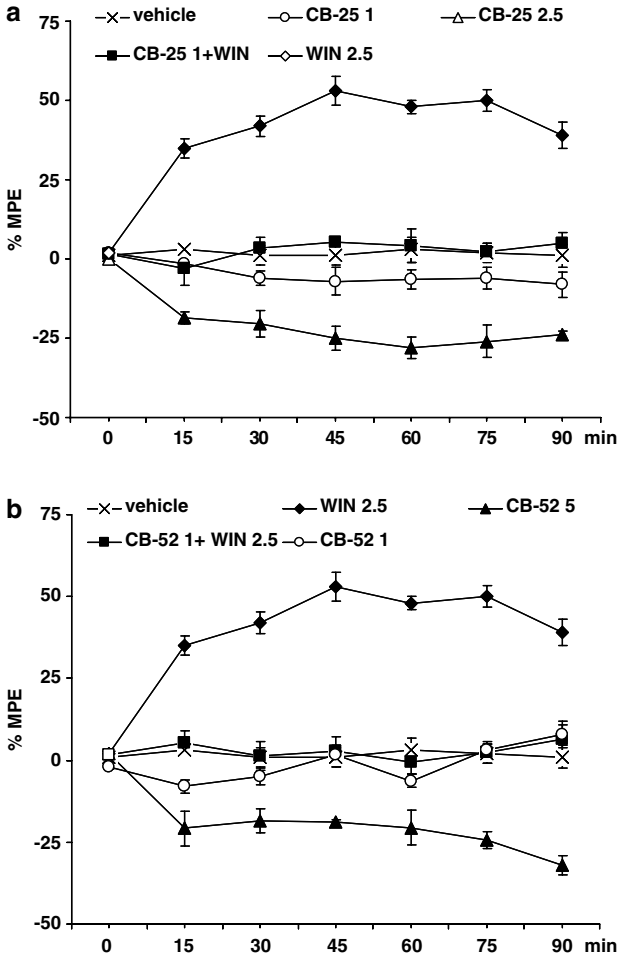

Figure 5 Nociception, expressed as percentage of the maximum possible effect (%MPE) after systemic administration of (**a**) CB-25 (1 and 2.5 mg kg^{-1}, i.p.) and (**b**) CB-52 (1 and 5 mg kg^{-1}, i.p.) either alone or in combination (at 1 mg kg^{-1}, i.p.) with WIN55,212-2 (2.5 mg kg^{-1}, i.p.) in the rat. Each data point represents the mean ± s.e.m. of 10 observations. Significant differences between groups are shown as filled symbols ($P<0.05$; ANOVA; Student–Newman–Keuls test). For treatments with a single compound, means of the treated groups were compared to those from the relevant vehicle. For combination treatments, means were compared to those from treatment with the corresponding single compounds.

