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Significance of Cannabinoid CB₁ Receptors in Impropgan Antinociception

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Abstract

Impropgan is a congener of the H₂ antagonist cimetidine which produces potent antinociception. Because a) the mechanism of action of impropgan remains unknown and b) this drug may indirectly activate cannabinoid CB₁ receptors, the effects of the CB₁ antagonist/inverse agonist rimonabant (SR141716A) and three congeners with varying CB₁ potencies were studied on impropgan antinociception after intracerebroventricular (icv) dosing in rats. Consistent with blockade of brain CB₁ receptors, rimonabant ($K_d=0.23$ nM) and O-1691 ($K_d=0.22$ nM) inhibited impropgan antinociception by 48% and 70% after icv doses of 43 nmol and 25 nmol, respectively. However, two other derivatives with much lower CB₁ affinity (O-1876, $K_d=139$ nM and O-848, $K_d=352$ nM) unexpectedly blocked impropgan antinociception by 65% and 50% after icv doses of 300 nmol and 30 nmol, respectively. These derivatives have 600-fold to 1,500-fold lower CB₁ potencies than that of rimonabant, yet they retained impropgan antagonist activity in vivo. In vitro dose-response curves with ³⁵S-GTPγS on CB₁ receptor-containing membranes confirmed the approximate relative potency of the derivatives at the CB₁ receptor. Although antagonism of impropgan antinociception by rimonabant has previously implicated a mechanistic role for the CB₁ receptor, current findings with rimonabant congeners suggest that receptors other than or in addition to CB₁ may participate in the pain-relieving mechanisms activated by this drug. The use of congeners such as O-848, which lack relevant CB₁-blocking properties, will help to identify these cannabinoid-like, non-CB₁ mechanisms.

Keywords

cannabinoid; CB₁ receptor; antinociception; analgesia; impropgan; rimonabant

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PERSPECTIVE: This article describes new pharmacological characteristics of impropgan, a pain-relieving drug which acts by an unknown mechanism. Impropgan may use a marijuana-like (cannabinoid) pain-relieving mechanism, but it is shown presently that the principal cannabinoid receptor in the brain (CB₁) is not solely responsible for impropgan analgesia.

Introduction

Improgan, a congener of the histamine H₂ antagonist cimetidine (Fig. 1), shows promise as a novel analgesic with an unknown target in the brain¹⁰. Cimetidine and improgan both act in the brain to inhibit supraspinal and intraspinal nociceptive responses in rats, suggesting a morphine-like analgesic profile¹⁰. However, the antinociceptive mechanism is not opioid receptor-mediated and cannot be attributed to any known histamine receptor, possibly suggesting that it is new. In fact, over 100 brain receptors have been screened with improgan and all have shown low affinity^{10, 13}. Improgan also shows no activity on GTPγS binding, adenylate cyclase, inositol phosphate, or MAP kinase assays in brain preparations (unpublished results). Extensive testing, including tail-pinch, tail flick, hot plate, tail-immersion, and rotarod methods suggests that improgan produces profound analgesia. Improgan has shown antinociceptive activity after intracerebral injection into the periaqueductal gray and raphe magnus²¹, both of which participate in endogenous descending analgesic mechanisms. Repeated administration of improgan did not result in tolerance¹⁰. These findings in rats suggest that improgan produces non-opioid analgesia with fewer side effects than morphine¹⁰.

The analgesic mechanisms of cannabinoids may be the key to understanding improgan actions. Both cannabinoids and improgan activate non-opioid descending pathways leading to analgesia¹². CB₁, the principal cannabinoid target in the brain, is found in the periaqueductal gray and the rostral ventral medulla^{17,19}, both of which are sites of improgan action²¹. Improgan and CB₁ agonists, such as WIN 55, 212-2 (WIN), produce antinociception that exhibits dose-dependent inhibition by rimonabant (SR141716A), a CB₁ antagonist/inverse agonist¹². Recently, improgan antinociception was found to be reduced by chronic cannabinoid administration, also supporting an improgan-cannabinoid (possibly CB₁) interaction²². However, radioligand binding assays have confirmed that improgan, unlike WIN, does not directly bind to CB₁ or CB₂ sites¹². Although WIN can activate both of these receptors, the existence of the latter in the CNS is very limited, and CB₂-mediated analgesia occurs outside of the brain¹⁵. Spinal CB₂ receptors may contribute to antihyperalgesic actions in inflammatory pain states, but not under control conditions³⁰. These findings imply that improgan indirectly activates CB₁ along its analgesic circuit. Improgan studies in CB₁ null mice gave equivocal results which neither support nor refute the involvement of CB₁ receptors¹² (also see discussion).

