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AM630 IS AN INVERSE AGONIST AT THE HUMAN CANNABINOID CB_1 RECEPTOR

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Abstract. The present investigation examines WIN 55,212-2 and AM630 at the cloned human cannabinoid CB_1 receptor stably expressed in Chinese hamster ovary (CHO) cells. The effect of various concentrations of WIN 55,212-2 and AM630 on basal [^{35}S]GTPγS binding to cell membranes was determined. WIN 55,212-2 (100 μM) stimulated basal [^{35}S]GTPγS binding 77.9% with an EC_{50} value of 0.36 μM. Conversely, AM630 (100 μM) inhibited basal [^{35}S]GTPγS binding by 20.9% with an EC_{50} value of 0.90 μM. These results show that WIN 55,212-2 is an agonist and AM630 is an inverse agonist in this system.

Key Words: **human cannabinoid CB_1 receptor, inverse agonism, AM630, WIN 55,212-2, GTPγS, CHO cells, inverse agonist, negative intrinsic activity**

Introduction

AM630 (6-iodopravadoline) is a synthetic compound which binds to cannabinoid receptors (1). Interestingly, AM630 has different pharmacological actions when tested in different biological systems. Initial studies by Pertwee et al., 1995 have shown that AM630 is an antagonist in the mouse isolated vas deferens as it attenuates the ability of the cannabinoid agonist WIN 55,212-2 (*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-napthalenyl)methanone mesylate) to inhibit electrically-evoked contractions in this tissue (1). The K_d value of AM630 in antagonizing WIN 55,212-2 in this assay was calculated to be 0.036 μM. However, later studies by Pertwee et al., 1996 showed that AM630 is an agonist in the guinea pig myenteric plexus with an IC_{50} value of 1.9 μM in inhibiting electrically-evoked contractions (2). Interestingly, additional studies by Hosohata et al., 1997 demonstrated that AM630 is an antagonist

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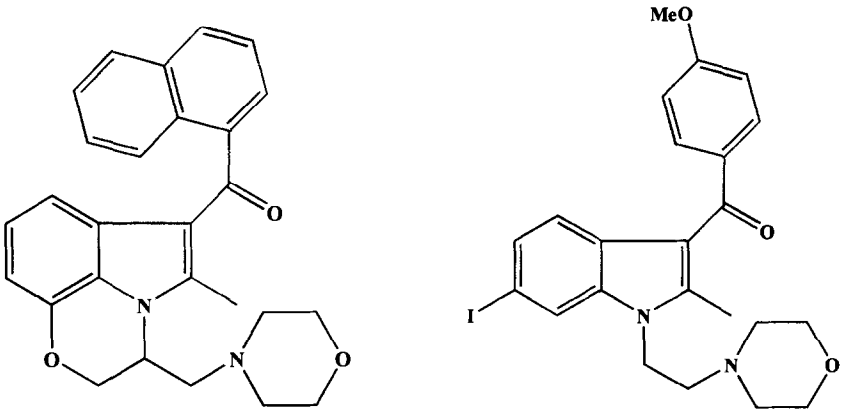
in the guinea pig brain with a K_e value of 9.3 μM (3). The potential therapeutic benefits of cannabinoids in man make it important to study the effects of these compounds at human cannabinoid receptors. The present investigation examines the effects of various concentrations of WIN 55,212-2 and AM630 at the cloned human cannabinoid CB_1 receptor stably expressed in CHO cells. An assay which measures the amount of ligand-activated [^{35}S]GTPγS binding to membranes of CB_1-tranfected CHO cells was used (4).



WIN 55,212-2 AM630

Fig. 1

Structures of WIN 55,212-2 (left) and AM630 (right)

Methods

Drugs.[^{3}H]SR141716A (52.0 Ci/mmol) was supplied by Amersham Life Sciences (Arlington Heights, IL). [^{35}S]GTPγS (1250 Ci/mmol) was obtained from DuPont NEN (Boston, MA). WIN 55,212-2 was supplied by Research Biochemicals International (Natick, MA) and AM630 was synthesized in Dr. Alexandros Makriyannis' laboratory (University of Connecticut, Storrs, CT).

