BML-190 and AM251 act as inverse agonists at the human cannabinoid CB_2 receptor: signalling via cAMP and inositol phosphates

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Abstract The aminoalkylindole BML-190 and diarylpyrazole AM251 ligands have previously been shown to bind to cannabinoid CB_2 and CB_1 receptors, respectively. In HEK-293 cells stably expressing the human CB_2 receptor, BML-190 and AM251 potentiated the forskolin-stimulated accumulation of cAMP. Moreover, the CB_2 receptor can interact productively with 16z44, a promiscuous $G\alpha_{16/z}$ chimera. BML-190 and AM251 reduce the basal levels of inositol phosphate production in cells expressing the CB_2 receptor and 16z44. These results demonstrate that BML-190 and AM251 act as inverse agonists at the human CB_2 receptor acting via $G\alpha_{i/o}$ and $G\alpha_q$ family-coupled pathways.

Key words: BML-190; AM251; Cannabinoid; CB_2; Inverse agonist

1. Introduction

The pharmacological targets of psychoactive cannabinoids and endogenous eicosanoids are the cannabinoid receptors. Two cannabinoid receptor subtypes have been identified and are termed CB_1 and CB_2 (for a review see [1]). The two subtypes share approximately 48% homology and utilize $G_{i/o}$ heterotrimeric G proteins to inhibit the activity of adenylyl cyclase and stimulate mitogen-activated protein kinases. CB_1 receptors also regulate the activity of potassium and calcium channels [2]. CB_1 receptors are widely distributed in the central and peripheral nervous system as well as in peripheral tissues. In contrast, CB_2 receptors are primarily expressed in immune cells and in numerous other tissues including the spleen, pancreas, thymus and tonsils [1].

Cannabinoids have been implicated in many physiological processes including the control of pain perception, motor function, memory, suppression of humoral immune responses, stimulation of lymphocyte proliferation as well as providing relief from the symptoms of multiple sclerosis and the side effects of chemotherapy [1]. Furthermore, cannabinoid agonists modulate cytokine expression and promote tumor growth [3]. CB_2 receptor agonists have also been demonstrated to induce apoptosis in tumors of immune origin [4]. This wide spectrum of effects has led to an intensive search for ligands that are able to selectively modulate the binding of endogenous cannabinoids at each receptor subtype and regulate their corresponding intracellular signalling pathways.

The library of ligands that are selective for either CB_1 or CB_2 receptors is growing rapidly [5]. Often the data accompanying the descriptions of the ligands' synthesis only detail the receptor subtype selectivity. One such ligand is indomethacin morpholinylamide, also known as BML-190 [6]. BML-190 was reported as having a K_i value for CB_2 receptors of 435 nM with 50-fold selectivity over CB_1 receptors. A subsequent report used BML-190 as an agonist in a study of the effect of cannabinoids on the proliferation of cancer cells [7]. However, no supporting data were provided to demonstrate the agonism of BML-190. AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,3-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) was produced as a derivative of the CB_1 receptor antagonist/inverse agonist SR141716A [8]. As AM251 has a high affinity for CB_1 receptors it has come to be widely used as a CB_1-selective antagonist in numerous pharmacological and functional studies [9,10] even though its efficacy at CB_2 receptors has not been clearly established.

With the increasing recognition that inverse agonists are useful therapeutic tools in the management of pathophysiological states [11–13] it is particularly important to distinguish between antagonists and inverse agonists in order that the effects of different classes of ligand can be thoroughly evaluated. The purpose of the present study was to establish the effect of BML-190 and AM251 on the $G_{i/o}$-mediated inhibition of cAMP accumulation in a human embryonic kidney 293 (HEK-293) cell line stably expressing the human CB_2 receptor (293/CB_2). In addition, following demonstration that the CB_2 receptor is able to functionally interact with a chimeric $G\alpha_{16/z}$ subunit, 16z44 [14], we showed that the pattern of inositol phosphate (IP) production elicited by the tested ligands mirrored their pattern of inhibition of cAMP accumulation, and supports the notion that they act as inverse agonists at the CB_2 receptor.