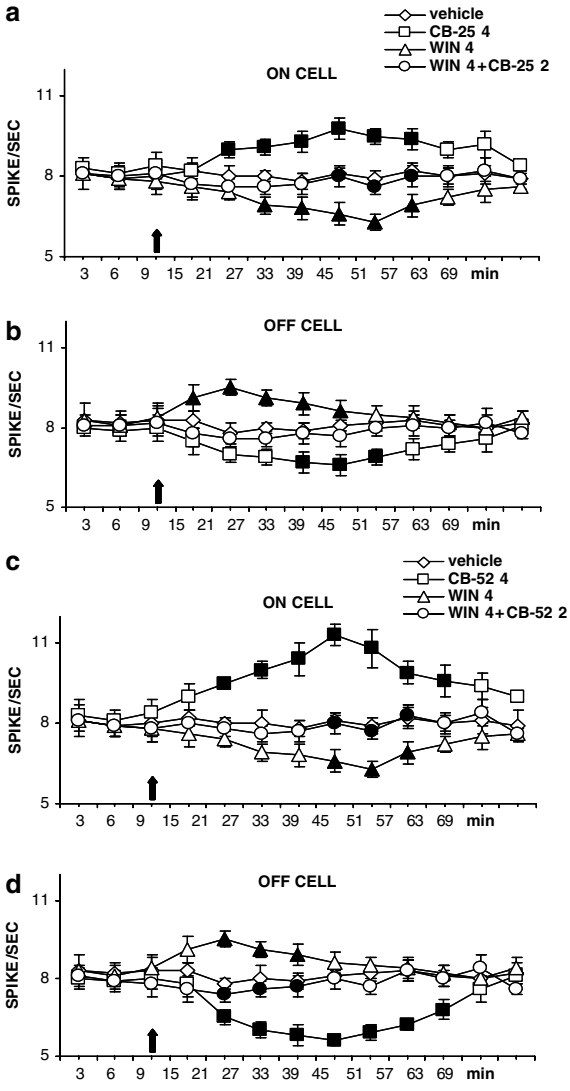

Figure 6 Spontaneous activities of RVM 'ON' (**a**, **c**) and 'OFF' (**b**, **d**) cells before and after microinjections into the ventrolateral PAG of CB-25 (4 nmol per rat), CB-52 (4 nmol per rat) or WIN55,212-2 (4 nmol per rat) alone, or of CB-25 or CB-52 (2 nmol per rat) in combination with WIN55,212-2 (4 nmol per rat). CB-25 and CB-52 at the lowest dose (2 nmol per rat) did not change pro-nociceptive 'ON' and anti-nociceptive 'OFF' cell firing (for clarity, these curves are not shown). CB-25 and CB-52 at the highest dose increased pro-nociceptive ON cell firing (**a**, **c**) and decreased anti-nociceptive OFF cell firing (**b**, **d**). WIN55,212-2 (4 nmol per rat) caused effects that were opposite to those induced by CB-25 and CB-52 and were blocked by co-injection with the low, inactive, doses of CB-25 and CB-52 (2 nmol per rat). The black arrow denotes the time of injections of drugs. Each point represents the mean ± s.e.m. of 10 recorded neurons. Significant differences between groups are shown as filled symbols ($P<0.05$; Wilcoxon signed rank test). For treatments with a single compound, means of the treated groups were compared to those from the relevant vehicle. For combination treatments, means were compared to those from treatment with the corresponding single compounds.

two assays were similar, although producing opposing effects on receptor functionality. In this case, we could not compare the potencies of the two compounds in the two assays with their affinity for mouse CB_1 receptors, as no binding data for CB-25 and CB-52 at the mouse CB_1 receptor are available. These findings support the concept that not only the potency but also the efficacy and the type (agonist, inverse agonist, partial agonist) of the functional activity of certain compounds at CB_1 receptors depend on the animal species as well as on the type of functional assay used. These differences may reflect the existence of CB_1 receptors that are pre-coupled or not to one or more types of G proteins, or of a higher or lower endogenous tone of endocannabinoids in the *in vitro* experimental model used. For example, one would expect to find higher pre-coupling of CB_1 receptors to G proteins in hCB_1-CHO cells (where the CB_1 receptor is overexpressed) than in N18TG2 cells (where the receptor is expressed constitutively), thus explaining why in the former cells one observes higher efficacy for both CB-52 and (although in opposing directions) CB-25. On the other hand, in intact cells (particularly N18TG2 cells, which produce high levels of the endocannabinoid 2-AG; Bisogno

