Published in final edited form as:

Neuropharmacology. 2012 October ; 63(5): 905–915. doi:10.1016/j.neuropharm.2012.06.046.

AM-251 and Rimonabant Act as Direct Antagonists at Mu-Opioid Receptors: Implications for Opioid/Cannabinoid Interaction Studies

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Abstract

Mu-opioid and CB1-cannabinoid agonists produce analgesia; however, adverse effects limit use of drugs in both classes. Additive or synergistic effects resulting from concurrent administration of low doses of mu- and CB1-agonists may produce analgesia with fewer side effects. Synergism potentially results from interaction between mu-opioid receptors (MORs) and CB1 receptors (CB1Rs). AM-251 and rimonabant are CB1R antagonist/inverse agonists employed to validate opioid-cannabinoid interactions, presumed to act selectively at CB1Rs. Therefore, the potential for direct action of these antagonists at MORs is rarely considered. This study determined if AM-251 and/or rimonabant directly bind and modulate the function of MORs. Surprisingly, AM-251 and rimonabant, but not a third CB1R inverse agonist AM-281, bind with mid-nanomolar affinity to human MORs with a rank order of affinity (K_i) of AM-251 (251 nM) > rimonabant (652 nM) > AM281 (2135 nM). AM-251 and rimonabant, but not AM-281, also competitively antagonize morphine induced G-protein activation in CHO-hMOR cell homogenates ($K_b = 719$ or 1310 nM, respectively). AM-251 and rimonabant block morphine inhibition of cAMP production, while only AM-251 elicits cAMP rebound in CHO-hMOR cells chronically exposed to morphine. AM-251 and rimonabant (10 mg/kg) attenuate morphine analgesia, whereas the same dose of AM-281 produces little effect. Therefore, in addition to high CB1R affinity, AM-251 and rimonabant bind to MORs with mid-nanomolar affinity and at higher doses may affect morphine analgesia via direct antagonism at MORs. Such CB1-independent actions of these antagonists may contribute to reported inconsistencies when CB1/MOR interactions are examined via pharmacological methods in CB1-knockout versus wild-type mice.

Keywords

Morphine; Marijuana; CB1 Receptors; G-protein; Adenylyl Cyclase; Analgesia

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1. Introduction

Two cannabinoid receptors have been well characterized: CB1 receptors (CB1Rs), one of the most abundantly expressed G-protein coupled receptors in the central nervous system, and CB2 receptors (CB2Rs) which are localized primarily on immune cells (Howlett et al., 2004). Both CB1Rs and CB2Rs are coupled to the $G_{i/o}$ -subtype of G-proteins that inhibit the activity of adenylyl cyclase (Kaminski, 1996) and activate mitogen-activated protein (MAP) kinases (Bouaboula et al., 1995; Bouaboula et al., 1996), CB1Rs (Mackie and Hille, 1992) and CB2Rs (Atwood et al., 2012) additionally inhibit the function of voltage gated calcium channels, while only CB1Rs have been shown to modulate the activity of potassium channels (Hampson et al., 1995). CB1R agonists produce a characteristic tetrad of *in vivo* effects in rodents, defined by a decline in locomotor activity, hypothermia, catalepsy, and analgesia (McLaughlin et al., 2005). Since compounds that activate CB1Rs produce analgesia, selective CB1R agonists are being investigated as potential novel analgesic agents (Jhaveri et al., 2007). CB2R agonists also appear to modulate inflammatory hyperalgesia and neuropathic pain (Fox and Bevan, 2005; Manzanares et al., 2006). Although CB1Rs may represent a novel therapeutic option for treatment of acute and chronic pain, psychoactive side effects associated with CB1R activation limits the potential usefulness of drugs in this class (Hosking and Zajicek, 2008).

It is well known that activation of mu-opioid receptors (MORs) by agonists such as morphine produces potent and efficacious analgesia (Trescot et al., 2008). Similar to CB1Rs, mu-opioid receptors (MORs) couple to pertussis toxin-sensitive $G_{i/o}$ -subtype of G-proteins to inhibit cAMP production, activate MAP-kinase activity, close voltage gated calcium channels, and open inwardly rectifying potassium channels (Waldhoer et al., 2004). Unfortunately, also like CB1R agonists, both acute and prolonged use of MOR-analgesics is associated with several therapeutically limiting adverse effects such as euphoria, tolerance and dependence (Waldhoer et al., 2004). It has long been observed that concurrent administration of MOR and CB1R agonists produces additive or synergistic analgesic effects (Welch and Eads, 1999). Therefore, combining lower doses of drugs from both classes might represent a means to reduce adverse effects while maintaining adequate analgesia (Cichewicz, 2004). A common mechanism proposed to explain the synergism of analgesia observed when opioids and cannabinoids are co-administered is a direct interaction between MORs and CB1Rs (Schoffelmeer et al., 2006a).

Interactions between CB1Rs and MORs might be predicted based on observations that MORs are frequently co-localized with CB1Rs on neurons (Rodriguez et al., 2001; Salio et al., 2001), and both receptors form functional heterodimers (Hojo et al., 2008) and utilize the same pool of G-proteins (Shapira et al., 2000). Indeed, recent *in vitro* studies demonstrate that the constitutive activity of the CB1Rs negatively regulates MOR function (Canals and Milligan, 2008). For example, the neutral CB1R antagonist O-2020 produces no effect on MOR activity, but the CB1R inverse agonist SR-141716A (*e.g.*, rimonabant) enhances MOR function. Interactions between CB1Rs and MORs have also been predicted to occur *in vivo*. For example, da Fonseca Pacheco *et al.* reported that AM-251, a selective CB1R inverse agonist, antagonizes peripheral analgesia produced by morphine (da Fonseca Pacheco et al., 2008). Co-administration of AM-251 with morphine also decreases the development of tolerance and dependence in chronically treated mice (Trang et al., 2007). Lastly, CB1 agonists intensify morphine analgesia (Reche et al., 1996; Vaysse et al., 1987; Williams et al., 2008). Importantly, these studies collectively suggest that co-administration with CB1R ligands might not only lower doses of chronically administered MOR agonists required to achieve adequate analgesia, but also delay or prevent development of opioid tolerance and/or dependence as well. Although these and additional pharmacologically based studies in wild-type mice do provide sufficient evidence for a role for CB1Rs in mediating MOR

analgesia, curiously, these results are often not validated when examined by similar studies employing CB1-knockout mice (Miller et al., 2011; Raffa and Ward, 2012).

