Biochemical and Biophysical Research Communications 435 (2013) 76–81

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of raloxifene as a novel CB2 inverse agonist

Pritesh Kumar, Zhao-Hui Song *

Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292, USA

ARTICLE INFO

Article history:
Received 30 March 2013
Available online 20 April 2013

Keywords:
Cannabinoid receptor
Inverse agonist
Drug repurposing
Raloxifene
Osteoporosis

ABSTRACT

The purpose of the current study was to apply a high throughput assay to systematically screen a library of food and drug administration (FDA)-approved drugs as potential ligands for the cannabinoid receptor 2 (CB2). A cell-based, homogenous time resolved fluorescence (HTRF) method for measuring changes in intracellular cAMP levels was validated and found to be suitable for testing ligands that may act on CB2. Among the 640 FDA-approved drugs screened, raloxifene, a drug used to treat/prevent post-menopausal osteoporosis, was identified for the first time to be a novel CB2 inverse agonist. Our results demonstrated that by acting on CB2, raloxifene enhances forskolin-stimulated cAMP accumulation in a concentration-dependant manner. Furthermore, our data showed that raloxifene competes concentration-dependently for specific [^{3}H]CP-55,940 binding to CB2. In addition, raloxifene pretreatment caused a rightward shift of the concentration–response curves of the cannabinoid agonists CP-55,940, HU-210, and WIN55,212-2. Raloxifene antagonism is most likely competitive in nature, as these rightward shifts were parallel and were not associated with any changes in the efficacy of cannabinoid agonists on CB2. Our discovery that raloxfiene is an inverse agonist for CB2 suggests that it might be possible to repurpose this FDA-approved drug for novel therapeutic indications for which CB2 is a target. Furthermore, identifying raloxifene as a CB2 inverse agonist also provides important novel mechanisms of actions to explain the known therapeutic effects of raloxifene.

1. Introduction

Two cannabinoid receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), have been identified and cloned [1,2]. Both CB1 and CB2 are coupled to $G_{i/o}$ proteins and the activation of these receptors leads to the inhibition of adenylate cyclase activity [3,4].

CB1 receptors are distributed in the central nervous system as well as several peripheral tissues [3,4]. CB2 receptors are primarily located in immune cells, including neutrophils, monocytes, natural killer cells, T cells, B cells, macrophages, mast cells and microglia cells [3,4]. This distribution suggests an important role for the CB2 receptor in mediating many of the immunomodulatory, but not the psychoactive effects produced by cannabinoids, for which CB1 receptor is the prime target.

Because CB2 ligands have a wide range of therapeutic potentials, many novel agonists and antagonists for CB2 receptors have been synthesized by pharmaceutical industry as well as academic laboratories [5,6]. However, it is estimated that pharmaceutical product development requires at least 10 to 15 years and costs between $500 million and $2 billion [7,8].

Virtually all clinically used drugs exhibit effects on biological targets other than those for which they were designed for. This property of drugs may result in drug repurposing, which refers to the process of finding new uses of existing drugs outside the scope of the original indication [9,10]. The benefits of drug repurposing include the existing approval by regulatory agencies for human use and the availability of human pharmacokinetics data and safety profiles for the approved drug. As a result, drug repurposing is potentially a time, cost-effective and low risk drug development approach. Therefore, systematically profiling food and drug administration (FDA)-approved drugs against a variety of novel targets will provide mechanistic insights into potentially novel therapeutic effects of the existing drugs for drug repurposing [9,10].

In the current study, first of all we validated a high throughput cAMP assay appropriate for testing novel ligands for CB2 receptor. There are many cAMP assays available for screening purposes [11,12]. Homogenous time resolved fluorescence (HTRF) is based on the principle of competition of antibody binding sites between the native cAMP produced by cells and the d2-labeled cAMP [11,13]. One distinct advantage of this assay over the other technologies is HTRF's ratiometric measurement. This feature is extremely advantageous because it allows the reduction of well-to-well

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

* Corresponding author. Fax: +1 502 852 7868.
E-mail address: zhsong@louisville.edu (Z.-H. Song).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.04.040

variation and it eliminates the interference of compound autofluorescence. This assay has been successfully miniaturized and still maintains accuracy and reproducibility. It is non-radioactive and does not require separation or washing steps. It is not labor intensive, is cost-effective and has high sensitivity in the upper femtomolar range. These qualities make the cell-based HTRF cAMP assay the assay of choice for this study [11,13].

In an attempt to rapidly and efficiently identify drugs that may act as agonist or inverse agonist for CB2, in this study we screened a library of compounds consisting 640 FDA-approved drugs using the validated high throughput cAMP assay. All of the compounds in the library have well-characterized bioactivity, bioavailability and safety profiles which could enhance drug repurposing. Our rational of screening this library of FDA-approved drugs is that if novel cannabinoid ligands are found from this library, this may provide novel therapeutic implications for these marketed drugs. In addition, identifying novel cannabinoid ligands from FDA-approved drugs can provide novel mechanisms of actions for the known therapeutic effects these drugs.

It is well known that raloxifene (Evista, Eli Lilly and Company), a selective estrogen receptor modulator (SERM), works as an agonist at estrogen receptors in the bone and acts as an antagonist at the estrogen receptors in the breast [14,15]. As a result, not only does raloxifene decrease the risk of vertebral fractures, it is also reduces the prevalence of hormone-positive breast cancer [14,15]. In the current study, our screening of FDA-approved drugs against CB2 identified raloxifene as a potential inverse agonist for the CB2 cannabinoid receptor. This initial finding prompted us to further characterize the pharmacological profile of raloxifene. In follow-up experiments, we investigated the pharmacological profiles of raloxifene for CB2 by conducting cell-based cAMP accumulation assays, as well as competitive radioligand binding assays. This study provides first evidence that raloxifene is a novel CB2 inverse agonist. Our discovery that raloxifene is an inverse agonist for CB2 suggests it might be possible to repurpose this FDA-approved drug for novel therapeutic indications for which CB2 is a target. Furthermore, identifying raloxifene as a novel CB2 inverse agonist also provides important novel mechanisms of actions to explain the known therapeutic effects of raloxifene.

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). The chemical library containing 640 FDA approved drugs were purchased from Enzo Life Sciences (Farmingdale, NY).

2.2. Cell transfection and culture

Human Embryonic Kidney 293 (HEK293) cells were maintained in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 g/ml streptomycin in a humidified atmosphere consisting of 5% CO_2, at 37 °C. Expression plasmids containing the CB2 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 g/ml geneticin. Having established cell lines stably expressing CB2 receptors, the cells were maintained in growth medium containing 400 g/ml of geneticin until needed for experiments.

2.3. Cell-based HTRF cAMP assay

Cellular cAMP levels were measured using reagents supplied by Cisbio International (HTRF cAMP HiRange 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 M). 5000 cells were added at 5 l 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 l 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 l 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 or raloxifene 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

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]CP55940 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]CP55940 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]CP55940 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.