Presently, the effects of several chemical congeners of rimonabant (Table 1) with varying CB₁ affinities were studied on cannabinoid and improgan antinociception. These studies are needed to further test the cannabinoid hypothesis of improgan action and to search for novel ligands relevant to the mechanism of improgan antinociception.

Methods

Animals

Male Sprague-Dawley rats (175 – 200 g) from Taconic Farms, (Germantown, NY) were maintained on a 12-hr light/ dark cycle (lights on from 7:00am to 7:00pm) and provided with food and water. Rats were housed in groups of three or four until the time of surgery and individually thereafter. All animal experiments were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

Surgery

For intracerebroventricular (icv) injections in rats, animals were anesthetized with pentobarbital sodium and supplemented with isoflurane. Cannulas were stereotactically

implanted into the left lateral ventricle and anchored to the skull with three stainless steel screws and cranioplast cement. The coordinates for the cannula (in mm from bregma) were: anterior-posterior -0.8 , medial-lateral $+1.5$, dorso-ventral -3.3 . After surgery, the animals were individually housed with food and water available and were allowed to recover for at least 5 to 7 days before testing. Each animal was only studied once.

Rat ICV Injections and Nociceptive Testing

Rats were tested with the tail flick test³. A randomly selected location 2–5 cm from the tip of the ventral surface of the tail was exposed to radiant heat and the latency for tail movement was recorded. The heat source was set so that baseline latencies are generally between 3 and 4 sec with a 15-sec cutoff. The heat source was not adjusted for individual animals. The animals were tested with three tail flick tests performed at one-min intervals, and the third test was used as the baseline score. Animals were then gently secured by wrapping with a laboratory pad, the stylet was removed, and the icv injection cannula inserted. This cannula extends 1 mm beyond the guide to penetrate the lateral ventricle. Icv injections were performed manually over a one-min period with 2 μ l of an antagonist solution or DMSO vehicle control. One min after the end of the infusion, the injection cannula was clipped approximately 2 mm above the juncture with the guide cannula. After a five min interval, a single tail flick test was performed, followed by a second icv injection of 10 μ l impropgan, WIN, or vehicle (60% DMSO). The second injection was performed by removing the first clipped injection cannula, and repeating the process above. The second cannula was clipped as before and single tail flick latencies were recorded five, ten, and thirty min later. Successful icv injections were assured by following the movement of an air bubble in the tubing between the syringe and the cannula and by the absence of leakage. After testing, animals received pentobarbital sodium (100 mg/kg, i.p.) and India Ink (5 μ l, icv). Proper distribution of the ink in the cerebroventricular system indicated successful icv injections. Data from animals with poor placements or unsuccessful injections were excluded.

Drugs and Solutions

Small amounts of rimonabant and three of its chemical congeners (Table 1) were available from a previous study³⁴. Additional amounts of O-848 were synthesized as given below. Additional rimonabant was provided by the National Institute on Drug Abuse Drug Supply Program. Impropgan base (kindly provided by Prof. R. Leurs, Vrije University, Amsterdam¹¹) and WIN (dosed as mesylate salt; RBI/Sigma, Natick, MA) were dissolved in 60% DMSO and 40% saline and all antagonists were dissolved in 100% DMSO. This icv vehicle has been widely used for cannabinoids and antagonists and has no adverse effects on motor behavior, nociceptive responses, or the ability to detect antagonism of antinociception¹⁴. In all cases, both the identity (experimental group) of the subject, and the identity of the solutions injected were blinded to the experimenters.