Many studies utilize the high affinity, “selective” CB1 antagonist/inverse agonists AM-251 (da Fonseca Pacheco et al., 2008; Haghighparast et al., 2009; Pacheco Dda et al., 2009; Trang et al., 2007) or rimonabant (Fattore et al., 2005; Kirkham and Williams, 2001; Miller et al., 2011; Schoffelmeer et al., 2006b) to investigate potential interactions between CB1Rs and MORs. AM-251 has been reported to exhibit approximately 306-fold selectivity for CB1Rs over CB2Rs, with a K_i value of 0.23–7.49 nM for CB1Rs (Gatley et al., 1997; Lan et al., 1999). However, AM-251 appears to also act as an agonist at the putative cannabinoid receptor GPR55 in the low to mid-nanomolar range (Sharir and Abood, 2010). Rimonabant similarly binds with high affinity to CB1Rs (*e.g.*, K_i = 1.98–11.8 nM), displaying roughly 82-fold selectivity when compared to CB2Rs (Felder et al., 1995; Rinaldi-Carmona et al., 1994). For most interaction studies, it is thus assumed that both antagonists bind selectively to CB1Rs and not to other receptors, most importantly to MORs. As such, few studies have actually examined whether AM-251 or rimonabant might also interact directly with MORs. The purpose of this study was to explore the possibility that AM-251 and/or rimonabant might indeed bind to and modulate the activity of MORs. Therefore, we tested the hypothesis that the frequently reported effects of these antagonists on MOR function are not entirely mediated by indirect action at CB1Rs, but also by via direct binding to MORs. If correct, this could help to explain the reported inconsistencies when CB1/MOR interactions are examined via pharmacological methods in CB1-knockout versus wild-type mice (Miller et al., 2011). We report that both AM-251 and rimonabant bind to MORs with mid-nanomolar affinity and function as antagonists. In addition, both antagonists (10 mg/kg, i.p.) attenuate morphine analgesia, whereas the same dose of AM-281, a third high affinity CB1 antagonist with much lower MOR affinity, does not.

2. Material and Methods

2.1 Drugs

AM-251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) and AM-281 (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide) are both CB1R antagonist/inverse agonists (Pertwee, 2005) and were purchased from Tocris Bioscience (Ellisville, MO). O-2050 ((6aR, 10aR)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran) is a neutral antagonist at CB1Rs (Gardner and Mallet, 2006) and was purchased from Tocris Bioscience (Ellisville, MO). The CB1R selective antagonist/inverse agonist rimonabant (originally reported as SR141715A) (Rinaldi-Carmona et al., 1994) was obtained from Cayman Chemical Company (Ann Arbor, MI). Naloxone is a non-selective antagonist at μ , δ and κ -opioid receptors (Spivak et al., 1997) and was purchased from Sigma-Aldrich (St. Louis, MO). Morphine sulfate was obtained through Cardinal Health (Dublin, OH) and acts as an agonist at MORs (Trescott et al., 2008). The highly selective MOR peptide agonist [3 H]DAMGO (Spivak et al., 1997), CB1R/CB2R agonist [3 H]CP-55,940 (Hojo et al., 2008) and [35 S]GTP γ S used in G-protein activation studies, were all purchased from Perkin-Elmer (Waltham, MA). Non-radiolabeled CP-55,940 and DAMGO were obtained from Tocris Bioscience (Ellisville, MO). Non-radioactive GTP γ S was purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Mice

The University of Arkansas for Medical Sciences IACUC committee approved animal use protocols employed in this study. All efforts were made to minimize animal suffering and to reduce the number of animals used. B6SJL mice were obtained from an in-house breeding

colony and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained on a 12 hr light/dark cycle with free access to food and water. Following anesthesia with isoflurane, B6SJL mice were sacrificed and brains were harvested by decapitation and snap frozen employing liquid nitrogen.

2.3 Cell Culture

Chinese hamster ovary (CHO-K1) cells were stably transfected in our laboratory with either the human CB2 receptor (CNR2; CHO-hCB2)(Shoemaker et al., 2005) or the human mu-opioid receptor (hMOR) containing an N-terminal cMyc epitope tag (OPRM1; CHO-hMOR) (described following). All cells were cultured in DMEM (Mediatech Inc., Manassas, VA) containing 10% fetal calf serum (Gemini Bioproducts, Sacramento, CA), 0.05 IU/mL penicillin, 50 µg/mL streptomycin (Invitrogen, Carlsbad, CA), and 250 µg/mL of Geneticin (G418; Sigma-Aldrich, St. Louis, MO). Cells were maintained in a humidified chamber at 37°C with 5% CO₂, harvested weekly and only cells from passages 5–15 were used in all experiments.

CHO-K1 cells stably expressing hMOR receptors (CHO-hMOR) were created using the cationic-lipid lipofectin as described previously for production of CHO-hCB2 cells (Shoemaker et al., 2005). Briefly, CHO cells were cultured to 80% confluence (3×10^6 cells in 100-mm dishes) and incubated for 6 hr with 5 µg of pcDNA3.1 plasmids containing the cDNA encoding for the human mu-opioid receptor (OPRM1) containing an N-terminal cMyc epitope tag, and 15 µg of lipofectin reagent in serum-free Opti-MEM. Selection antibiotic (1 mg/ml geneticin) was added to the cell culture medium 48 hr after transfection, and surviving colonies were picked 14 days after beginning selection. To confirm hMOR expression, competition binding using whole cells obtained from each colony was performed by employing [³H]DAMGO (1.0 nM) displaced by nonradioactive naloxone (10 µM) as described below. The clone expressing the highest level of specific hMOR binding was selected for all described studies, designated as CHO-hMOR cells, and maintained in DMEM containing 250 µg/ml geneticin throughout the study.

2.4 Membrane Preparation

CHO-hMOR cells, CHO-hCB2 cells, CHO-WT cells and whole B6SJL mouse brains were homogenized with 10 complete strokes utilizing a 7ml Dounce glass homogenizer in an ice-cold buffer containing 50 mM HEPES pH 7.4, 3 mM MgCl₂, and 1 mM EDTA as described previously (Shoemaker et al., 2005). The homogenized samples were centrifuged at $40,000 \times g$ for 10 min at 4°C. Supernatants were discarded; the pellets re-suspended in the buffer, homogenized again, and centrifuged similarly twice more. After the final centrifugation step, supernatants were discarded and pellets were re-suspended in ice-cold 50 mM HEPES, pH 7.4 to achieve an approximate protein concentration of 10 mg/ml. Membrane homogenates were divided into aliquots and stored at –80°C for future use. A small aliquot of each membrane preparation was removed prior to freezing and the protein concentration was determined using BCATM Protein Assay (Thermo Fisher Scientific, Waltham, MA).