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Abbreviations: AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,3-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM630, 6-iodopravadoline; BML-190, indomethacin morpholinylamide; FBS, fetal bovine serum; HEK, human embryonic kidney; IP, inositol phosphates; JWH 015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; MEM, minimum essential medium; PLC, phospholipase Cβ; WIN 55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

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2. Materials and methods

2.1. Materials

The cDNA encoding the human CB_2 receptor was provided by Dr. David Shire (Sanofi Recherche, Labège, France). HEK-293 cells were obtained from the American Type Culture Collection (ATCC CRL-1573; Rockville, MD, USA). Ligands were purchased from Sigma-RBI (St. Louis, MO, USA) and Tocris Cookson (Bristol, UK). [^{3}H]Adenine and *myo*-[^{3}H]inositol were purchased from Amersham Biosciences (Bucks, UK). Cell culture reagents, Lipofectamine™ and Plus™ reagent were from Invitrogen Life Technologies (Carlsbad, CA, USA). Scintillation fluid was from EG&G Wallac (Finland). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2. Generation of stable cell lines

HEK-293 cells were seeded into 25 cm^2 culture flasks at a density of 3×10^5 cells per flask. The following day, 2 h before transfection, fresh minimal essential medium (MEM) with 10% (v/v) fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin was added to the cells. 10 μg of receptor cDNA was introduced into the cells using the calcium phosphate precipitation method [15]. Transfected cells were cultured for 16 h at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. Cells were washed with phosphate-buffered saline and cultured in normal growth medium for a further 24 h before selection by the addition of 400 μg/ml G418. Following the death of all mock-transfected cells grown in the same selection medium, transfected cells were re-plated at a low density and individual colonies were isolated and maintained in growth medium containing 150 μg/ml G418.

2.3. cAMP and IP accumulation assays

293/CB_2 cells were labeled with [^{3}H]adenine (1 μCi/ml) in MEM with 1% FBS for 20–24 h. Labelled cells were challenged with 50 μM forskolin and appropriate drugs for 30 min at 37°C and assayed for cAMP accumulation as described previously [16]. For IP assays, 2×10^5 293/CB_2 cells were transiently transfected with 16z44 and/or pcDNA3 using Lipofectamine™ and Plus™ reagents according to the manufacturer's instructions. Cells were labelled, challenged with drugs and assayed for IP production as previously described [14]. Triplicates were performed for each data point and at least three separate trials were done for each ligand. Data were analyzed by non-linear least-squares regression using the computer-fitting program Prism 3.02 (GraphPad Software, San Diego, CA, USA).

3. Results

We generated a HEK-293 cell line stably expressing the human CB_2 receptor (293/CB_2). Following the selection process using G418, resistant cells were replated at a low density and 12 individual clones were isolated. To identify clonal cell lines expressing the receptor, intracellular cAMP accumulation was determined following challenge with the ligands JWH 015 ((2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone) and BML-190. In each of the 12 clonal cell lines, JWH 015 elicited a reduction in the forskolin-stimulated cAMP accumulation, however cAMP levels were potentiated when challenged with 10 μM BML-190. This effect was not observed in wild-type HEK-293 cells. The level of forskolin-stimulated cAMP accumulation varied between clones, as did the degree of potentiation by BML-190. BML-190 stimulation of several of the clones increased forskolin-stimulated cAMP accumulation approximately two-fold. One of these clones was chosen for further investigation.

To characterize the response of 293/CB_2 cells to JWH 015, BML-190 and other cannabinoid ligands, a series of dose–response curves were constructed (Fig. 1). Agonists JWH 015 and WIN 55,212-2 (*R*(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate) caused reductions of 44 ± 7.5% and 37 ± 5% in the forskolin-stimulated accumulation of cAMP with EC_{50} values of 4.5 ± 4.2 nM and 1.2 ± 0.5 nM ($n=3$), respectively. BML-190 caused an increase in the forskolin-stimulated cAMP levels of 103 ± 17% with an EC_{50} value of 980 ± 70 nM ($n=3$). To compare the properties of BML-190 with a known cannabinoid inverse agonist, dose–response curves were constructed using 6-iodopravadoline (AM630). This ligand increased the level of cAMP accumulation by 127 ± 7% with an EC_{50} value of 90.3 ± 19.6 nM ($n=3$). AM251 also behaved as an inverse agonist in our system, potentiating the forskolin response by 99 ± 7.5% with an EC_{50} value of 650 ± 30 nM ($n=3$). The CB_1-selective agonist, ACEA, at a concentration of 10 μM had no effect on the cAMP accumulation in 293/CB_2 cells (data not shown).

