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CB2 cannabinoid receptor is a novel target for third-generation selective estrogen receptor modulators bazedoxifene and lasofoxifene

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ABSTRACT

The purpose of the current study was to investigate the ability of the third-generation selective estrogen receptor modulators (SERMs) bazedoxifene and lasofoxifene to bind and act on CB2 cannabinoid receptor. We have identified, for the first time, that CB2 is a novel target for bazedoxifene and lasofoxifene. Our results showed that bazedoxifene and lasofoxifene were able to compete for specific [^{3}H]CP-55,940 binding to CB2 in a concentration-dependent manner. Our data also demonstrated that by acting on CB2, bazedoxifene and lasofoxifene concentration-dependently enhanced forskolin-stimulated cAMP accumulation. Furthermore, bazedoxifene and lasofoxifene caused parallel, rightward shifts of the CP-55,940, HU-210, and WIN55,212-2 concentration–response curves without altering the efficacy of these cannabinoid agonists on CB2, which indicates that bazedoxifene- and lasofoxifene-induced CB2 antagonism is most likely competitive in nature. Our discovery that CB2 is a novel target for bazedoxifene and lasofoxifene suggests that these third-generation SERMs can potentially be repurposed for novel therapeutic indications for which CB2 is a target. In addition, identifying bazedoxifene and lasofoxifene as CB2 inverse agonists also provides important novel mechanisms of actions to explain the known therapeutic effects of these SERMs.

1. Introduction

Selective estrogen receptor modulators (SERMs) exhibit a unique pharmacological profile [1,2]. In contrast to estrogens, which are classified as agonists, and antiestrogens, which are classified as antagonists, SERMs are characterized by having estrogen agonist action in some tissues while acting as estrogen antagonists in others [1,2].

Based on the timing of their clinical development, SERMs can be divided into three generations: (1) tamoxifen, a triphenylethlene, is considered a first generation SERM [1,2], (2) raloxifene, a benzothiophene, is a member of second generation SERMs [1,2], and (3) third generation SERMs are typified by indole-based bazeoxifene [1–3] and napthalene derivative lasofoxifene [1,2,4].

Both first generation SERM tamoxifen and second generation SERM raloxifene have been approved by FDA to be used in the United States [1,2]. Tamoxifen is prescribed frequently for the prevention and treatment of breast cancer, and raloxifene is used mainly for the prevention and treatment of osteoporosis in post-menopausal women [1,2]. In 2009, third generation SERMs bazedoxifene and lasofoxifene were approved for use in Europe to prevent and treat post-menopausal osteoporosis under the trade names Conbriza and Fablyn, respectively [1–4].

Cannabinoids exert their activity by activating cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), which are two inhibitory G-protein-coupled receptors that were cloned and identified in the early 1990's [5–8]. CB1 is expressed in the central nervous system (CNS) and peripheral organs, whereas CB2 is primarily expressed in periphery tissues such as immune cells with limited distribution in the CNS [5–8]. Since CB2 receptor expression is minimal in the CNS, this receptor has emerged as a highly attractive therapeutic target, as CB2 ligands would, in theory, lack psychoactivity [7,8].

Because CB2 ligands have a wide range of therapeutic potentials, many novel agonists and antagonists for CB2 receptors have been synthesized and patented by pharmaceutical industry as well as academic laboratories [9,10]. However, bringing a new drug to market is a highly expensive and time consuming process which could cost anywhere from $500 million to $2 billion and could take 10–15 years [11,12]. In contrast, drug repurposing, i.e. discovering novel uses for marketed drugs outside of its original scope of indication, has emerged as a time, cost-effective, and low risk drug development approach [13,14]. The advantages of drug

Abbreviations: SERM, selective estrogen receptor modulator; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; GPCR, G protein-coupled receptor; FDA, food and drug administration; HTRF, homogenous time resolved fluorescence.

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repurposing include: (1) Existing approval by regulatory agencies for human use, and (2) Existing human pharmacokinetic and safety data [13,14].

Previously, in an attempt to rapidly and efficiently identify drugs that may act as agonists or inverse agonists for CB2, we screened a library of 640 FDA-approved drugs using a validated high throughput cAMP assay [15]. Our efforts resulted in the identification of raloxifene (Evista), a second generation SERM, as a novel CB2 inverse agonist [15].

Our previous finding that raloxifene is an inverse agonist for the CB2 cannabinoid receptor prompted us to hypothesize that third-generation SERMs bazedoxifene and lasofoxifene may also act as inverse agonists for CB2. To test this hypothesis, in the current study, we investigated the actions of these two drugs on heterologously expressed human CB2 receptors, as well as the effects of these two drugs on the actions of known cannbinoids by conducting both competitive radioligand binding assays and cell-based cAMP accumulation assays.