2.5 Competition Receptor Binding

Each reaction mixture contained 100 µg of membrane homogenates, 0.1 nM [³H]-CP-55,940 or 1 nM [³H]-DAMGO, 5 mM MgCl₂, and increasing concentrations of the non-radioactive competitive ligands AM-251, AM-281 or morphine in a 50 mM Tris-HCl buffer (pH 7.4) with 0.1% bovine serum albumin. The total volume of the incubation mixture was 1 ml. The reaction mixtures were briefly mixed and allowed to reach equilibrium binding by incubation at room temperature for 90 min. Non-specific binding was defined as the amount of radioligand binding remaining in the presence of a single 10 µM concentration of either non-radioactive CP-55,940 or naloxone. Binding was terminated by rapid vacuum filtration

through glass fiber filters (Brandel, Gaithersburg, MD), followed by three 5 ml washes of ice-cold 50 mM Tris-HCl (pH 7.4) buffer containing 0.1% bovine serum albumin. Four ml of scintiverse© scintillation fluid (Fisher Scientific, Waltham, MA) was added to the filters and the amount of radioactivity was quantified 24 hr later utilizing liquid scintillation spectrophotometry.

2.6 [³⁵S]GTPγS Binding

The GTPγS binding assay to measure G-protein activation was conducted as previously described (Prather et al., 2000a). Briefly, in a total volume of 1 ml, 10 ug of membrane homogenates was added to a reaction mixture containing 0.1 nM [³⁵S]GTPγS, 20 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, 10 μM GDP, 0.1% bovine serum albumin and the indicated concentrations of morphine, AM-251, AM-281, naloxone and/or O-2050. After mixing, reaction mixtures were incubated at 30°C for 2 hr (an interval shown to produce optimal agonist-induced [³⁵S]GTPγS binding levels, data not shown). Nonspecific binding was defined by the amount of radioactivity remaining in the presence of a 10 μM concentration of non-radiolabeled GTPγS. Reactions were terminated by rapid vacuum filtration through glass fiber filters followed by three washes with ice cold 50 mM HEPES (pH 7.4) containing 0.1% bovine serum albumin. Four ml of scintiverse© scintillation fluid (Fisher Scientific, Waltham, MA) was added to the filters and the amount of radioactivity was quantified 24 hr later utilizing liquid scintillation spectrophotometry. For experiments examining the reversal or attenuation of agonist-stimulated G-protein activation, antagonists and agonists were added to the incubation mixtures at the same time.

2.7 Intracellular cAMP Assay in Intact CHO-hMOR cells

CHO-hMOR cells were plated into 24-well culture plates at a density of 6×10^6 cells/plate and incubated overnight at 37°C in 5% CO₂. As previously described (Prather et al., 2000), the following day culture media was removed from each well and replaced with 500 μl pre-incubation mixture containing DMEM with 0.9% NaCl, 500 μM 3-isobutyl-1-methylxanthine (IBMX), and 2 μCi/well [³H]adenine. Cells were incubated for 1 hr at 37°C. The pre-incubation mix was removed and cannabinoid agonists or inverse agonists were added to the cells for 15 min in a Krebs-Ringer-HEPES solution (10 mM HEPES, 110mM NaCl, 25mM Glucose, 55mM Sucrose, 5mM KCl, 1mM MgCl₂, 1.8 mM CaCl₂, pH 7.4) containing IBMX, 10μM forskolin and the test compound. Reactions were terminated by adding 50 μl 2.2N HCl and [³H]cAMP was isolated by employing alumina column chromatography. Radioactivity contained in 4 ml of the final column eluate was counted by a Packard-Tri-carb 2100/TR liquid scintillation counter after adding 10 ml of liquid scintillation cocktail. Similar methods were utilized for chronic studies except that cells were incubated with morphine (1 μM) overnight prior to experiments.

2.8 Measurement of Analgesia

Each experimental group consisted of four to six B6SJL or C57BL/6J mice. Mice were injected i.p. with the indicated drug dose or vehicle (50% polyethylene glycol/50% saline). Thirty min after drug or vehicle injections, analgesia was measured by a modified tail-flick procedure employing a radiant heat apparatus (model TF-6; Emdie Instruments, Richmond, VA) previously published by D'Amour and Smith (D'Amour, 1941). Briefly, the tail of a mouse was placed over a photocell and exposed to a beam of intense light, which initiated a timer. When the tail was "flicked" in response to the noxious stimulus, the light beam was broken, stopping the timer and extinguishing the light beam. An average latency for each mouse was calculated from a series of two tail-flicks. A maximal cut-off time of 15 sec was employed to avoid tail injury. The baseline tail-flick latency prior to drug administration for all mice was maintained at 5–6 sec by optimizing the light intensity. Immediately after determining basal tail-flick latencies, increasing doses of morphine were administered s.c.

Following a second 30 min inter-trial interval, tail-flick latencies were again determined to quantify the degree of analgesia by determining the percent of the maximal possible effect (%MPE). %MPE was calculated by subtracting the basal tail-flick latency from the latency in the presence of drug, divided by the difference between the cut-off time (15 sec) minus the basal latency, multiplied by 100. For experiments examining antagonism of morphine analgesia, an injection of either naloxone (4 mg/kg, i.p.), AM-251 (10 mg/kg, i.p.), rimonabant (10 mg/kg, i.p.) or AM-281 (10 mg/kg, i.p.) was administered 30 min prior to morphine administration.

2.9 Statistical Analysis

All data are expressed as the mean $\pm$ SEM and results were obtained from a minimum of three separate experiments, each performed in triplicate. The *in vivo* experiments were conducted by employing a minimum of N = 4–6 mice. Curve fitting and statistical analyses for *in vitro* experiments were performed using GraphPad Prism version 4.0b (GraphPad Software Inc., San Diego, CA). The Cheng-Prusoff equation (Cheng and Prusoff, 1973) was used to convert the experimental IC₅₀ values obtained from competition receptor binding experiments to K_i values, a quantitative measure of receptor affinity. Non-linear regression for one-site competition was used to determine the IC₅₀ for competition receptor binding. The affinity (*e.g.*, K_d) values of [³H]CP-55,940 for mCB1 and hCB2 receptors, used to calculate the K_i values presented, were 0.37 (Brents et al., 2011) and 0.38 nM (Shoemaker et al., 2005), respectively. The K_d values determined of [³H]DAMGO for mMORs and hMORs were 0.5 and 0.5 nM, respectively (data not shown). Curve fitting of concentration-effect curves via non-linear regression was employed to determine the ED₅₀ (a measure of potency) and E_{max} (a measure of efficacy) for the GTP γ S binding and adenylyl cyclase experiments. Schild analysis as previously described by Thomas, *et. al.* (Thomas et al., 2005) was performed to determine K_b values and the Schild slope concerning the ability of AM-251, rimonabant and AM-281 to shift the morphine concentration-effect curves for G-protein activation. A one-way ANOVA, followed by the Newman-Keuls *post-hoc* test, was used to determine statistical significance ($P < 0.05$) between three or more groups. When only two groups were compared, the Students t-test was employed, with ($P < 0.05$) as the minimal level of significance.