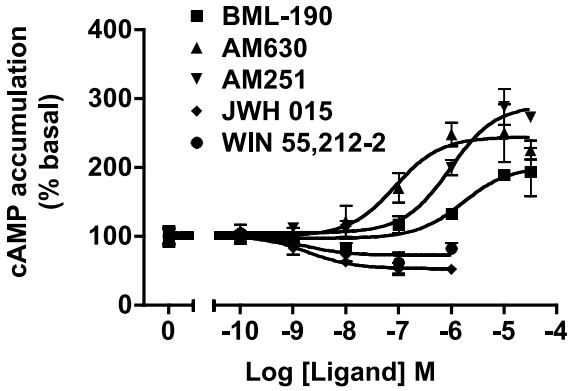

Fig. 1. Effects of cannabinoid receptor ligands on the forskolin-induced cAMP accumulation in 293/CB_2 cells. 293/CB_2 cells were labeled with 1 μCi/ml [^{3}H]adenine for 20–24 h before the assay. cAMP accumulation was assayed following 50 μM forskolin and drug treatments as indicated for 30 min. Data points are mean ± S.D. values of triplicate determinations in a single experiment.

To further confirm the characteristics of these ligands we tested their ability to activate intracellular IP production via $G_{q/11}$ family member α-subunits. None of the five ligands were able to alter basal IP levels in 293/CB_2 cells, suggesting that the CB_2 receptor is unable to couple to endogenously expressed $G\alpha_{q/11}$ subunits (data not shown). Similarly, no response was observed in 293/CB_2 cells transiently transfected with the $G\alpha_{16}$ subunit. However, 293/CB_2 cells transiently transfected with the cDNA encoding a promiscuous $G\alpha_{16/z}$ chimera, 16z44 (0.5 μg DNA/well) [14], showed increased levels of IP accumulation in the absence of CB_2 ligands (approximately 17-fold higher than basal responses in the same cell line transfected with pcDNA3). Following challenge with 10 μM WIN 55,212-2 or JWH 015, the IP levels increased by 28 ± 4% and 43 ± 13% ($n=3$), respectively. In contrast, 10 μM of BML-190, AM630 and AM251 decreased IP accumulation by 38 ± 9%, 62 ± 9% and 73 ± 4% ($n=3$), respectively. These ligands had no effect on the basal IP levels in 293/CB_2 cells transfected with pcDNA3 (data not shown).

Given the high degree of basal activity in 293/CB_2 cells transiently expressing 16z44, the effect of cDNA amounts used in transfections was examined for subsequent basal activity as well as cell responsiveness to the agonist JWH-015 and inverse agonist AM251 (Fig. 2). As the amount of cDNA increased from 62.5 ng/well to 1 μg/well, the basal activity increased approximately 12-fold. At cDNA concentrations of 0.25–0.5 μg/well an increase in the basal level IP accumu-

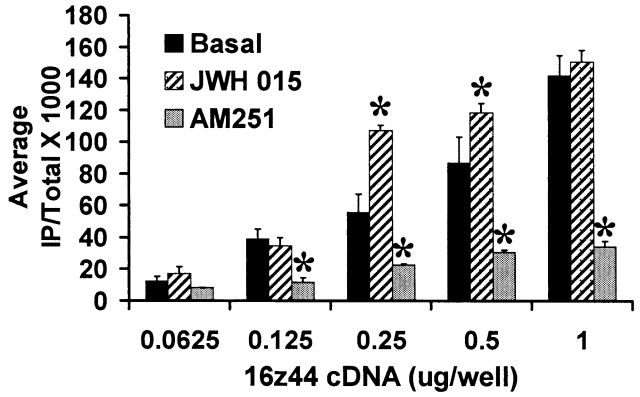

Fig. 2. Effects of cannabinoid receptor ligands on IP levels in 293/CB_2 cells transiently transfected with varying amounts of 16z44. 293/CB_2 cells were transiently transfected with 1 μg of total cDNA (varying amounts of 16z44 and balanced with pcDNA3). The following day cells were labelled with *myo*-[^{3}H]inositol. Forty-eight hours following transfection, cells were challenged with 10 μM ligands and assayed for IP accumulation. IP accumulation is presented as the ratio of [^{3}H]IPs to total cellular [^{3}H]inositol-containing compounds multiplied by 1000. Data points are mean ± S.D. values of triplicate determinations in a single experiment. *Significantly different from the basal level; $P < 0.05$ by unpaired Student's *t*-test.

