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Searching for novel ligands for the cannabinoid and related receptors.

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SEARCHING FOR NOVEL LIGANDS FOR THE CANNABINOID AND RELATED RECEPTORS

By

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B.S., University of Kentucky, 2009

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A Thesis Approved on

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DEDICATION

This thesis is dedicated to my parents

Mr. Prakash Patel

And

Mrs. Umadevi Patel

For their support, and

For the sacrifices they made for me to be where I am today.

For that I am eternally grateful.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Zhao-Hui Song, for his patience and guidance. My gratitude also goes to the members of my committee, Drs. Walter Williams, Frederick Benz, Paul Epstein, and Albert Cunningham for agreeing to offer their expertise and insight into my project. Finally, I would like to thank Justin Hallgren, Drs. Akhilesh Kumar, Donald Nerland, and Zhuanhong Qiao for passing on their technical knowledge.

ABSTRACT

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A cell-based, Homogenous Time Resolved Fluorescence (HTRF) method was optimized and used to test a library of 60 putative endocannabinoids for activity towards CB1 or CB2, and to test cannabinoid ligands and fatty acid amides for GPR119 by measuring cAMP levels in this study. The Z' factors for the assay were greater than 0.5 for all three receptors and the assay was able to can tolerate up to 1% DMSO demonstrating a robust and suitable technology for screening. The known cannabinoid and GPR119 agonists exhibited the rank order of potency expected for CB1/CB2/GPR119. Our data demonstrate that none of the amides, N-acyl amino acids (glycine and alanine) Acyl-dopamines, and Acyl-GABAs was able to activate either CB1 or CB2. However the ethanoamides Dihomo-gamma-linolenoyl ethanolamide (DLEA) and DTEA Docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA) were found to activate CB1 and CB2. Our data provide direct evidence to support the hypothesis that unsaturation in the acyl chain of fatty acid ethanolamides affects the ability of these

compounds to activate GPR119. Our results suggested that GPR119 activation requires certain structural requirements for the charged head groups of the fatty acid amides.

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INTRODUCTION

Marijuana (*Cannabis sativa*) is one of the oldest and most widely abused drugs, which has also been used for medicinal purposes by various cultures. The primary psychoactive constituent of marijuana is Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam, 1971). The recognized central nervous system (CNS) responses to cannabinoids include alterations in cognition, memory, and motor function and dysphoria/euphoria, and sedation (Hollister, 1986).

In addition to psychotropic activity, Δ^9-THC and other cannabinoids produce a variety of effects with therapeutic potential, e.g., analgesia, anti-nausea, anti-convulsion, anti-inflammation and lowering intraocular pressure (Goutopoulos and Makriyannis, 2002; Hollister, 1986). During the past two decades, a major investigative effort on the mechanisms of action of cannabinoids has been launched. Cannabinoids have been found to act through G-protein coupled receptors on cell membranes (Childers and Breivogel, 1998; Childers and Deadwyler, 1996; Devane et al., 1988; Howlett, 1995). Several cDNAs and genes encoding cannabinoid (CB) receptors have been cloned, including CB1 and CB2 (Matsuda et al., 1990; Munro et al., 1993). Endogenous cannabinoid ligands have been isolated from the brain (Devane et al., 1992a); high affinity cannabinoid mimetics with a variety of chemical structures have been synthesized, and subtype-selective ligands for cannabinoid receptors are becoming available (Huffman, 2000; Palmer et al., 2002).

Cannabinoid Receptor Expression

CB1 receptors are primarily distributed in the CNS (brain and spinal cord) and peripheral nervous system (Grotenhermen, 2004). CB1 receptor expression has also been found in several peripheral organs and tissues including endocrine glands, leukocytes, spleen, heart and parts of the reproductive, urinary and gastrointestinal tracts (Grotenhermen, 2004). In the CNS, CB1 is highly expressed in the basal ganglia, globus pallidus, entopeduncular nucleus, substantia nigra pars reticulata, caudate-putamen, cerebral cortex, cerebellum, hippocampus and dorsal primary afferent spinal cord regions (Pertwee, 2005).

CB2 receptors are primarily located in immune cells, which include neutrophils, monocytes, natural killer cells, T cells, B cells, macrophages, mast cells, and microglia (Pertwee, 2005). CB2 receptors have also been detected in the spleen and tonsils (Pertwee, 2005). CB2 is thought to mediate many of the immumnomodulatory properties produced by cannabinoids.

Cannabinoid Receptor Signaling

The cannabinoid receptors activate multiple signal transduction pathways. CB1 and CB2 receptor agonists inhibit forskolin-stimulated adenylyl cyclase by activation of a pertussis toxin-sensitive G-protein (Felder et al., 1995). Stimulation of adenylyl cyclase has been reported in pertussis toxin-treated cells, suggesting that in the absence of functional $G_{i/o}$ coupling, the CB1 receptor can activate G_s (Felder et al., 1998; Glass and Felder, 1997; Maneuf and Brotchie, 1997). It has been reported that activation of the CB2 receptor can produce stimulation of cAMP formation, as well (Rhee et al., 1998). Both CB1 and CB2 receptors are also coupled to the MAP kinase cascade via $G_{i/o}$ proteins