3. Results

3.1 Morphine and AM-251, but not AM-281, bind to mMORs in mouse brain homogenates with affinity in the nanomolar range

The chemical structures of all drugs examined in this study are presented in Figure 1. Competition receptor binding studies employed [³H]DAMGO or [³H]CP-55,940 to characterize the binding of ligands to MORs or CB1Rs in mouse brain homogenates, respectively. Morphine was determined to bind with high affinity (0.17 ± 0.08 nM, N=3) to mMORs, but not to mCB1Rs (Figure 2A, Table 1). Unexpectedly, in addition to having high affinity for mCB1Rs, the CB1R inverse agonist AM-251 was found to also bind to mMORs with a relatively high mid-nanomolar affinity (327 ± 98 nM, N=5). The affinity of AM-251 for MORs is similar to its affinity for hCB2Rs, also determined by competition receptor binding utilizing [³H]CP-55,940 in CHO-hCB2 cell membranes (Figure 2B, Table 1). In marked contrast, a second CB1R inverse agonist examined, AM-281, only binds to mMORs (Figure 2C, Table 1) with low micromolar affinity. Therefore, in addition to high CB1R affinity, AM-251, but not AM-281, binds to mMORs with mid-nanomolar affinity.

3.2 AM-251, but not AM-281, antagonizes G-protein activation produced by the MOR agonist morphine in mouse brain homogenates

Consistent with an agonist profile, morphine potently activates G-proteins in mouse brain as determined by [35 S]GTP γ S binding ($ED_{50} = 157 \pm 18$ nM, $N=9$, Figure 3A). In contrast, AM-251 produces a concentration-dependent decrease in basal G-protein activity in mouse brain homogenates ($IC_{50} = 88 \pm 29$ nM, $N=3$), a property consistent with that of an inverse agonist (Mackie, 2008). AM-281 only decreases basal G-protein activity in mouse brain homogenates at a high concentration of 10 μ M (Figure 3A). AM-251 may act as an inverse agonist to reduce basal GTP γ S binding by stabilizing the constitutively active state of CB1Rs, MORs or a number of other G-protein coupled receptors present in mouse brain homogenates. To determine if AM-251 acts as an inverse agonist specifically at MORs, CB1Rs or both receptors, AM-251 (1 μ M) was co-incubated with 1 μ M of the neutral MOR antagonist naloxone ($N=8$), 1 μ M of the neutral CB1R antagonist O-2050 ($N=13$) or a combination of naloxone and O-2050 ($N=5$). Interestingly, neither co-incubation with each antagonist alone, nor their combination, significantly diminishes the reduction in basal G-protein activity produced by AM-251 (Figure 3B). The lack of antagonism by naloxone suggests that AM-251 likely reduces basal GTP γ S binding by a mechanism that does not involve MORs. Since AM-251 is a known inverse agonist at CB1Rs (Pertwee, 2005), it is somewhat surprising that O-2050 did not attenuate a portion of the AM-251-mediated reduction in basal G-protein activity. However, it is possible that the 5-fold lower affinity of O-2050 for CB1Rs [2.4 nM, (Gardner and Mallet, 2006)], when compared to AM-251 (0.49 nM, Table 1), precludes significant antagonism when both ligands were co-incubated at the equal molar concentrations as employed here. Co-incubation of AM-251 with higher concentrations of O-2050 was considered, but concentrations of O-2050 greater than 1 μ M alone were found to increase basal GTP γ S binding (data not shown). As such, due to potential confounding of experimental interpretation, co-incubation with concentrations of >1 μ M of O-2050 was not attempted.

Although AM-251 does not appear to function as an inverse agonist at MORs (Figure 3B), data presented in Figure 3C demonstrate that AM-251 (10 μ M; $N=5$) antagonizes morphine activation of G-proteins in mouse brain homogenates by shifting the concentration-effect curve 9-fold to the right (from $ED_{50} = 157 \pm 18$ nM to 1378 ± 395 nM, $K_b = 1282$ nM). Co-administration with AM-251 did not alter the efficacy of G-protein activation produced by morphine and the Hill slope for this curve was not significantly different than 1. In contrast to AM-251, co-administration with AM-281 (10 μ M; $N=3$) has no significant effect on the ability of morphine to activate G-proteins in mouse brain ($ED_{50} = 194 \pm 72$ nM, $N=3$).

3.3 Morphine, AM-251 and rimonabant, but not AM-281, bind to hMORs in transfected CHO cell homogenates with affinity in the nanomolar range

To determine if AM-251 and a second, more structurally similar CB1R antagonist/inverse rimonabant bind to MORs directly, in the absence of CB1Rs, similar studies were performed in CHO cells stably expressing only hMORs (*e.g.*, CHO-hMOR cells). Consistent with results observed in mouse brain homogenates, AM-251 and rimonabant bind to hMORs with relatively high mid-nanomolar affinities of 251 ± 69 nM ($N=4$) and 652 ± 103 (N=4) nM in CHO-hMOR cells, respectively (Figure 4, Table 1). Also similar to results observed in mouse brain homogenates, AM-281 binds to hMORs with approximately 10-fold less affinity relative to AM-251, in the low micromolar range.

3.4 AM-251 and rimonabant, but not AM-281, competitively antagonize G-protein activation by the MOR agonist morphine in CHO-hMOR cell homogenates

To determine whether AM-251, rimonabant or AM-281 modulate the activity of MOR directly, in the absence of CB1Rs, GTP γ S binding studies similar to those conducted using

mouse brain membranes were performed in CHO-hMOR cell homogenates. As anticipated, morphine produces a concentration-dependent activation of G-proteins in CHO-hMOR cell homogenates ($ED_{50} = 96 \pm 18$ nM, $N = 4$, Figure 5A). Similar to that observed in mouse brain homogenates, AM-251 ($IC_{50} = 265 \pm 69$ nM, $N = 3$), rimonabant ($IC_{50} = 1100 \pm 270$ nM, $N = 5$) and AM-281 ($IC_{50} = 974 \pm 285$ nM, $N = 3$) concentration-dependently decrease basal G-protein activity in CHO-hMOR membranes (Figure 5A). To determine if these ligands act as inverse agonists at MORs, in the absence of CB1Rs, $10 \mu\text{M}$ of each compound was co-incubated with $1 \mu\text{M}$ of the neutral MOR antagonist naloxone. As observed for AM-251 in mouse brain membranes, co-incubation with naloxone did not significantly alter the reduction in basal G-protein activity produced by AM-251, rimonabant or AM-281 (Figure 5B). In contrast, the agonist activity produced by the MOR agonist morphine (100 nM) was completely reversed by naloxone. Interestingly, a $10 \mu\text{M}$ concentration of AM-251, rimonabant or AM-281 also produces $\sim 20\%$ reduction in basal G-protein activity in CHO-WT cells that are devoid of MORs (Figure 5C). Collectively, these results indicate that in both mouse brain and CHO-hMOR membranes, all three ligands examined likely reduce basal GTP γ S binding by a mechanism that does not involve MORs. Therefore, AM-251, rimonabant and AM-281 are not inverse agonists at MORs.