Synthesis of O-848

Although O-848 has been described in the literature³⁴, synthesis of this compound has not been previously published (Fig. 2). Ester **1** was prepared following a literature procedure¹⁶. This compound was then reduced to alcohol **2** using LiBH_4 in THF according to another literature procedure²⁷. A mixture of alcohol **2** (93 mg, 0.25 mmol) and PBr_3 (4 mL) was stirred for 24 h. The reaction mixture was poured onto crushed ice and extracted with CH_2Cl_2 . The organic layer was washed with water and brine, dried over sodium sulfate and concentrated to give intermediate **3**, which was dissolved in 5 mL of DMF and added to a mixture of NaH (61 mg, 2.5 mmol), 1-(2-hydroxyethyl) piperidine (49 mg, 0.38 mmol) and DMF (5 mL). The resulting mixture was stirred for 24 h. The reaction was poured onto crushed ice and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium

sulfate and concentrated to give a brown oil, which was purified by combiflash ($\text{CH}_2\text{Cl}_2:\text{MeOH}:\text{NH}_4\text{OH}$ 40:1:0.1 to 10:1:0.1) to give final product **4** (O-848, 90 mg, 74% from **2**): (CDCl_3) δ 7.39 (m, 1H), 7.28 (d, 2H, $J = 8.6$ Hz), 7.26 (m, 2H), 7.05 (d, 2H, $J = 8.6$ Hz), 4.63 (s, 2H), 3.67 (t, 2H, $J = 6.1$ Hz), 2.60 (t, 2H, $J = 6.1$ Hz), 2.42 (m, 4H), 2.14 (s, 3H), 1.58 (m, 4H), 1.42 (m, 2H); MS (ESI, MH^+).

Analysis of Antinociceptive Data

Results are expressed as latencies (sec, mean $\pm$ SEM). Analysis of variance (between groups: drug treatment, within groups [repeated measures]: time) yielded highly significant ($p < 0.001$) drug by time interactions from all nociception studies performed (Fig. 3-6). Bonferroni post-hoc tests were performed to determine significant differences between groups (Graphpad Prism 4.0, San Diego, CA).

CB₁ receptor-stimulated [³⁵S]GTP γ S binding assay

Membrane fractions from a CB₁-HEK 293 stable cell line were used to assess the activity at CB₁ receptors in a manner similar to previously described studies^{1,18}. Cells were suspended in phosphate buffered saline containing 1 mM EDTA and centrifuged at $500 \times g$ for 5 min. The pellet was homogenized in homogenate buffer (50 mM Tris-HCl, 1 mM EDTA, 3 mM MgCl_2 , pH 7.4) and centrifuged ($42,000 \times g$, 15 min, 4°C). The resulting pellet was resuspended in homogenate buffer and aliquots stored at -80°C . On the day of assay, aliquots were thawed on ice, centrifuged, and the pellet resuspended in assay buffer (50 mM Tris-HCl, 100 mM NaCl, 3 mM MgCl_2 , 0.2 mM EGTA, 0.1% bovine serum albumin, pH 7.4). Briefly, binding was initiated with addition of 20 μg of membrane protein into glass tubes containing 0.1 nM [³⁵S]GTP γ S (Perkin Elmer/NEN, Boston, MA) and 10 μM GDP in binding buffer. Nonspecific binding was assessed in the presence of 20 μM unlabeled GTP γ S. Samples were incubated for 90 min at 30°C with various concentrations of WIN, along with candidate antagonists in a total volume of 500 μl , followed by rapid filtration through Whatman GF/B filters. Filters were mixed with scintillation cocktail, remained at room temperature for 3 hr, and were counted in a Beckman scintillation counter.

Results

As observed previously, icv improgan (388 nmol, 80 μg) produced a large increase in tail flick latency in rats at 5 and 10 min after injection, which returned to baseline 30 min later. This dose was chosen to produce near-maximal, but not supramaximal responses, based on recent dose-response studies¹⁴. The CB₁ antagonist/inverse agonist rimonabant produced dose-dependent inhibition of improgan antinociception (Fig. 3A). The 43 nmol dose of rimonabant partially blocked, while the 108 nmol dose completely blocked improgan antinociception. The CB₁ agonist WIN showed activity identical to that of improgan when injected alone. This effect was also blocked by the 43 nmol and the 108 nmol pretreatment with rimonabant (Fig. 3B). Neither dose of rimonabant had antinociceptive properties when injected alone.