Production of a stable CHO cell line. The human cannabinoid CB_1 receptor cDNA in the pSVL vector (a gift from Dr. Marc Parmentier, Bruxelles, Belgium) was cut with XbaI and BamHI and inserted into the corresponding site of the shuttle vector pGEM -3Z(-) (Promega, Madison, WI). The cDNA was then inserted into the SalI - BamHI site of the pHβAPr-1-neo vector (a gift from Dr. L. Kedes, Stanford University) for stable expression in CHO cells. DNA (5 μg) was diluted to 0.1 μg/μl in 20 mM HEPES buffer (sterile, cell culture grade). Separately, 30 μl of the cationic lipid, DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate, Boehringer Mannheim, Indianapolis, IN) was mixed with 70 μl HEPES buffer. The DNA solution was added to the DOTAP solution and gently mixed. The transfection mixture was incubated for 15 min at room temperature then added to the cells. The media used for the CHO cells was Ham's F12 media containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown for 48 hours at 37°C in a humidified environment containing 5% CO_2 and 95% air before being selected with 500 μg/ml G418 (geneticin). Saturation binding studies using [^{3}H]SR141716A revealed one clone (CHO/CB_1f) which expressed 3.2 pmol receptor/mg protein. This clone was used in these studies.

Membrane preparation. Confluent monolayers of CHO/CB_1f cells were washed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PD) and incubated with PD containing 0.02% EDTA for 5 min at 37°C. CHO/CB_1f cells were then harvested and centrifuged at 1520 x g for 5 min. The pellet was resuspended by pipetting in 20 volumes of 50 mM Tris-HCl buffer containing 5.0 mM $MgCl_2$, pH=7.4, and centrifuged again at 1520 x g for 5 min. Pellets were stored at -70°C until use. All membrane preparations were used within 4 weeks. On the day of use, pellets were homogenized in 20 volumes of assay buffer containing 25 mM Tris-HCl, 150 mM NaCl, 2.5 mM $MgCl_2$, 1.0 mM EDTA, 0.25% BSA, 50 μM GDP, 30 μM bestatin, 10 μM captopril and 0.1 mM phenylmethylsulfonyl fluoride, pH=7.4 using 10 strokes of a glass/teflon homogenizer and incubated for 30 min at 30°C. Cells were then centrifuged at 40,000 x g for 15 minutes then homogenized as above in assay buffer to give a stock tissue concentration of 1.0% (w/v). One hundred μl of tissue was used in each assay tube. This gave a final CB_1 receptor concentration of approximately 0.21 pmol receptor/tube as determined by the Lowry assay (5).

Activation of [^{35}S]GTPγS binding in CHO/CB_1f membranes by cannabinoid ligands . Assay tubes contained final concentrations of 0.1 nM [^{35}S]GTPγS, 0.1% tissue (w/v), and either 0.1 nM - 100,000 nM WIN 55,212-2, or 0.1 nM - 100,000 nM AM630 and were brought to a final volume of 1.0 ml in assay buffer. WIN 55,212-2 and AM630 were initially dissolved in 100% ethanol to produce a 2.0 mM stock solution. Further dilutions were done using assay buffer. The amount of [^{35}S]GTPγS bound at the lowest concentration of each ligand was referred to as basal binding for that ligand and was assigned a value of 100%. Tubes were incubated at 30°C for 90 min and filtered using a Brandel harvester onto Whatman GF/B filters presoaked for 90 min in assay buffer. Filters were washed 4 times with ice-cold GTPγS wash buffer (25 mM Tris-HCl, 120 mM NaCl, pH=7.4). Scintillation cocktail was added and samples were stored at 4°C overnight and counted in a Beckman LS 6000SC Scintillation counter.