To determine if these compounds act as antagonists at MORs, the effect of co-incubation with each ligand on the ability of morphine to activate G-proteins in CHO-hMOR membranes was next examined. Co-incubation with AM-281 (Figure 6A; $ED_{50} = 94 \pm 19$ nM, $N = 3$) fails to alter morphine-induced G-protein activation in CHO-hMOR cell homogenates ($ED_{50} = 93.6 \pm 14.0$ nM, $N = 5$). However, significant antagonism is observed upon co-administration with either rimonabant (Figure 6B) or AM-251 (Figure 6C). Specifically, the potency of morphine to activate G-proteins is reduced 6-fold by co-incubation with rimonabant ($10 \mu\text{M}$; $ED_{50} = 575 \pm 58.3$ nM, $K_b = 1310$ nM, $N = 3$). AM-251 also produces a concentration-dependent shift-to-the-right in the morphine concentration-effect curve, with a relatively low $1 \mu\text{M}$ concentration ($ED_{50} = 496 \pm 130$ nM, $N = 5$) producing a 5-fold, and a higher $10 \mu\text{M}$ concentration ($ED_{50} = 1496 \pm 123$ nM, $N = 4$) resulting in a 16-fold reduction in the potency of morphine to activate G-proteins. Co-administration with rimonabant or AM-251 does not alter the maximal efficacy of G-protein activation produced by morphine and the Hill slopes for all curves are not significantly different than 1. Additionally, Schild-analysis of the shift in potency produced by AM-251 reveals that the antagonism at hMORs is competitive, as indicated by a Schild plot slope of 1.02, and K_b value of 719 nM (Figure 6C inset). Collectively, these studies suggest that AM-251 and rimonabant, but not AM-281, competitively antagonize morphine-induced activation of G-proteins via MORs expressed in CHO-hMOR cells.

3.3 AM-251, rimonabant and AM-281 attenuate acute inhibition of adenylyl cyclase activity produced by morphine in intact CHO-hMOR cells, but only AM-251 induces cAMP rebound in cells chronically treated with morphine

After determining that AM-251 and rimonabant antagonize G-protein activation produced by the MOR agonist morphine, experiments were designed to examine the effect of these ligands on MOR modulation of the intracellular effector adenylyl cyclase (Figures 7 & 8). MORs are coupled to the $G_{i/o}$ -subtype of G-proteins (Murthy and Makhoulf, 1996) and thus agonists such as morphine produce a decrease in cAMP production. As expected, morphine produces a concentration-dependent reduction in intracellular cAMP concentrations of approximately an 80% in intact CHO-hMOR cells, with half-maximal inhibition occurring at 24.0 ± 8.0 nM ($N = 6$; Figure 7A). Naloxone, AM-251, rimonabant and AM-281 act as neutral antagonists regarding adenylyl cyclase activity in CHO-hMOR cells, producing no effect on intracellular cAMP levels when a single, receptor saturating concentration ($10 \mu\text{M}$) of each compound alone is examined (Figure 7B). However, the inhibitory actions of

morphine 10 nM are completely reversed by co-incubation with naloxone (1 μ M; $N=3$) and significant attenuation is also observed in the presence of AM-251 (10 μ M; $N=6$), rimonabant (10 μ M; $N=4$) or AM-281 (10 μ M; $N=3$) (Figure 7C). The incomplete antagonism of morphine produced by AM-251 and rimonabant is somewhat surprising, as near full receptor occupancy at the 10 μ M concentration employed would be predicted, based on affinity of these compounds for hMORs (Table 1). However, these unanticipated observations could be explained, in part, by relatively poor solubility observed for these compounds in the Krebs-Ringer-HEPES buffer used for these assays. It is also possible that the test compounds may exhibit a lower affinity for MOR in the adenylyl cyclase assays conducted in whole cells, due to the presence of intracellular guanine nucleotides, when compared to assay conditions employed for receptor binding studies to determine K_i values.

Overnight treatment of CHO-hMOR cells with morphine produces desensitization of MORs as demonstrated by a significant reduction in the ability of morphine to inhibit cAMP production upon acute challenge following chronic treatment (Figure 8). Characteristic of the effects produced by antagonists/inverse agonists when administered to cells following chronic treatment with MOR agonists (Liu and Prather, 2001), naloxone (10 μ M; $N=4$) and AM-251 (10 μ M; $N=4$), but not rimonabant (10 μ M; $N=3$) or AM-281 (10 μ M; $N=4$), produce a rebound in cAMP levels above basal levels in CHO-hMOR cells exposed overnight to morphine. Interestingly, the degree of cAMP rebound produced by the test compounds examined (*e.g.*, naloxone > AM-251 > rimonabant > AM-281), directly correlates with the affinity of these compounds for hMORs (Table 1).

3.4 Naloxone, AM-251, rimonabant and AM-281 antagonize morphine analgesia in a manner that correlates closely with their affinity for MORs

Using separate groups of five or more mice per dose, as anticipated, morphine injected *s.c.* produces dose-dependent analgesia ($ED_{50} = 4.2 \pm 0.6$ mg/kg) in B6/SJL mice (Figure 9A). Employing a dose of morphine similar to the ED_{50} required to produce analgesia in B6/SJL mice (5 mg/kg), 30 min *i.p.* pre-injection with naloxone (4 mg/kg) completely antagonizes morphine analgesia in both B6/SJL (Figure 9B) and C57BL/6J (Figure 9C) mice. Likewise, AM-251 and rimonabant (10 mg/kg) injected *i.p.* 30 min prior to morphine significantly antagonizes analgesia by 60–72% in both strains of mice (Figure 9B and 9C). However, pre-injection of an equivalent 10 mg/kg dose of AM-281 produces no effect on morphine analgesia in B6/SJL (Figure 9B) and less than 40% antagonism in C57BL/6J (Figure 9C) mice.

4. Discussion

This study demonstrates that two commonly employed selective CB1R antagonist/inverse agonists AM-251 and rimonabant also bind to MORs with relatively high, mid-nanomolar affinity and act directly as MOR antagonists in both *in vitro* and *in vivo* assays. Interestingly, both AM-251 and rimonabant bind to MORs with significantly higher affinity when compared to a third, less commonly utilized, CB1R antagonist/inverse agonist AM-281. Functional assays examining G-protein activity demonstrate that both compounds act as competitive antagonists at hMORs. AM-251 and rimonabant also antagonize the ability of morphine to acutely inhibit activity of the intracellular effector adenylyl cyclase in intact CHO-hMOR cells, while only AM-251 (the compound with the highest MOR affinity) produces cAMP rebound when administered to cells chronically exposed to morphine. Most importantly, pretreatment of mice with 10 mg/kg (*i.p.*) of AM-251 or rimonabant attenuate morphine analgesia in two different strains of mice, whereas a similar dose of AM-281 produces little, or no, antagonism. Collectively, our results indicate that effects of relatively high, but often employed, concentrations and/or doses of AM-251 and rimonabant on MOR signaling observed in various *in vitro* and *in vivo* assays should be carefully interpreted due

to potential confounding antagonist actions produced by these ligands acting directly at both MORs and CB1Rs. Specifically, these findings could have important implications for interpretation of data obtained from past reports that used these compounds and also in choice of CB1R antagonists and concentrations to employ for future studies to examine MOR and CB1R interactions.