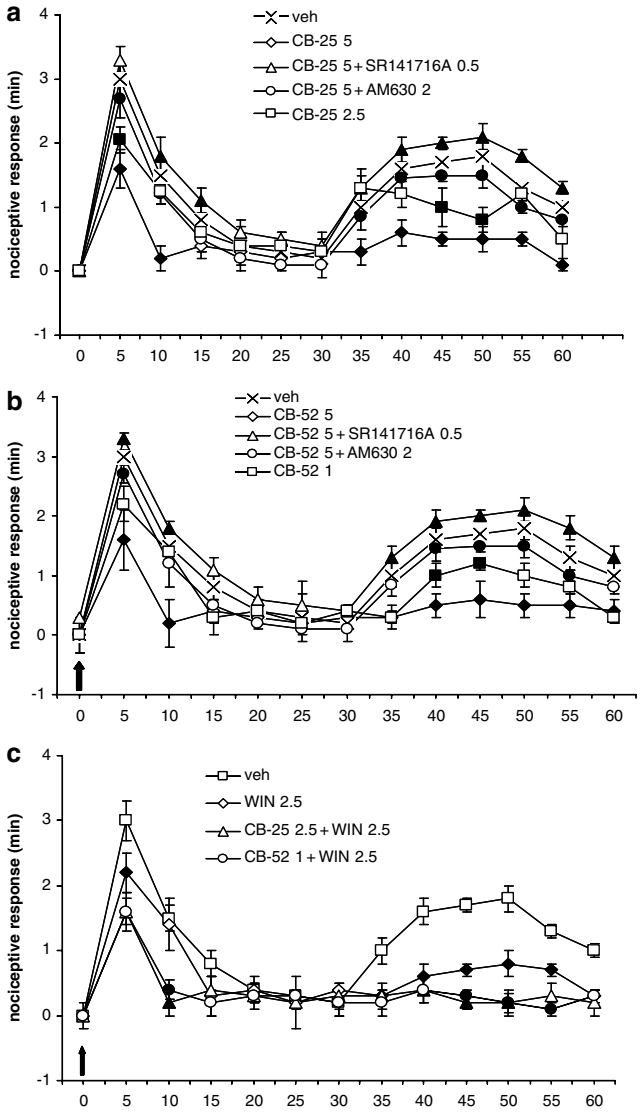

Figure 7 Effect of subcutaneous formalin (1.2%, 50 μl) on the time course of the nociceptive behaviour. (**a**, **b**) Formalin was injected into the hind paw of mice 15 min after the systemic administration of vehicle (10% DMSO in 0.9% NaCl, i.p.), CB-25 (2.5 and 5 mg kg^{-1}, i.p.) or CB-52 (1.0 and 5 mg kg^{-1}, i.p.). CB-25 and CB-52, at highest dose (5 mg kg^{-1}, i.p.), were also administered in combination with SR141716A (0.5 mg kg^{-1}, i.p.), or AM630 (2 mg kg^{-1}, i.p.), which *per se* did not change the formalin-induced nocifensive behaviour (not shown). (**c**) The effect of WIN55,212-2 (WIN, 2.5 mg kg^{-1}, i.p.) either alone or in combination with the low dose of either CB-25 (2.5mg kg^{-1}, i.p.) or CB-52 (1 mg kg^{-1}, i.p.). The data represent the total time of the nociceptive responses (mean$\pm$s.e.m.) of 10 mice per group, measured every 5 min. Significant differences between groups are shown as filled symbols ($P<0.05$; ANOVA; Student–Newman–Keuls test). For treatments with a single compound, means of the treated groups were compared to those from the relevant vehicle. For combination treatments, means were compared to those from treatment with the corresponding single compounds.

et al., 1997), one expects to find more endocannabinoids tonically activating the receptors than in cell membranes, and this might possibly explain why a potent but inefficacious agonist like CB-25 acts as inverse agonist in N18TG2 cells. Finally, differences between the ability to inhibit forskolin-stimulated cAMP in N18TG2 cells and to stimulate GTP-γ-S binding to mouse brain membranes might also be due to possibility that the two compounds, apart from CB_1, bind to and activate other G-protein-coupled receptors, which might be expressed (and assayed) to different extents in the two systems.