Results

Figure 1 shows the structures of WIN 55,212-2 and AM630. The effects of WIN 55,212-2 and AM630 at the human cannabinoid CB_1 receptor in the [^{35}S]GTPγS assay are shown in figure 2. WIN 55,212-2 (100 μM) stimulated [^{35}S]GTPγS binding 77.9% above basal levels with an EC_{50} value of 0.36 μM. In contrast, AM630 inhibited basal [^{35}S]GTPγS binding by 20.9% with an EC_{50} value of 0.90 μM. Non-linear regression analysis determined that all data best fit a one-site model (Prism, GraphPad Software Inc., San Diego, CA). These results show that WIN 55,212-2 is an agonist (positive intrinsic activity) and AM630 is an inverse agonist (negative intrinsic activity) at the human cannabinoid CB_1 receptor. One-way ANOVA and the Newman-Keuls' Multiple-Range test showed that [^{35}S]GTPγS binding using 10,000 and 100,000 nM AM630 was significantly different than basal [^{35}S]GTPγS binding ($p<0.01$). No effect of WIN 55,212-2 or AM630 was seen in wild-type CHO cells (data not shown).

Discussion

The major finding of this study is that AM630 is an inverse agonist in membranes of CHO cells expressing 3.2 pmol receptor/mg protein of the human cannabinoid CB_1 receptor. Incubation of membranes of CHO cells expressing the human cannabinoid CB_1 receptor with WIN 55,212-2 demonstrated a dose-dependent increase in [^{35}S]GTPγS binding. In contrast, AM630 dose-dependently decreased basal [^{35}S]GTPγS binding in these membrane preparations. The finding that WIN 55,212-2 is a cannabinoid agonist is in agreement with other reports in the literature (6,7). The finding that AM630 is an inverse cannabinoid agonist is novel. However, indirect evidence for

inverse cannabinoid agonists has been shown. Compton et al., 1996 have shown that, as expected, intravenous injection of the cannabinoid ligand, SR141716A, in mice inhibited Δ^9-THC-induced hypoactivity, hypothermia and antinociception at doses well below 3 mg/kg. However, above this dose, SR141716A alone stimulated locomotor activity (8). In addition, Richardson et al., 1997 have shown that intrathecal injection of 1.0 pM SR141716A evoked a significant thermal hyperalgesia in mice (9). In the above studies, SR141716A was suggested to be inhibiting an endogenous cannabinoid system or acting as an inverse agonist.

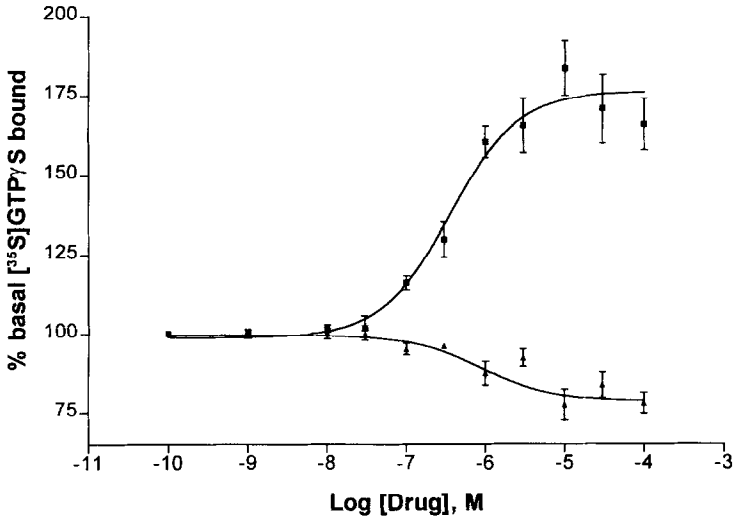


Fig. 2

Effects of WIN 55,212-2 (closed squares) and AM630 (closed triangles) on 0.1 nM [^{35}S]GTPγS binding to membranes prepared from CHO cells stably expressing the human cannabinoid CB_1 receptor. WIN 55,212-2 stimulated [^{35}S]GTPγS binding 77.9% above basal levels with an EC_{50} value of 0.36 μM. In contrast, AM630 inhibited basal [^{35}S]GTPγS binding by 20.9% with an EC_{50} value of 0.90 μM. Symbols represent the mean response and the vertical lines indicate the S.E.M. Drugs were initially dissolved in 100% ethanol to produce a 2.0 mM stock solution. Further dilutions were done using assay buffer to give final WIN 55,212-2 concentrations of 0.1 nM - 100,000 nM and AM630 concentrations of 0.1 nM - 100,000 nM. Experiments were done at least 6 times.