O-1691, another potent CB₁ antagonist, also inhibited improgan antinociception (Fig. 4A). Two doses of this drug (10 and 25 nmol) produced dose-related inhibition of antinociception. A higher dose (50 nmol) did not produce complete antagonism. A 25 nmol dose of O-1691 completely blocked WIN antinociception (Fig. 4B). This dose of O-1691 did not show antinociceptive properties when injected alone.

O-1876, a congener with 100-fold lower CB₁ potency than rimonabant, also reduced improgan antinociception (Fig. 5A). The pattern of inhibition with this drug was remarkably similar to that seen after O-1691, except in this case maximal (but incomplete) antagonism was observed after 300 nmol of O-1876. This antagonist treatment also blocked WIN activity with similar

potency (Fig. 5B). A 300 nmol dose of O-1876 did not show antinociceptive properties when injected alone.

When given alone, O-848 tended to show a dose-related increase in tail flick latencies 5 and 10 min after injection (Fig. 6). However, repeated measures ANOVA of data from O-848-treated subjects in the absence of impropgan or WIN failed to substantiate a statistical significance to this trend (O-848 treatment: $P=0.11$, time: $P<0.01$, interaction: $P=0.53$). When combined with impropgan, O-848 reduced impropgan antinociception at 5 and 10 min after injection, but a slight enhancement was observed in the 30 min group (left panels, Fig. 6). ANOVA of data from impropgan-treated subjects showed that O-848 significantly modified tail flick latencies (O-848 treatment: $P=0.38$, time: $P<0.001$, interaction: $P<0.01$). Since this significant interaction term could be due to either antagonism of impropgan at 5 or 10 min, or due to enhancement at 30 min, a separate ANOVA was performed without the 30 min data (O-848 treatment: $P=0.21$, time: $P<0.001$, interaction: $P=0.021$). This second analysis confirms that O-848 inhibits impropgan antinociception in either the 5 or 10 min groups (or both). Post-hoc analysis did not identify which groups show this inhibition. The magnitude of the inhibition (50%) was maximal in the 10 min data after 30 nmol of O-848. Latencies measured in these groups closely matched the effects of O-848 alone (especially at the higher doses), possibly consistent with a partial agonist profile.

In contrast to the effects of O-848 on impropgan actions, this drug did not significantly inhibit WIN antinociception (Fig. 6, right panels). Because 20 μg (38.3 nmol) of WIN did not achieve maximal antinociception in this experiment, the effects of a larger dose (30 μg , 57.5 nmol) were also studied. When O-848 was combined with either dose of WIN, a tendency toward enhancement (not inhibition) of latencies was observed. ANOVA of WIN (20 μg)-treated subjects found significant main effects of O-848 ($P<0.05$), time ($P<0.001$) but no significant interaction ($P=0.13$). A similar analysis of the WIN (30 μg) treatment groups did not find significant changes.

^{35}S -GTP γS -binding studies were used to quantify CB₁-mediated responses and assess CB₁ antagonist potencies (Fig. 7). The CB₁ agonist WIN produced concentration-dependent increases in specific binding, which were competitively blocked by rimonabant and its congeners. All compounds produced concentration-dependent shifts in the WIN concentration-response curves, although antagonist concentrations needed to block the responses varied considerably. Thus, low concentrations (10–100 nM) of rimonabant and O-1691 were highly effective, yet larger concentrations of O-1876 (30–300 nM) produced only slight changes. Even larger concentrations of O-848 (1,000 – 3,000 nM) were needed to alter WIN responses. O-848 was also a very weak CB₁ antagonist in membranes prepared from rat brain. Non-linear regression of all curves produced agonist dose ratios for each antagonist treatment, permitting Schild analyses, and estimates of CB₁ antagonist K_d values (Fig. 7 and Table 2).