To the best of our knowledge, this is the first report to demonstrate that bazedoxifene and lasofoxifene are inverse agonists for the CB2 cannabinoid receptor. Our findings indicate that these two marketed drugs can potentially be repurposed for novel therapeutic indications for which CB2 is a target. Our discovery that CB2 is a novel target for bazedoxifene and lasofoxifene suggests novel mechanisms of actions for these third-generation SERMs.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagles's Medium (DMEM), penicillin/streptomycin, L-glutamine, trypsin, and geneticin were purchased from Mediatech (Manassas, VA). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). Glass tubes used for cAMP accumulation assays were obtained from Kimble Chase (Vineland, NJ). These tubes were silanized by exposure to dichlorodimethylsilane (Sigma–Aldrich, St. Louis, MO) vapor for 3 h under vacuum. 384-well, round bottom, low volume white plates were purchased from Grenier Bio One (Monroe, NC). The cell-based HTRF cAMP HiRange assay kits were purchased from CisBio International (Bedford, MA). Forskolin was obtained from Sigma (St. Louis, MO). Bazedoxifene was purchased from Cayman Chemical (Ann Arbor, MI). Lasofoxifene was purchased from Toronto research Chemicals (Toronto, Ontario).

2.2. Cell transfection and culture

Human Embryonic Kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2, at 37 °C. Expression plasmids containing the wildtype human cannabinoid receptors were stably transfected into HEK293 cells using lipofectamine, according to manufacturer's instructions. Stably transfected cells were selected in culture medium containing 800 μg/ml geneticin. Having established cell lines stably expressing wildtype human CB2 receptors, the cells were maintained in growth medium containing 400 μg/ml of geneticin until needed for experiments.

2.3. Cell-based homogenous time resolved fluorescence (HTRF) cAMP assay

Cellular cAMP levels were measured using reagents supplied by Cisbio International (HTRF HiRange cAMP kit). Cultured cells were washed twice with phosphate-buffered saline (8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, and 2.7 mM KCl, pH 7.2), and then dissociated in phosphate-buffered saline containing 1 mM EDTA. Dissociated cells were collected by centrifugation for 5 min at 2000*g*. The cells were resuspended in cell buffer (DMEM plus 0.2% fatty acid free bovine serum albumin) and centrifuged a second time at 2000*g* for 5 min at 4 °C. Subsequently, the cells were resuspended in an appropriate final volume of cell buffer plus the phosphodiesterase inhibitor Ro 20-1724 (2 μM). 5000 cells were added at 5 μl per well into 384-well, round bottom, low volume white plates (Grenier Bio One, Monroe, NC). Compounds were diluted in drug buffer (DMEM plus 2.5% fatty acid free bovine serum albumin) and added to the assay plate at 5 μl per well. Following incubation of cells with the drugs or vehicle for 7 min at room temperature, d2-conjugated cAMP and Europium cryptate-conjugated anti-cAMP antibody were added to the assay plate at 5 μl per well. After 2 h incubation at room temperature, the plate was read on a TECAN GENious Pro microplate reader with excitation at 337 nm and emissions at 665 nm and 620 nm. To assess receptor antagonism, HEK293 cells stably expressing CB2 were pre-incubated for 20 min with vehicle (DMSO) or drug (bazedoxifene or lasofoxifene) at a concentration of 1 or 10 μM before subject to stimulation with cannabinoid agonists.

2.4. Cell harvesting and membrane preparation

Cells were washed twice with cold phosphate-buffered saline (PBS) consisting of 8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH 7.2, and scraped off the tissue culture plates. Subsequently, the cells were homogenized in membrane buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4) with a Polytron homogenizer. After the homogenate was centrifuged at 46,000*g* for 30 min at 4 °C, the pellet was resuspended in membrane buffer and stored at −80 °C. Protein concentrations were determined by Bradford assay using a BioRad protein reagent kit.

2.5. Ligand binding assays

The protocol for the equilibrium ligand binding assay can be found in our published papers [16–19] and are briefly described below. Drug dilutions were made in binding buffer (membrane buffer containing 0.5 mg/ml fatty acid free BSA) and then added to the assay tubes. [^{3}H]CP-55,940 was used as a labeled ligand for competition binding assays for CB2. Binding assays were performed in 0.5 ml of binding buffer containing 0.1 mg/ml BSA for 60 min at 30 °C. Membranes (80 μg) were incubated with [^{3}H]CP-55,940 in siliconized culture tubes, with unlabeled ligands at various concentrations. Free and bound radioligands were separated by rapid filtration through GF/B filters (Whatman International, Florham Park, New Jersey, USA). The filters were washed three times with 3 ml of cold wash buffer (50 mmol/l Tris–HCl, pH 7.4, containing 1 mg/ml of BSA). The bound [^{3}H] CP-55,940 was determined by liquid scintillation counting in 5 ml of CytoScint liquid scintillation fluid (MP Biomedicals, Solon, Ohio, USA). The assays were performed in duplicate, and the results represent the averaged data from at least three independent experiments.

2.6. Data analysis

Data analyses for cell-based HTRF cAMP assays were performed based on the ratio of fluorescence intensity of each well at 620 and 665 nm. Data are expressed as delta F%, which is defined as [(standard or sample ratio − ratio of the negative control)/ratio of the negative control] × 100. The standard curves were generated by plotting delta F% versus cAMP concentrations using non-linear least squares fit (Prism software, GraphPad, San Diego, CA).