Uses of an inverse cannabinoid agonist can be seen in studies by Terranova et al., 1996 where SR141716A reduced memory deficits in aged rats and improved social recognition in adult rats (10). Two studies have shown that SR141716A is an inverse agonist in CHO cell membranes expressing the human cannabinoid CB_1 receptor (11,12). Therefore, inverse cannabinoid agonists may be beneficial in the treatment of memory disorders in humans. It is important to note that CHO cells do not endogenously express cannabinoid receptors. Therefore, it is not known if the receptor's tertiary conformation in the plasma membrane and its coupling mechanisms mimic that of endogenously-expressed cannabinoid CB_1 receptors in human neurons. Nevertheless, the development of other cannabinoids with greater negative intrinsic activity can be investigated using SR141716A and AM630 as prototypes.

In conclusion, the data presented here show that WIN 55,212-2 is an agonist whereas AM630 is inverse agonist in membranes of CHO cells expressing the human cannabinoid CB_1 receptor. Furthermore, the consistency of these data with other reports in the cannabinoid literature supports the use of CHO-transfected cells in studying the human cannabinoid CB_1 receptor. Further studies need to be done to determine the full potential of inverse cannabinoid agonists for use in man.

Acknowledgements

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References

1. R.G. PERTWEE, G. GRIFFIN, S. FERNANDO, X. LI, A. HILL and A. MAKRIYANNIS, Life Sci. **56** 1949-1955 (1995).
2. R.G. PERTWEE, S.R. FERNANDO, J.E. NASH and A.A. COUTTS, J. Pharmacol. **118** 2199-2205 (1996).
3. K. HOSOHATA, R.M. QUOCK, Y. HOSOHATA, T.H. BURKEY, A. MAKRIYANNIS, P. CONSROE, W.R. ROESKE and H.I. YAMAMURA, Life Sci., **61** PL 115-PL 118 (1997).
4. J.R. TRAYNOR and S.R. NAHORSKI, Mol. Pharmacol. **47** 848-854 (1995).
5. O.H. LOWRY, N.J. ROSEBROUGH, A.L. FARR and R.J. RANDALL, J. Biol. Chem. **193** 265-275 (1951)
6. M. PACHECO, S.R. CHILDERS, R. ARNOLD, F. CASIANO and S.J. WARD, J. Pharmacol. Exp. Ther. **257** 170-183 (1991).
7. D.R. COMPTON, L.H. GOLD, S.J. WARD, R.J. BALSTER and B.R. MARTIN, J. Pharmacol. Exp. Ther. **263** 1118-1126 (1992).
8. D.R. COMPTON, M.D. ACETO, J. LOWE and B.R. MARTIN, J. Pharmacol. Exp. Ther. **277** 586-594 (1996).
9. J.D. RICHARDSON, L. AANONSEN and K.M. HARGREAVES, Eur. J. Pharmacol. **319** R3-R4 (1997).
10. J.P. TERRANOVA, J.J. STORME, N. LAFON, A. PERIO, M. RINALDI-CARMONA, G. LE FUR and P. SOUBRIE, Psychopharmacology **126** 165-172 (1996).
11. M. BOUABOULA, S. PERRACHON, L. MILLIGAN, X. CANAT, M. RINALDI-CARMONA, M. PORTIER, F. BARTH, B. CALANDRA, F. PECCEU, J. LUPKER, J-P. MAFFRAND, G. LE FUR and P. CASELLAS, J. Biol. Chem. **272** 22330-22339 (1997).
12. R.S. LANDSMAN, T.H. BURKEY, P. CONSROE, W.R. ROESKE and H.I. YAMAMURA, Eur. J. Pharmacol. **334** R1-R2 (1997).