Discussion

Although impropgan antinociception is reduced by WIN-blocking doses of rimonabant¹² (confirmed in Fig. 3), radioligand experiments showed that impropgan lacks affinity for CB₁ and CB₂ receptors from various biological sources assessed with several different radioligands¹². In addition, impropgan has virtually no effect on GTP γS activation in either the presence or absence of WIN in CB₁-containing membranes¹⁴. Taken together, these studies show that CB₁ is not a direct target for impropgan. If CB₁ receptors participate in impropgan action, then an indirect mechanism must be involved. Impropgan may act through an endocannabinoid mechanism, but this has not been explored. The compound has no effect on fatty acid amide hydrolase, an enzyme of endocannabinoid metabolism (unpublished).

Studies with impropen in CB₁ knockout mice gave complex results. If the CB₁ receptor mediates impropen effects, then impropen-induced antinociception should not have been observed in CB₁ (−/−) animals. Surprisingly, impropen antinociception *was* observed in CB₁ (−/−) mice¹². One possible explanation for these data may be that rimonabant is blocking impropen at a receptor other than CB₁. If so, then rimonabant should still have prevented impropen antinociception in CB₁ (−/−) mice. However, impropen antinociception in CB₁ (−/−) mice was not blocked by rimonabant, even though the same treatment was effective in wild-type control mice¹². Thus, results with CB₁ (−/−) mice failed to substantiate the importance of CB₁ mechanisms in impropen antinociception. However, these results also do not disprove the hypothesis. Thus these findings provide neither positive nor negative evidence on the role of CB₁ mechanisms. Several studies suggest the up-regulation of a non-CB₁, non-CB₂ cannabinoid receptors in the CNS of germ line CB₁ (−/−) mice (e.g. see²⁰). Thus, impropen antinociception may utilize the CB₁ receptor in normal mice, but the germ line knockout animal may acquire alternate circuits during development⁶. Clearly, additional studies were needed in order to learn the role of CB₁ in impropen antinociception.

Rimonabant is the first potent and selective CB₁ antagonist which is active in vivo²⁹. However, rimonabant can act at non-CB₁ sites as well^{8,24,26}. Thus, blockade of impropen by rimonabant (even at pharmacologically relevant doses) does not absolutely prove a CB₁ mechanism. Therefore, the use of rimonabant congeners with varying potencies toward the CB₁ receptor was designed to provide a powerful test of the CB₁ cannabinoid hypothesis of impropen antinociception. If rimonabant acts at the CB₁ receptor to block impropen antinociception, then various congeners of rimonabant should block impropen antinociception at doses commensurate with their CB₁ potencies. Accordingly, drugs like O-1876 and O-848 should not have been blockers of impropen antinociception in vivo. If, on the other hand, rimonabant acts at a non-CB₁ receptor to block impropen antinociception, there should have been no correlation between the potencies of congeners toward the CB₁ receptor and the doses of these drugs needed to block impropen.

Previously several congeners of rimonabant were synthesized to determine structure-activity relationships at the CB₁ receptor³⁴. This study concluded that N(1)- and C(5) substituents in rimonabant which retain the central pyrazole structure (Fig. 1) are primarily involved in receptor antagonism, whereas replacements at the C(3) position (R4 in Table 1) can contribute to CB₁ receptor recognition and agonism³⁴. Of the many congeners made and tested, O-1691, O-1876, and O-848 were chosen for the present studies because of their close chemical similarities to rimonabant, and their widely divergent CB₁ receptor affinities (Table 1).