The complexity of the functional activity at mouse CB_1 receptors of CB-25 and CB-52 is also illustrated by the surprising effects observed with the two compounds in two assays of anti-nociceptive activity, a typical property of cannabinoid receptor ligands, carried out in normal rats. The anti-nociceptive activity of cannabinoid receptor ligands in these assays was previously shown to be mediated only by CB_1 receptors, whereas CB_1, but not CB_2, receptor antagonists were found to induce hyperalgesia (Palazzo *et al*., 2001; de Novellis *et al*., 2005). When measuring either the threshold of thermal pain sensitivity in the plantar test or the spontaneous firing activity of pro- and anti-nociceptive neurons in the RVM, we observed that both CB-25 and CB-52, administered systemically or directly into the ventrolateral PAG, respectively, behaved as inverse agonists or as potent albeit partial agonists (see above), that is, as if they acted by counteracting a strong cannabinoid receptor-mediated, and possibly supraspinal, anti-nociceptive endogenous tone determined by the noxious stimulus (Walker *et al*., 1999). Indeed, the two compounds in both assays, and at doses inactive *per se*, also counteracted the anti-nociceptive effect of WIN55,212. Although it might fit with the *in vitro* functional profile observed here with CB-25 at mouse CB_1 receptors, or with CB-52 in the GTP-γ-S assay in mouse brain membranes (where the compound exhibited a very potent but inefficacious stimulation of binding), this interpretation of these *in vivo* pharmacological data, however, does not agree with some of the other data discussed above, that is, with (1) CB-52 activity in mouse N18TG2 cells (low potency and little efficacy at inhibiting cAMP formation) and (2) both CB-25 and CB-52 activity at human CB_1 receptors (inefficacy but also little potency at inhibiting cAMP formation). The two compounds investigated in the present study were previously shown (Brizzi *et al*., 2005) to be selective for cannabinoid receptors over enzymes that catalyse endocannabinoid inactivation (and hence potentially inactive as 'indirect' agonists of cannabinoid receptors) or over vanilloid TRPV1 receptors. However, we cannot rule out at this stage that they might induce their pro-nociceptive effects also via as yet uncharacterized non-CB_1, non-TRPV1 receptors (see for example Di Marzo *et al*., 2001).

The picture of the functional activity of CB-25 and CB-52 at human CB_2 receptors was far less complicated. Both compounds exhibited little or no efficacy when using either forskolin-stimulated cAMP formation in intact hCB_2-CHO cells or GTP-γ-S binding to membranes from similar cells, thus underscoring the importance of using similar cell systems, even when using different functional assays, to investigate the functional activity of cannabinoid receptor ligands *in vitro*. Furthermore, we found that the potency of CB-25 and CB-52 as antagonists of CP55,940-induced GTP-γ-S binding, either expressed as K_b values or as the minimal concentration required to exert a significant rightward shift of the dose–response curve of CP55,940, re-

flected their relative affinities for human recombinant CB_2 receptors.

