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The tamoxifen derivative ridaifen-B is a high affinity selective CB_2 receptor inverse agonist exhibiting anti-inflammatory and anti-osteoclastogenic effects

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ABSTRACT

Selective estrogen receptor modulators (SERMs) target estrogen receptors (ERs) to treat breast cancer and osteoporosis. Several SERMs exhibit anti-cancer activity not related to ERs. To discover novel anti-cancer drugs acting via ER-independent mechanisms, derivatives of the SERM tamoxifen, known as the "ridaifen" compounds, have been developed that exhibit reduced or no ER affinity, while maintaining cytotoxicity. Tamoxifen and other SERMs bind to cannabinoid receptors with moderate affinity. Therefore, ER-independent effects of SERMs might be mediated via cannabinoid receptors. This study determined whether RID-B, a first generation ridaifen compound, exhibits affinity and/or activity at CB_1 and/or CB_2 cannabinoid receptors. RID-B binds with high affinity (K_i = 43.7 nM) and 17-fold selectivity to CB_2 over CB_1 receptors. RID-B acts as an inverse agonist at CB_2 receptors, modulating G-protein and adenylyl cyclase activity with potency values predicted by CB_2 affinity. Characteristic of an antagonist, RID-B co-incubation produces a parallel-rightward shift in the concentration-effect curve of CB_2 agonist WIN-55,212-2 to inhibit adenylyl cyclase activity. CB_2 inverse agonists are reported to exhibit anti-inflammatory and anti-ostoeclastogenic effects. In LPS-activated macrophages, RID-B exhibits anti-inflammatory effects by reducing levels of nitric oxide (NO), IL-6 and IL-1α, but not TNFα. Only reduction of NO concentration by RID-B is mediated by cannabinoid receptors. RID-B also exhibits pronounced anti-osteoclastogenic effects, reducing the number of osteoclasts differentiating from primary bone marrow macrophages in a cannabinoid receptor-dependent manner. In summary, the tamoxifen derivative RID-B, developed with reduced affinity for ERs, is a high affinity selective CB_2 inverse agonist with anti-inflammatory and anti-osteoclastogenic properties.

1. Introduction

Cannabinoids have been used for medicinal purposes for centuries; however, there are only two FDA-approved cannabinoids that are currently used therapeutically (Grotenhermen and Muller-Vahl, 2012). Cannabinoids can be endogenously produced (endocannabinoids), extracted from plants (phytocannabinoids) or synthetically produced (synthetic cannabinoids) (Pertwee, 2006). They act at two major types of receptors, CB_1 and CB_2 receptors, which are $G_{i/o}$-coupled seven transmembrane receptors. Cannabinoids that are FDA-approved for therapeutic use act at both CB_1 and CB_2 receptors (Pertwee, 2010); however, unfortunately produce many adverse effects, including euphoria, lightheadedness, sedation, tolerance and dependence (Clark et al., 2005). Thus, there is a need to develop drugs acting via cannabinoid receptors with fewer side effects.

There is significant scientific evidence supporting the development of drugs that act at either CB_1 or CB_2 receptors. For example, CB_1 receptors are densely expressed in the central nervous system (CNS), making these receptors potential targets for many CNS-related disorders, such as epilepsy, Parkinson's disease, Huntington's disease, pain,

Abbreviations: AC, adenylyl cyclase; CB_1R, cannabinoid receptor type 1; CB_2R, cannabinoid receptor type 2; CHO, Chinese hamster ovary; CP-55,940, 5-(1,1-dimethylheptyl)-2-[5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]phenol; DMEM, Dulbecco's Modification of Eagle's Medium; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; GPCR, G-protein coupled receptor; [^{35}S]GTPγS, guanosine 5′-O-(3-[^{35}S]thio)triphosphate; hCB_2, human CB_2 receptors; IBMX, isobutyl-methyl-xanthine; RID, ridaifen; SERM, selective estrogen receptor modulator; TRAP, tartrate-resistant acid phosphatase; WIN-55,212-2, [(3*R*)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone

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nausea and decreased appetite (Pertwee, 2010; Garcia et al., 2011; Valdeolivas et al., 2012). Although CB_2 receptors primarily modulate immune function, they are also expressed in the CNS, reproductive system and bone (Bhutani and Gupta, 2013; Cabral et al., 2015; Hojnik et al., 2015). Drugs acting via CB_2 receptors could prove therapeutically useful for treatment of diseases related to immune function, including inflammatory disorders (Cabral and Griffin-Thomas, 2009) and neuropathic pain (Wilkerson et al., 2012), but also have been shown to produce anti-angiogenic and anti-proliferative effects in several types of cancer (Vidinsky et al., 2012).

Our laboratory and others have recently reported that several selective estrogen receptor modulators (SERMs) exhibit moderate affinity for cannabinoid receptors and act as inverse agonists (Kumar and Song, 2013; Prather et al., 2013; Ford et al., 2016). SERMs are agonists and antagonists at estrogen receptors (ERs) and have traditionally been used therapeutically to treat ER-positive breast cancer, post-menopausal conditions, and osteoporosis (Maximov et al., 2013). Interestingly, some SERMs demonstrate anti-cancer activity in cancers devoid of ERs (Martinkovich et al., 2014). A potential mechanism to explain these ER-independent effects of SERMs might be due to inverse agonist activity at cannabinoid receptors. Thus, development of cannabinoid ligands based on a SERM scaffold might result in efficacious anti-cancer drugs acting via a novel mechanism with reduced side effects. However, to avoid potential ER-related adverse effects, it will first be important to develop SERM-based cannabinoids that act selectively at CB_1 and/or CB_2 receptors and lack affinity for ERs.

Tamoxifen, a SERM in the triphenylethylene class, has been used for decades for treatment of ER-positive breast cancer. However, due to tissue-specific action at ERs not expressed in breast, tamoxifen also unfortunately produces several adverse effects, including increased incidence of endometrial cancer, stroke, hot flashes, and ocular changes (Maximov et al., 2013; Martinkovich et al., 2014). A class of pseudo-symmetrical derivatives of tamoxifen has been synthesized with the goal of decreasing affinity for ER receptors, to reduce these adverse effects, while maintaining cytotoxic actions in cancer via novel ER-independent mechanisms of action (Shiina et al., 2008; Guo et al., 2013b). This novel class is referred to as the ridaifen (RID) compounds, and a first generation compound, ridaifen-B (RID-B), appears to have promise against some forms of cancer by inhibiting cell proliferation more efficaciously than tamoxifen (Nagahara et al., 2008; Tsukuda et al., 2013). Importantly for this study, RID-B is equally cytotoxic in both ER-positive and ER-negative cancer cells. Furthermore, the mechanism of action of RID-B has been shown to be different from that of tamoxifen, as well as 200 other existing cancer medications (Tsukuda et al., 2013). Since cannabinoids and RID-B share cytotoxicity in several types of ER-negative cancers (Alexander et al., 2009; Nagahara et al., 2013), it is possible that the ER-independent actions of RID-B are mediated via cannabinoids receptors.

As an initial step to determine if cannabinoid receptors may contribute to the effects of RID-B, this study characterized the affinity and activity of RID-B at cannabinoid receptors. Based on our previous studies with other SERMs, it was hypothesized that RID-B exhibits moderate to high affinity for the cannabinoid receptors and demonstrates inverse agonist activity with anti-inflammatory and anti-osteoclastogenic activity.