Because O-1876 and O-848 were reported to be more than 100-fold less potent than rimonabant on CB₁ receptors (Table 1), the in vivo inhibition of impropen action by these drugs was unexpected (Figs. 5, 6). Since only a limited amount of data on these compounds was available³⁴, it seemed important to re-examine the CB₁ antagonist affinity of these drugs. In addition, significant discrepancies in CB₁ K_i values for compounds related to rimonabant have been noted, depending on the radioligand used^{28,32}. For the present work, it seemed that the most unambiguous determination of CB₁ potencies would be measurement of antagonism of cannabinoid responses in vitro (Fig. 7). The use of the same cannabinoid agonist (WIN) both in vivo and in vitro was thought to be an added strength of the experimental design. The K_d values of the four compounds obtained by this method (Fig. 7 & Table 2) confirm the earlier conclusions³⁴ (Table 1) that rimonabant and O-1691 are both high potency CB₁ antagonists, whereas O-1876 and O-848 are several hundred-fold lower in potency. However, the CB₁ antagonist K_d values for all of the drugs (Table 2) were considerably lower (4–30 fold) than the previously-reported corresponding K_i values³⁴ (Table 1). Notwithstanding these differences, both studies show that O-1876 and O-848 are 106-fold (Table 1) to 604-fold (Table

2) and 395-fold (Table 1) to 1,530-fold (Table 2) less potent, respectively, than rimonabant. Literature K_i or K_d values for rimonabant are 0.16 – 0.33 nM^{1,4,7}.

In this study, two compounds with high CB₁ potency (rimonabant and O-1691) blocked both impropgan and WIN activity, thus yielding the hypothesized results (Figs 3-4, Table 2). The findings show that comparable doses of these two compounds have similar actions against both impropgan and WIN antinociception, consistent with the CB₁ hypothesis of impropgan analgesia. Both present (Fig. 4) and previous³⁴ data confirm that O-1691, which does not increase tail flick latency when injected alone, is a high potency CB₁ antagonist that blocks cannabinoid antinociception.

Results with the remaining two compounds (O-1876 and O-848) are not consistent with the CB₁ hypothesis. Both of these compounds have a very low CB₁ affinity (Table 2) and they blocked impropgan antinociception at doses not consistent with their ability to act on CB₁. Table 2 shows that 604 times the active dose of rimonabant would be needed in order for O-1876 to produce comparable antagonism of impropgan. However, a dose of O-1876 that is 6 times the dose of rimonabant was active. This discrepancy is even larger with O-848. A dose of O-848 that is 1,530 times the active dose of rimonabant should be needed to block impropgan (Table 2). Surprisingly, nearly identical doses of O-848 and rimonabant reduced impropgan antinociception by about 50% (Table 2). If rimonabant and O-848 block impropgan antinociception by the same mechanism, this finding strongly suggests that the CB₁ receptor does not mediate this response.

A number of possible non-CB₁ sites of rimonabant action need to be considered when searching for the impropgan mechanism. For example, rimonabant has activity in hippocampal slices of the CB₁ –/– mouse brain⁹. This drug also inhibits anandamide-induced vasodilation in rat coronary and mesenteric arteries^{5,33,35}. However, in these and other studies²⁴, rimonabant's non-CB₁ activity occurs at concentrations ranging from 0.5 to 10 μ M, which is 1,000-fold larger than the concentrations needed to block CB₁ (0.5 – 2 nM). The potency of rimonabant to block impropgan activity is similar to its potency to block WIN antinociception (Fig. 3). Assuming that WIN antinociception is mediated through the CB₁ receptor²³, this argues that rimonabant must be acting in the nanomolar range to block both WIN-induced and impropgan-induced antinociception (Fig. 7). Thus, targets for rimonabant acting in the micromolar range are not likely to be relevant to the antagonism of impropgan.

One non-CB₁ target of potential further interest for both WIN and impropgan antinociception may be the TRPV1 receptor. Although best studied in the spinal cord, this site exists in brain, may have relevance for analgesia³¹, and may be a target for rimonabant at higher concentrations⁸. However, binding studies found that impropgan (10 μ M) has no affinity at these sites (unpublished).

Although many of the above studies show non-CB₁ targets for rimonabant in the micromolar range, one report found evidence that rimonabant acts at a non-CB₁, non-CB₂ target in mouse vas deferens at a concentration in the nanomolar range²⁴. Rimnabant's K_B for antagonism at this novel receptor was reported to be 15.4 nM. In contrast, the K_B of rimnabant for antagonism of WIN in the same assay (which is CB₁-mediated) was 2.4 nM²⁵, suggesting. T that rimnabant has a non-CB₁ target in the relevant concentration range. It is possible that the impropgan-blocking effect of rimnabant is due to its ability to block this novel receptor, and not its ability to block CB₁. This could explain why O-1876 and O-848, two compounds with virtually no CB₁ activity, are blockers of impropgan antinociception.