In vitro studies indicate that direct interactions between MORs and CB1Rs are likely because both receptors are often co-localized (Pickel et al., 2004; Rodriguez et al., 2001; Salio et al., 2001) and form functional heterodimers (Hojo et al., 2008). Furthermore, in cells expressing both receptors, activation of CB1Rs by WIN-55,212-2 produces non-competitive antagonism of morphine-induced G-protein stimulation (Rios et al., 2006). These results indicate that CB1R agonists act at a receptor distinct from MOR, in this example presumably CB1Rs, to negatively modulate the activation of MOR by morphine. Another elegant *in vitro* report demonstrated that, opposite to that produced by agonists, relief of constitutive CB1R activity by selective inverse agonists enhances agonist activation of MORs (Canals and Milligan, 2008). These observations suggest that at the cellular level, CB1R agonists may negatively modulate, while CB1R inverse agonists appear to enhance MOR function. Importantly, these observations are in opposition to those obtained in the present study in which the CB1R inverse agonists AM-251 and rimonabant, but not AM-281, inhibit morphine activation of MORs.

Such apparent discrepancies might be explained by potential confounding actions produced by AM-251 and/or rimonabant acting simultaneously as an antagonist-inverse agonist at CB1Rs and as an antagonist at MORs. For example, on one hand at lower concentrations these ligands might be expected to enhance MOR activation via their action as CB1R inverse agonists (Canals and Milligan, 2008). However, results from this study indicate that, particularly at higher concentrations, AM-251 and rimonabant might also be anticipated to simultaneously decrease MOR function due to direct antagonism. Such opposing actions of these compounds on MOR function might thus be anticipated to lead to a “canceling out” of each individual effect, contributing to a diminished overall response. As such, determination of the “actual” effect of CB1R antagonism on MOR function would likely be complicated if high concentrations of these or similar antagonists exhibiting dual CB1R/MOR affinity are employed.

Current literature also suggests that CB1R agonists and antagonists appear to have potentially opposite roles in opioid dependence and production of withdrawal. For example, blocking endocannabinoid breakdown or direct administration of Δ^9 -THC attenuate naloxone-precipitated signs of opioid withdrawal in morphine dependent mice in a CB1R-dependent manner (Ramesh et al., 2011). In contrast, when 3 mg/kg rimonabant is administered acutely to morphine dependent rats, it produces an opioid-like withdrawal syndrome similar to that produced by naloxone (Navarro et al., 1998). Although experiments employing transgenic mice lacking CB1Rs to determine the exact mechanism responsible for rimonabant- or AM251-precipitated opioid withdrawal have not been reported, other studies utilizing CB1R knockout mice show that CB1Rs must be intact to achieve acute morphine-induced conditioned place preference, chronic morphine-induced sensitization to locomotor responses (Martin et al., 2000), and morphine self-administration (Ledent et al., 1999). Lastly, acute rimonabant administration (3 mg/kg) decreases heroin self administration (Navarro et al., 2001), whereas doses as low as 1 mg/kg of the CB1R agonists WIN-55,212-2 and Δ^9 -THC enhance the reinforcing effects of heroin in dependent rats (Solinas et al., 2005).

To determine if AM-251 and/or rimonabant produce effects consistent with direct antagonism of MORs following chronic opioid administration, we examined the actions of

these compounds in a cellular model of opioid-induced tolerance and dependence (Avidor-Reiss et al., 1995) by employing CHO-hMOR cells. Cellular tolerance develops in response to chronic opioid treatment resulting in adaptations within cells to maintain homeostasis. Acute treatment with morphine or other opioid agonists decreases cAMP production; however, upon chronic administration, cells adapt by up-regulating cAMP levels. When opioids are abruptly removed or cells are exposed to an antagonist like naloxone, a rebound of increased cAMP production above basal levels occurs (Liu and Prather, 2001). In the present study, although AM-251, rimonabant and AM-281 all attenuate the ability of morphine to acutely reduce intracellular cAMP levels, only AM-251 produces significant levels of cAMP rebound similar to that produced by naloxone in CHO-hMOR cells chronically treated with morphine. This suggests that under situations involving chronic administration of opioids, only compounds with relatively high affinity for MOR, like naloxone and AM-251, are able to efficaciously produce cAMP rebound. Furthermore, it appears that AM-251 produces effects that are consistent with direct MOR antagonism when evaluated in assays examining either acute actions of MOR agonists or following chronic opioid administration.

Finally, results reported here demonstrate that AM-251 and rimonabant attenuate morphine analgesia in mice to a much greater extent than does AM-281. Although the exact mechanism(s) responsible for these *in vivo* results are unknown, the degree of morphine antagonism produced by AM-251, rimonabant and AM-281 appears to be directly correlated to the affinity of these compounds for MORs (*e.g.*, AM-251 > rimonabant >> AM-281; Table 1). In contrast to differing affinities for MORs, all three ligands possess relatively similar high affinity for CB1Rs (*e.g.*, 0.49–1.8 nM; Table 1). These observations coupled with the additional *in vitro* findings reported here indicate that the effects of these compounds on morphine analgesia may result not only from inverse agonist actions at CB1Rs, but also from direct antagonism of MORs as well. Other studies similarly report that AM-251 antagonizes morphine analgesia mediated by both central and peripheral mechanisms. For example, AM-251 injected intra-plantarly (20 – 80 µg) dose-dependently attenuates morphine analgesia in hyperalgesic paws (da Fonseca Pacheco et al., 2008). This same group also demonstrate that 2–4 µg of AM-251 administered i.c.v. produces a dose-dependent reversal of centrally-mediated morphine analgesia (Pacheco Dda et al., 2009). Lastly, Haghparast *et al.* (Haghparast et al., 2009) show that intra-accumbal administration of AM-251 (5–125 ng) daily for 3 days results in sensitization to morphine analgesia in a dose-dependent manner. These studies suggest that CB1Rs indirectly modulate morphine-mediated analgesia and are based on a critical assumption that the doses of AM-251 examined produce selective antagonism of CB1Rs. However, since concentrations of AM-251 actually achieved at the site(s) of action were not determined in these studies, it is also possible that levels of AM-251 attained via intra-plantar, intra-accumbal or i.c.v. routes of administration were sufficiently high to result in direct MOR antagonism, in addition to CB1R antagonism.