2. Materials and methods

2.1. Materials

Ridaifen-B, CP-55,940, and JWH-015 were purchased from Sigma Aldrich (St. Louis, MO). WIN-55,212-2, AM-630, and AM-281 were obtained from Tocris Bioscience (Minneapolis, MN). SR-144528 was procured from Cayman Chemicals (Ann Arbor, MI). The radioligand [^{3}H]CP-55,940 (131.4 Ci/mmol) was purchased from Perkin Elmer (Waltham, MA) and [^{35}S]GTPγS (1250 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO). All compounds were diluted to 10^{-2} M in 100% DMSO and stored at −20 °C. N-1 napthylethlene, sulfanilamide, and lipopolysaccharide from *E. coli* 0111:B4 were purchased from Sigma Aldrich (St. Louis, MO). ELISA kits to measure levels of mouse TNFα and mouse IL-6 were obtained from R&D systems (Minneapolis, MN). Mouse IL-1α release was quantified by ELISA kits procured from Ray Biotech (Norcross, GA). The WST-1 cell proliferation reagent from CellPro Roche was obtained from Sigma Adrich (St. Louis, MO). Reagents for TRAP staining were purchased from Sigma Aldrich (St. Louis, MO). All other supplies were purchased from Fisher Scientific.

3. Methods

3.1. Animals

Bone marrow was extracted from the tibiae and femora of eight to ten week-old C57/BL6J mice. Animals were maintained according to guidelines of UAMS Institutional Animal Care and Use Committee, animal use protocol number 3661.

3.2. Cell culture

Chinese hamster ovary (CHO-K1) cells stably expressing human CB_1 receptors (CHO-hCB_1) were purchased from the DiscoverRx Corporation (Fremont, CA). CHO-K1 cells stably expressing human CB_2 receptors (CHO-hCB_2) were produced in our laboratory as described previously (Shoemaker et al., 2005a). CHO-hCB_1 cells were cultured in Kaighn's modification of Ham's F-12 media (Sigma Aldrich, St. Louis, MO) while Dulbecco's Modification of Eagles's Medium (DMEM; Cellgro, Manassas, VA) was used for CHO-hCB_2 cells. Both types of growth medium contained 10% FetalPlex animal serum complex (Gemini Bio Products, West Sacramento, CA) and 1% penicillin/streptomycin (10,000 IU/ml penicillin, 10,000 μg/ml streptomycin; Cellgro, Manassas, VA) and 0.5 mg/ml of G418 geneticin (Sigma Aldrich, St. Louis, MO), a selection antibiotic used to maintain the stable expression of transfected receptors. Cells were used between passages 4–18 and were maintained in a humidified incubator at 37 °C with 5% CO_2. Cells were harvested using PBS (10 mM)/EDTA (1 mM) upon reaching 90–100% confluency. Cells were then either plated for assessment of adenylyl cyclase activity (see following for complete Methods), frozen at −80 °C for future membrane preparation or reseeded into flasks for further culturing of the cell line.

3.3. Membrane preparation

Cell pellets were harvested from CHO cells stably expressing human CB_1 and CB_2 receptors (CHO-hCB_1 and CHO-hCB_2) and stored in −80 °C. Pellets were thawed on ice, pooled and suspended in ice-cold homogenization buffer (50 mM HEPES pH 7.4, 3 mM $MgCl_2$, and 1 mM EGTA). Suspended pellets were placed in a 40 ml Dounce glass homogenizer and subjected to 10 strokes followed by centrifugation at 40,000 × *g* for 10 min at 4 °C. Supernatants were discarded and the homogenization-centrifugation steps were repeated twice more. Pellets were then resuspended with ice-cold 50 mM HEPES, pH 7.4 and aliquoted for storage at −80 °C. Protein concentration was determined using the BCA™ Protein Assay (Thermo Scientific, Rockford IL).

3.4. Competition receptor binding

Increasing concentrations of RID-B (10^{-10} to 10^{-5} M) were co-incubated with 0.2 nM of the CB_1/CB_2 agonist [^{3}H]CP-55,940 as previously reported (Prather et al., 2013). Each sample also contained 50 μg of CHO-hCB_1 or CHO-hCB_2 membrane homogenates, 5 mM $MgCl_2$ and an incubation mix containing 50 mM Tris-HCl buffer (pH 7.4) with 0.05% bovine serum albumin. Nonspecific binding was defined as