Antagonism of impropgan antinociception by rimnabant initially suggested a role for the CB₁ receptor in the impropgan analgesic circuit. However, the present data with rimnabant congeners argue against this hypothesis, suggesting the importance of non-CB₁ receptors. It

is possible that impropen may have both a CB₁ and non-CB₁ mediated component. In this scenario, drugs that could exclusively block either CB₁ or a non-CB₁ target would be able to partially block impropen antinociception. Only drugs with the ability to block both targets would be able to completely block impropen activity. The present findings are consistent with this prediction. O-1691, O-1876, and O-848 were able to partially block impropen antinociception at the maximum dose tested (Fig. 4-6), fitting the profile for a drug that blocks only one of the two targets. The action of O-1691 is most likely due to its ability to block CB₁, because this drug has a high affinity towards this receptor. In contrast, O-1876 and O-848 most likely act on a non-CB₁ target because both of these drugs have a very low affinity for the CB₁ receptor. The complete inhibition of impropen activity by rimonabant (Fig. 3) could be explained by blockade of both CB₁ and non-CB₁ targets. A previous study has also suggested a dual CB₁ and non-CB₁ mechanism of action for a novel compound with cannabinoid properties²⁴.

In the present study, the CB₁ agonist WIN was used as a control to assess the ability of rimonabant and its congeners to block CB₁ mediated antinociception. It was expected that drugs with a high CB₁ potency (rimonabant and O-1691) would block WIN antinociception, and drugs with a low CB₁ potency (O-1876 and O-848) would not. Three of the drugs (rimonabant, O-1691, and O-848) gave the predicted results. However, the unexpected antagonism of WIN antinociception by O-1876 (Fig. 5B) is not consistent with the CB₁ receptor's role in mediating WIN responses. Even though WIN clearly acts at CB₁ receptors, non-CB₁ actions of this drug have also been reported^{2,9,20,26}. Because some of the reported effects of WIN may be blocked by rimonabant, but not by all CB₁ antagonists⁸, further studies are required to delineate this potential non-CB₁ mechanism for WIN antinociception. O-848 will be a useful tool in searching for this mechanism and in the further characterization of impropen antinociception.

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Impropgan

Rimonabant

Figure 1.
Chemical structures of impropgan and rimonabant.

Figure 2.
Chemical synthesis of O-848.

Figure 3.

Effects of rimonabant (SR) on improgan (Imp, A) or WIN 55,212-2 (WIN, B) antinociception in rats. Animals were tested (ordinate, mean $\pm$ S.E.M. tail-flick latencies for the number of subjects in parentheses) for baseline responses (BL) then received an icv injection of 100% DMSO or rimonabant (doses in brackets are in nmol). Latencies were reassessed 5 min later (Post), followed by a second injection of 60% DMSO, improgan (388 nmol, 80 μ g), or WIN (38.3 nmol, 20 μ g). Latencies were then determined 5, 10, and 30 min after the second injection (abscissa). The same DMSO/DMSO and rimonabant [108]/DMSO groups are shown in Fig. 3A and 3B. Data were combined with some results which were previously published¹². +, ++ $p < 0.05$, < 0.01 vs. DMSO/DMSO group at the same time point, respectively. *, ** $P < 0.05$, $p < 0.01$ vs. DMSO/Imp group at the same time point, respectively. #, ## $P < 0.05$, $P < 0.01$ vs. DMSO/WIN group at the same time point, respectively.

Figure 4.

Effects of O-1691 on improgan (A) or WIN 55,212-2 (B) antinociception in rats. Subjects were treated and tested as in Fig. 3, except O-1691 was injected instead of rimonabant. The same DMSO/DMSO, DMSO/Imp, and DMSO/WIN data are shown in Fig. 3. Footnotes indicating statistical significances are the same as in Fig. 3.