lation was measured upon challenge with 10 μM JWH 015. However, it is clear that at the highest concentration of cDNA tested the agonist was unable to promote any further increase in IP accumulation over basal levels (Fig. 2). In contrast, AM251 was able to reduce the IP accumulation with increasing efficacy (approximately three-fold reduction of basal at 0.125 μg/well of DNA to four-fold reduction at 1 μg/well) as the amount of cDNA rose (Fig. 2). Dose–response curves were constructed for JWH 015, AM251 and BML-190 to determine the potencies of these ligands acting through the 16z44-coupled pathway. The EC_{50} values were determined to be 10.3 ± 1.2 nM, 261 ± 37 nM and 494 ± 207 nM ($n = 3$), respectively (Fig. 3).

4. Discussion

Our results show that both BML-190 and AM251 behave as inverse agonists at the human cannabinoid CB_2 receptor. This was demonstrated by their ability to dose-dependently increase the forskolin-stimulated levels of cAMP in HEK-293 cells stably expressing the receptor (Fig. 1). In the absence of forskolin stimulation, BML-190 caused no change in cAMP levels (data not shown), suggesting that this effect occurs because of the ability of BML-190 to stabilize a form of the receptor that does not freely activate $G_{i/o}$ proteins, rather than by a direct activation of G_s proteins. We confirmed the functionality of our heterologous expression system by demonstrating that the non-selective agonist, WIN 55,212-2 [5], and the CB_2 receptor selective agonist, JWH 015 [17], both inhibited forskolin-stimulated cAMP accumulation. The potencies and efficacies that we observed for WIN 55,212-2 are in agreement with previously published values for heterologously expressed human CB_2 receptors [18,19]. Furthermore, as has been previously reported for preparations of CB_2 receptors, WIN 55,212-2 was a more potent agonist than JWH 015 [20].

The inverse agonist AM630 has previously been shown to enhance forskolin-stimulated cAMP production in CB_2-transfected cells ($EC_{50} = 230$ nM) and to inhibit [^{35}S]GTPγS binding to CB_2 receptor membranes ($EC_{50} = 76.6$ nM) [21]. Our data confirm the inverse agonist activity of this ligand at CB_2 receptors and demonstrate that AM630 acts with approximately 10-fold higher potency and approximately 1.3-fold greater efficacy than BML-190. Similarly, AM251, which has previously been classified as a CB_1 receptor antagonist [1], acted as a CB_2 receptor inverse agonist in our system. The potencies and efficacies were of a similar magnitude as those of BML-190. This suggests that results obtained using AM251 to pharmacologically define receptor subtypes and responses should be confirmed with other ligands.

In order to confirm the inverse agonist activity of BML-190 and AM251 in a second system, we have shown that the human CB_2 receptor is able to functionally interact with the 16z44 chimera. This mutant harbors 44 $G\alpha_z$-specific sequences at the C-termini of $G\alpha_{16}$ and is more promiscuous in its ability to couple to $G_{i/o}$- and G_s-linked receptors [14]. Our experiments also determined that the human CB_2 receptor is unable to activate phospholipase Cβ (PLC) via endogenous $G_{q/11}$ or $G\alpha_{16}$ proteins. This confirms previous observations that the human CB_2 receptor does not functionally couple to members of the $G\alpha_q$ subfamily [22].