As might be predicted, CB1 agonists appear to produce opposite effects relative to antagonist inverse-agonists such as AM-251 or rimonabant, resulting in an intensification of morphine analgesia (Reche et al., 1996; Vaysse et al., 1987; Williams et al., 2008). Interestingly, when ineffective doses of Δ^9 -THC and morphine are administered together, a synergistic analgesia results (Cichewicz, 2004). Although these studies indicate that the effects of CB1 agonists and inverse agonists on morphine analgesia may have important clinical ramifications, results reported here suggest that design of future studies should include initial assays to assure that the concentrations of the cannabinoid ligands to be tested do indeed bind selectively to CB1Rs and do not produce off-target effects such as direct action at MORs. This is indeed potentially clinically relevant because while no pharmacokinetic studies have yet measured levels of AM-251 in chronically treated animals

or humans, plasma levels of rimonabant in patients after chronic dosing have been reported to be in the mid-nanomolar range (Heness et al., 2006).

It should be made clear that the findings presented here are not in opposition to the reported “selective” CB1R binding properties of AM-251 (Gatley et al., 1997; Lan et al., 1999) and rimonabant (Felder et al., 1995; Rinaldi-Carmona et al., 1994). Indeed, observations reported in the current study demonstrate that AM-251 and rimonabant bind to CB1Rs with approximately 500 and 300-fold higher affinity relative to hCB2Rs, mMORs or hMORs, respectively (Table 1). However, additional observations presented here detailing novel direct antagonistic actions of AM-251 and rimonabant at MORs with relatively high, mid-nanomolar affinity are nonetheless significant because they may help to explain many conflicting reports in the literature concerning a lack of agreement between evidence for CB1-MOR interactions when investigated by pharmacological methods in wild-type versus CB1-knockout mice [reviewed in (Raffa and Ward, 2012)].

Similar to findings presented in the current study, rimonabant has also been shown to competitively antagonize G-protein activation produced by MOR agonists and to compete with [³H]DAMGO for binding to MORs, although with a relatively high IC₅₀ value of 5.7 μM (Cinar and Szucs, 2009). While not determined by the authors, it would be predicted based the IC₅₀ value presented, that the affinity of rimonabant for MORs would correspond to a K_i value of ~2 μM in this previously published study. Finally, a very recent study published while this manuscript was in review (Zador et al., 2012) demonstrated that rimonabant similarly binds to MORs with an affinity in the mid-nanomolar range and antagonizes MOR signaling in mouse forebrain membranes in a CB1-independent manner. Results from these studies, along with the detailed findings reported here, suggest that caution should be exercised when interpreting data from *in vitro* or *in vivo* experiments in which high concentrations of AM-281, rimonabant, and in particular, AM-251 are employed.

5. Conclusions

This study demonstrates that the commonly employed selective CB1R antagonist/inverse agonists, AM-251 and rimonabant, also directly bind to MORs with relatively high affinity in the mid-nanomolar range to produce antagonist actions in both *in vitro* and *in vivo* assays. As such, effects on MOR signaling resulting from relatively high concentrations and/or doses of AM-251 and/or rimonabant should be carefully interpreted, considering potential confounding antagonist/inverse actions produced by these ligands at both MORs and CB1Rs.

Acknowledgments

This work was supported in part by National Institute of Neurological Disorders and Stroke (NINDS) grant 5R21NS058430 (P.L.P.) and National Institutes of Environmental Health Sciences (NIEHS) pre-doctoral training grant 5T32ES01952 (K.A.S.).

Abbreviations

AM-251	(N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide)
AM-281	(1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide)
ANOVA	analysis of variance

cAMP	cyclic adenosine monophosphate
CHO-hCB2 cells	Chinese hamster ovary cells stably expressing human cannabinoid 2 receptors
CHO-hMOR cells	Chinese hamster ovary cells stably expressing human mu-opioid receptors
CP-55, 950	[(–)- <i>cis</i> -3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol]
DAMGO	[D-Ala ² , N-MePhe ⁴ , Gly-ol]-enkephalin
DMEM	Dulbecco's modified Eagle's medium
GTPγS	guanosine 5'-O-[gamma-thio]triphosphate
hCB2Rs	human cannabinoid 2 receptors
hMOR	human mu-opioid receptors
mCB1Rs	mouse cannabinoid 1 receptors
mMORs	mouse mu-opioid receptors
O-2050	((6aR,10aR)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran)

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Highlights

- CB1 antagonists AM-251 and rimonabant bind with mid-nanomolar affinity to MORs.
- AM-251 and rimonabant antagonize morphine G-protein activation and cAMP inhibition.
- AM-251, but not rimonabant, elicits cAMP rebound in morphine-treated cells.
- AM-251 and rimonabant, but not CB1 antagonist AM-281, attenuate morphine analgesia.
- AM-251 and rimonabant off-target actions at MORs confound CB1/MOR interaction studies.

Figure 1. Chemical structures of test compounds used in this study
The colored boxes highlight structural differences between the drugs examined.

Figure 2. Determination of the affinity of test compounds for mMORs, mCB1Rs and hCB2s by competition receptor binding performed with membrane homogenates

Specific binding was determined as described in the Materials and Methods by incubating 0.1 nM of [3 H]CP 55,950 or 1 nM of [3 H]DAMGO with increasing concentrations of A) morphine, B) AM-251 or C) AM-281 and 100 μ g of membranes prepared from mouse brains or CHO-hCB2 cells. The Cheng-Prusoff equation (Cheng and Prusoff, 1973) was used to convert the experimental IC_{50} values obtained from competition receptor binding experiments to K_i values, a quantitative measure of receptor affinity (presented in Table 1).

Figure 3. Modulation of basal and morphine-stimulated G-protein activity by test compounds utilizing [³⁵S]GTP γ S binding in mouse brain homogenates

A) Mouse brain membranes (10 μ g) were incubated in the presence of 0.1 nM [³⁵S]GTP γ S with increasing concentrations of morphine (filled squares), AM-251 (open triangles) or AM-281 (open squares) as described in the Materials and Methods. B) The effect of AM-251 (1 μ M) alone or in the presence of 1 μ M of the neutral MOR antagonist naloxone (NX), 1 μ M of the neutral CB1R antagonist O-2050 or both naloxone and O-2050 in combination on basal G-protein activity in mouse brain membranes was determined. The reduction in basal G-protein activity produced by AM-251 alone was not significantly attenuated by co-incubation with any drug or drug combination. Values designated with

different letters above the error bars are significantly different ($P < 0.05$, one-way ANOVA followed by a Newman-Keuls post-hoc test, mean $\pm$ SEM). C) Morphine produced a concentration-related activation of G-proteins in mouse membrane homogenates that was significantly shifted-to-the-right by co-incubation with AM-251 (10 μ M), but not AM-281 (10 μ M). Data are expressed as percent specific [35 S]GTP γ S binding normalized to the average maximal response produced by morphine and individual ED₅₀ values are discussed in the Results section.