Figure 5.

Effects of O-1876 on improgan (A) or WIN 55,212-2 (B) antinociception in rats. Subjects were treated and tested as in Fig. 3, except O-1876 was injected instead of rimonabant. The same DMSO/DMSO, DMSO/Imp, and DMSO/WIN data are shown in Fig. 3. Footnotes indicating statistical significances are the same as in Fig. 3.

Fig. 6.

Effects of O-848 on impropen and WIN 55,212–2 antinociception in rats. Subjects were treated and tested as in Fig. 3, except that several doses of O-848 were given instead of rimonabant, and multiple doses of WIN were studied. Nociceptive testing (ordinate, tail-flick latencies in sec, mean $\pm$ S.E.M., number of subjects given in parentheses) was performed 5 (top), 10 (middle) and 30 (bottom) min after injections. Treatment groups consisted of impropen (388 nmol, 80 μ g, left panels), WIN (20 μ g [38.3 nmol] or 30 μ g [57.5 nmol] as labeled, right panels), or DMSO vehicles (Veh, all panels), along with the specified dose of O-848 (abscissa, nmol, all panels). The “vehicle” group (Veh) in the O-848/DMSO curve are pooled baseline latencies from 30, 100 and 300 nmol-treated groups. This does not affect the statistical analyses, which included baseline values from all groups. ** $P < 0.01$ for O-848 by time interaction term among impropen-treated subjects by ANOVA.

Fig. 7.

Effects of CB₁ antagonists on CB₁-mediated activation of GTP γ S synthesis. Membranes from CB₁-HEK 293 cells or rat brain were prepared and incubated as described in the presence of ³⁵S-GTP γ S, antagonists, and increasing concentrations of the CB₁ agonist WIN55,212 (abscissa). Specific binding of GTP γ S (ordinate, fmol/mg protein, or %baseline, mean $\pm$ S.E.M.) is shown. Each point is the mean $\pm$ S.E.M. of 3 separate experiments each performed in triplicate. Five panels show the effects of varying concentrations of each antagonist on agonist concentration-response curves. Non-linear regression was used to estimate the apparent EC₅₀ of each curve. Lower right: Schild plots are shown for each of the five antagonist experiments. For each plot, the log of antagonist concentration (abscissa) is plotted vs. log of dose ratio (DR) minus one (ordinate). The dose ratio was calculated as the ratio of the estimated agonist ED₅₀ in the presence of antagonist divided by the agonist ED₅₀ in the absence of

antagonist. For each antagonist experiment, the slope of the Schild curve (S , expected to be 1.0) and the regression K_d (antilog of x-intercept) are given.

Table 1

Structures and CB₁ activity of selected congeners of rimonabant.

Drug	CB ₁ K _i (nM)	R1	R2	R3	R4
Rimonabant	6.2	2,4-dichloro	4-chloro	methyl
O-1691	1.5	2,4-dichloro	4-(n-penyl)	bromo
O-1876	657	2,4-dichloro	4-chloro	methyl
O-848	2450	2,4-dichloro	4-chloro	methyl

Literature K_i values are shown³⁴ for drugs synthesized and tested presently.

Table 2

Anti-Imprugan activity vs. CB₁ activity.

Drug	Active Dose (icv, nmol)	% Inhibition of Imp	Rel. Active Dose (SR)	CB ₁ K _d (nM)	Rel. CB ₁ K _d (SR)
Rimonabant	43	48	1.0	0.23	1.00
O-1691	25	70	0.6	0.22	0.96
O-1876	300	65	7.0	139	604
O-848	30	50	0.7	352	1,530

For each drug, the dose and degree of inhibition of imprugan antinociception is given. Relative active dose refers to the quotient of the active dose of the specified drug divided by the active dose of rimonabant (43 nmol). CB₁ K_d values were calculated from Schild plots (Fig. 7F). Relative CB₁ K_d refers to the quotient of the calculated CB₁ K_d of the drug divided by the calculated CB₁ K_d of rimonabant (0.23 nM). All percent inhibition values were calculated using 10 minute post imprugan injection data.