With this apparently simpler scenario in mind, we tested CB-25 and CB-52 in a widely employed assay of peripheral pain, where not only CB_1 but also CB_2 receptor agonists and antagonists have been shown to produce effects, that is, the biphasic nocifensive behaviour observed in rodents following intra-paw injection of formalin (Calignano *et al*., 1998; Hanus *et al*., 1999; Beaulieu *et al*., 2000). Quite surprisingly, in consideration of the pro-nociceptive effects observed here in the plantar test in normal rats, both CB-25 and CB-52 behaved as analgesics against the two phases of formalin-induced behaviours in mice. As it has been often reported that cannabimimetic drugs can produce biphasic effects (see for example Sulcova *et al*., 1998), these seemingly paradoxical results might be simply due to the fact that we tested *in vivo* only two doses of the compounds, and hence might have missed observing either anti-nociceptive actions in the above-mentioned plantar test or pro-nociceptive effects in the formalin test. Alternatively, some of our data might suggest that the endogenous tone of endocannabinoids, hypothesized to be at the supraspinal level to explain the effects observed in the plantar test and on RVM neurons (see above), is absent in mice treated with formalin. In fact, although it was previously shown in formalin-treated mice that CB_1 and CB_2 receptor inverse agonists cause a pro-nociceptive effect, no evidence for an elevated endogenous tone of skin endocannabinoids has ever been reported following formalin injection in either mice or rats (Calignano *et al*., 1998; Beaulieu *et al*., 2000). Therefore, it is possible that in the absence of an endocannabinoid-induced CB_1/CB_2 tone in the paw skin of formalin-injected rats, CB-25 and CB-52, by being neutral CB_2 antagonists, act uniquely as CB_1 receptor partial agonists (see above) (a neutral antagonist will only exhibit pharmacological activity in the presence of an endogenous tone arising from endocannabinoid release, whereas an inverse agonist will also be active if the receptor is pre-coupled to G-proteins). This hypothesis is supported by our observations that (1) SR141716A (rimonabant), a selective CB_1 receptor inverse agonist, at a dose inactive *per se*, totally abolished CB-25 and CB-52 anti-nociceptive effects in formalin-treated rats, (2) these compounds affected significantly also the first phase of formalin-induced nociception, as one would expect from a partial CB_1 agonist, and (3) submaximal doses of CB-25 or CB-52 and WIN55,212-2 produced additive anti-nociceptive effects, thus suggesting a possible similar mechanism of action for the three compounds. However, the rank of potency/efficacy of CB-25 and CB-52 in this *in vivo* assay, where CB-52 was fully efficacious at doses lower than those necessary to CB-25 to exert a similar anti-hyperalgesic action, did not coincide with their rank of potency/efficacy at mouse CB_1 receptors observed here *in vitro* (see above), although this discrepancy might also be due to pharmacokinetic factors. More importantly, the effects of CB-25 and CB-52 were also abolished by the selective CB_2 receptor inverse agonist AM630, at a dose that is not expected to block CB_1 receptors and that was inactive *per se*. Therefore, additional mechanisms must be taken into account to explain the effects of the two compounds in the formalin test. For example, it is possible that CB-25 and CB-52, through some as yet unknown late compensatory mechanism, trigger the formation of anti-nociceptive endocannabinoids acting preferentially at CB_2 receptors at a stage and site when and where the two compounds cannot antagonize these receptors any longer. In other words, the two compounds might produce anti-nociception via as yet undefined CB_2-mediated mechanisms (that might indirectly result also in the activation of CB_1-mediated pathways). On the other hand, blockade by a CB_2 receptor antagonist is not always diagnostic of the direct participation of CB_2 receptors in the effect of a given compound, as shown by the finding that the analgesic actions of the cannabinoid receptor-inactive mediator, palmitoylethanolamide, are still reversed by the CB_2 antagonist/inverse agonist SR144528 (Calignano *et al*., 1998).

In conclusion, we have provided here the first functional data on CB-25 and CB-52, two ligands of cannabinoid CB_1 and CB_2 receptors derived from olivetol or resorcinol and fatty acids, previously developed by us and shown to be characterized by high metabolic stability with respect to both plant and endogenous cannabinoids (Brizzi *et al*., 2005). Our overall *in vitro* pharmacological data indicate that these compounds act *in vitro* mostly as partial agonists at CB_1 receptors and as neutral antagonists at CB_2 receptors. The *in vivo* data presented here, if interpreted in the light of the quantitatively and qualitatively different types of endogenous cannabinoid tone that are likely to occur in different types of pain, support in part these conclusions but need to be substantiated by further studies, while leaving open the possibility of additional mechanisms of action. Apart from describing the complex pharmacology of two potential new tools for the study of the endocannabinoid system, our data also highlight how different animal species and experimental models might lead to even opposing results in studies with cannabinoid drugs.

Acknowledgements

We are grateful to the National Institute on Drug Abuse (Grant DA-9789 to RR and RP) and GW Pharmaceuticals (for a research grant to AT and RP) for support.

Conflict of interest

The authors state no conflict of interest.

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