Figure 4. Determination of the affinity of test compounds for hMORs stably expressed in CHO cells by competition receptor binding performed with membrane homogenates
Specific binding was determined as described in the Materials and Methods by incubating 1 nM of [³H]DAMGO with increasing concentrations of morphine (filled squares), AM-251 (open triangles), rimonabant (open circles) or AM-281 (open squares) and 100 μg of membranes prepared from CHO-hMOR cells. The Cheng-Prusoff equation (Cheng and Prusoff, 1973) was used to convert the experimental IC₅₀ values obtained from competition receptor binding experiments to K_i values, a quantitative measure of receptor affinity (presented in Table 1).

Figure 5. Modulation of basal G-protein activity by test compounds utilizing [³⁵S]GTP_γS binding in CHO-hMOR cell homogenates

A) CHO-hMOR membranes (10 μg) were incubated in the presence of 0.1 nM [³⁵S]GTP_γS with increasing concentrations of morphine (filled squares), AM-251 (open triangles), rimobant (open circles) or AM-281 (open squares) as described in the Materials and Methods. B) The effect of morphine (100 nM), AM-251 (1 μM), rimobant (1 μM) or AM-281 (1 μM) alone or in the presence of 1 μM of the neutral MOR antagonist naloxone (NX) on basal G-protein activity in membranes prepared from CHO-hMOR cells was determined. G-protein stimulation by morphine was significantly antagonized by naloxone; however, the reduction in basal G-protein activity produced by AM-251, rimobant or

AM-281 alone was not significantly attenuated by co-incubation with naloxone. An asterisk (*) above the histogram denotes a significant difference between the mean value of the indicate drug alone and the drug in the presence of naloxone ($P < 0.05$, Student's t-test, mean $\pm$ SEM). C) Morphine (10 μ M) and naloxone (10 μ M) alone produce no effect on basal G-protein activity, but AM-251 (10 μ M), rimonabant (10 μ M) and AM-281 (10 μ M) all produce approximately 20% inhibition of GTP γ S binding in membranes prepared from CHO-WT cells. Values designated with different letters above the error bars are significantly different ($P < 0.05$, one-way ANOVA followed by a Newman-Keuls post-hoc test, mean $\pm$ SEM).

Figure 6. Modulation of morphine-stimulated G-protein activity by test compounds utilizing [³⁵S]GTPγS binding in CHO-hMOR cell homogenates

Morphine produced a concentration-related activation of G-proteins in CHO-hMOR membrane homogenates that was significantly shifted-to-the-right by co-incubation with rimonabant (10 μM; Panel B) and AM-251 (1 and 10 μM; Panel C), but not by AM-281 (10 μM; Panel A). AM-251 competitively antagonizes G-protein activation by morphine with a Schild slope of 1.02 and a K_b value of 719 nM (Panel C; inset). Data are expressed as percent specific [³⁵S]GTPγS binding normalized to the average maximal response produced by morphine and individual ED₅₀ values are discussed in the Results section.

Figure 7. Modulation of forskolin-stimulated adenylyl cyclase activity by acute administration of test compounds in intact CHO-hMOR cells

A) Intact CHO-hMOR cells, pre-loaded with [3 H]adenine, were incubated with forskolin (10 μ M) and increasing concentrations of morphine (filled squares) as described in the Materials and Methods. B) Naloxone (1 μ M), AM-251 (10 μ M), rimonabant (10 μ M) or AM-281 (10 μ M) administered alone had no significant effect on forskolin-stimulated cAMP levels. C) Inhibition of adenylyl cyclase activity produced by 10 nM morphine was significantly attenuated by co-incubation with either the neutral MOR antagonist naloxone (1 μ M), AM-251 (10 μ M), rimonabant (10 μ M) or AM-281 (10 μ M). Values designated with

different letters above the error bars are significantly different ($P < 0.05$, one-way ANOVA followed by a Newman-Keuls post-hoc test, mean $\pm$ SEM).

Figure 8. Modulation of forskolin-stimulated adenylyl cyclase activity by test compounds following chronic morphine exposure

Following overnight exposure to 1 μ M morphine, adenylyl cyclase inhibition by morphine (100 nM) was reduced (e.g., desensitization), while levels of cAMP were elevated above baseline by naloxone (1 μ M) and AM-251 (10 μ M), but not by rimonabant (10 μ M) or AM281 (10 μ M) (e.g., cAMP rebound). Data are expressed as the percent control of forskolin-stimulated cAMP production. Values designated with different letters above the error bars are significantly different ($P < 0.05$, one-way ANOVA followed by a Newman-Keuls post-hoc test, mean $\pm$ SEM).

Figure 9. Antagonism of in vivo morphine analgesia in two different strains of mice by test compounds utilizing the tail-flick procedure

A) Morphine was administered subcutaneously (s.c.) to B6/SJL mice in doses ranging from 0.5 to 10 mg/kg to determine an ED_{50} for analgesia of 4.2 ± 0.61 mg/kg, using the tail-flick technique. Thirty-min pre-treatment of B) B6SJL or C) C57BL/6J mice by intra-peritoneal (i.p.) injection of naloxone (4 mg/kg) or AM-251 (10 mg/kg), but not AM-281 (10 mg/kg) significantly reduced analgesia produced by 5 mg/kg morphine. The test doses of naloxone, AM-251, rimobabant or AM-281 had no effect on basal tail-flick latencies when administered alone (data not shown). All data are expressed as the percent of maximum possible effect (% MPE). Values designated with different letters above the error bars are

significantly different ($P < 0.05$, one-way ANOVA followed by a Newman-Keuls post-hoc test, mean $\pm$ SEM).

Table 1
The affinity of Morphine, AM-251, AM-281 and Rimonabant for mu-opioid, CB1 and CB2 receptors

All measures of affinity are presented as K_i values, calculated by conversion of the IC_{50} values obtained from competition receptor binding, utilizing the Cheng-Prusoff equation as described in the Materials and Methods section. Data are expressed as mean $\pm$ SEM, with the number of experimental replications conducted for each condition indicated in parentheses, (N). The selectivity of each drug for mCB1Rs was calculated by dividing the affinity for the indicated receptor, by the affinity of the same ligand for the respective mCB1R.

	mCB1R		hCB2R		mMOR		hMOR	
	K_i (nM)		K_i (nM)	hCB2/mCB1	K_i (nM)	mMOR/mCB1	K_i (nM)	hMOR/mCB1
Morphine	> 10,000 (3)	-----	-----	-----	0.17 $\pm$ 0.08 (3)	-----	0.51 $\pm$ 0.15 (3)	-----
AM-251	0.49 $\pm$ 0.14 (6)	293 $\pm$ 52 (3)	598		327 $\pm$ 98 (5)	667	251 $\pm$ 69 (4)	512
AM-281	1.6 $\pm$ 0.4 (4)	684 $\pm$ 35 (3)	428		> 10,000 (3)	>6250	2135 $\pm$ 612 (3)	1334
Rimonabant	1.8 ^a	-----	-----		-----	-----	652 $\pm$ 103 (4)	362

^a K_i value obtained from [Rinaldi-Carmona, 1994 #1292].