When transiently expressed in 293/CB_2 cells the basal IP levels increased with increasing amounts of 16z44 used. At a concentration of 62.5 ng/well of 16z44 cDNA, the level of receptor/G protein coupling was inadequate to reproducibly observe significant modulation by cannabinoid ligands (Fig. 2). At 0.125 μg/well of 16z44 cDNA, a higher basal level of IP accumulation was observed. JWH 015 was unable to further stimulate IP accumulation but AM251 did significantly inhibit basal levels (Fig. 2). We propose that at a limiting concentration of 16z44, this α-subunit preferentially interacted with an active form of the CB_2 receptor rather than the inactive form, accounting for the increased basal activity. However, addition of agonist produced no further increase in signalling via 16z44, as all available α-subunits were already coupled to active receptors. Under these conditions the inverse agonist,

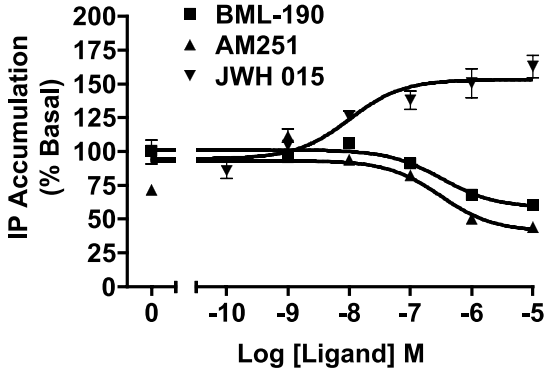

Fig. 3. Dose-dependent ligand stimulation of IP levels in 293/CB_2 cells transiently transfected with 16z44. 293/CB_2 cells were transiently transfected with 0.25 μg/well (JWH-015) or 0.5 μg/well (AM251 and BML-190) 16z44 cDNA. Transfected cells were labelled and assayed for IP formation in the presence of increasing concentrations of the indicated ligands. Data points are mean ± S.D. values of triplicate determinations in a single experiment. Basal IP levels are normalized to 100%.

AM251, was still able to stabilize the inactive, uncoupled form of the receptor and thus, a reduction of basal activity was measured. As the amounts of cDNA were increased to 0.25 μg/well and above, the basal activity increased and we were able to further stimulate IP accumulation with JWH 015 and inhibit basal activity with AM251. This suggests that as the 16z44 concentration increased this α-subunit was bound to both active and inactive forms of the receptor to allow measurement of the activities of both agonists and inverse agonists. At the highest level of DNA tested (1 μg/well) the basal activity was so high that further IP stimulation by JWH 015 was not observed. A functional coupling between CB_2 and 16z44 was still in operation as AM251 was able to reduce the basal activity (Fig. 2). Dose–response curves established that JWH 015, BML-190 and AM251 activated or inhibited IP accumulation through 16z44 in a dose-dependent manner (Fig. 3). The EC_{50} values for these three ligands are of the same order of magnitude whether signalling through $G_{i/o}$ or 16z44.

These data not only confirm the inverse agonistic activity of ligands BML-190 and AM251 in a second independent assay system but also establish for the first time that the human CB_2 receptor is able to signal via a $G\alpha_{16/z}$ chimera to modulate PLC-coupled intracellular signalling pathways. This will now facilitate the incorporation of the CB_2 receptor into high-throughput screening assays by allowing it to be assayed alongside other G protein-coupled receptors in assay platforms that detect changes in the PLC-coupled signalling pathways [23]. The basal activity observed in cells expressing both the CB_2 receptor and 16z44 will allow assays to be established that are capable of detecting agonists and inverse agonists.

Such ligands will have applications in the management and treatment of many clinical conditions. Of particular interest are the recent observations that the in vivo administration of a CB_2-selective antagonist reduced the promotion of tumor growth in mice treated with a cannabinoid [3]. It has also been demonstrated that the first CB_2 receptor inverse agonist produced, SR144528 [24], and the inverse agonist JTE-907 have anti-inflammatory effects in a mouse model when administered orally [25]. The isolation and characterization of specific, potent and efficacious inverse agonists of the CB_2 receptor will contribute towards the development of the management of tumor growth and inflammation by manipulation of cannabinoid receptor signalling activity.

In conclusion, our results demonstrate that both BML-190 and AM251 have negative intrinsic activity at the human CB_2 receptor stably expressed in HEK-293 cells. These observations will allow both ligands to be used with more precision in the pharmacological characterization of cannabinoid receptors and the analysis of the intracellular and physiological responses that they induce. Finally, our finding that the human CB_2 receptor is able to signal via 16z44-coupled pathways will contribute to the search for new CB_2 receptor ligands.

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