(Bouaboula et al., 1995). In heterologous cells, CB1 *but not CB2* receptors inhibit L-, N-, P-, and Q- type calcium channels and activate inwardly rectifying potassium channels (Caulfield and Brown, 1992; Felder et al., 1995; Gebremedhin et al., 1999; Henry and Chavkin, 1995; Mackie and Hille, 1992; Mackie et al., 1995; Pan et al., 1996). Exogenously expressed CB1 receptors couple to the inwardly rectifying GIRK channels in AtT-20 pituitary tumor cells in a pertussis toxin-sensitive manner, indicating that $G_{i/o}$ proteins serve as transducers of the response (Henry and Chavkin, 1995; Mackie et al., 1995). Inhibition of calcium channels and enhancement of inwardly rectifying potassium currents are pertussis toxin-sensitive, but independent of cAMP inhibition, suggestive of a direct G protein mechanism (Mackie and Hille, 1992; Mackie et al., 1995).

Cannabinoid Receptor Agonists

Based on their chemical structures, cannabinoid agonists can be classified into at least four groups: the classical cannabinoids such as (-)-Δ^9-THC and HU-210 (Little et al., 1989; Mechoulam et al., 1988), the non-classical cannabinoids typified by CP-55,940 (D'Ambra et al., 1992; Melvin et al., 1995), the aminoalkylindoles (AAIs) typified by WIN-55,212-2 (Compton et al., 1992; Ward et al., 1990) and the endogenous cannabinoids. The non-classical cannabinoids clearly share many structural features with the classical cannabinoids, e.g. a phenolic hydroxyl at C-1 (C2'), and alkyl side chain at C-3 (C-4'), as well as, the ability to adopt the same orientation of the carbocyclic ring as that in classical cannabinoids (Reggio et al., 1993). The AAIs, on the other hand, bear no obvious structural similarities with the classical/non-classical cannabinoids.

The first identified endogenous cannabinoid ligand, isolated first from brain, was arachidonylethanolamide (AEA, also called anandamide) (Devane et al., 1992b). sn-2-

arachidonyl glycerol (2-AG); was first isolated from intestinal tissue and shown to be a second endogenous cannabinoid ligand (Mechoulam et al., 1995). 2-AG has been found to be present at concentrations 170 times greater than anandamide in the brain (Stella et al., 1997). In addition, the fatty acid glycerol ether, 2-arachidonyl glyceryl ether has been suggested to be another endogenous cannabinoid ligand (Stella et al., 1997).

The cannabinoid agonists have been shown to have potential therapeutic uses as appetite stimulants, analgesics, anti-emetics, anti-spasmodic, anti-proliferative, anti-inflammatory, and anti-glaucoma agents (Goutopoulos and Makriyannis, 2002; Hollister, 1986; Pertwee, 2000; 2001a; b; Piomelli et al., 2000; Sanchez et al., 2001). The side effects accompanying the therapeutic responses of cannabinoid agonists include alterations in cognition, memory, and motor functions, dysphoria/euphoria, and sedation (Abood and Martin, 1992; Hollister, 1986).

Cannabinoid Receptor Antagonists

Rinaldi-Carmona and co-workers at Sanofi developed the first CB1 antagonist, SR141716A (Rinaldi-Carmona et al., 1994). SR141716A displays nanomolar CB1 affinity (Ki =1.98± 0.13 nM), but very low affinity for CB2. *In vitro*, SR141716A antagonizes the inhibitory effects of cannabinoid agonists on adenylyl cyclase activity in rat brain membranes. SR141716A also antagonizes the pharmacological and behavioral effects produced by CB_1 agonists after interperitoneal or oral administration (Barth and Rinaldi-Carmona, 1999). Other CB_1 antagonists have been reported, including AM-630 (Hosohata et al., 1997a; Hosohata et al., 1997b; Pertwee et al., 1995), LY-320135 (Felder et al., 1998) and O-1184 (Ross et al., 1998).

Rinaldi-Carmona and co-workers at Sanofi also reported the first CB2 antagonist, SR144528 (Barth and Rinaldi-Carmona, 1999; Rinaldi-Carmona et al., 1998). SR144528 displays sub-nanomolar affinity for both the rat spleen and cloned human CB2 receptors (K_i = 0.60±0.13 nM). SR-144528 displays a 700-fold lower affinity for both the rat brain and cloned human CB1 receptors.

There is strong evidence in the cannabinoid literature that SR141716A and SR144528 can act as inverse agonists. Moreover, both CB1 and CB2 receptor-transfected cells exhibit high constitutive activity (Bouaboula et al., 1999; Bouaboula et al., 1997). This constitutive activity can be blocked by the CB1-selective SR141716A and CB2-selective SR144528, respectively. Recently, therapeutic applications for cannabinoid inverse agonists are emerging in the literature. For example, the CB1 inverse agonist, SR141716A has been developed as an appetite suppressant.

The Cannabinoid Related Receptor: GPR119

Type 2 diabetes (T2D) and associated obesity are growing public health concerns (Shah, 2009). As a result, many pharmaceutical companies have focused their efforts to discover novel, orally effective agents that can modulate glucose homeostasis with a concurrent reduction in body weight. GPR119 is a member of the rhodopsin family of G protein-coupled receptors. Recently GPR119 has emerged as a promising therapeutic target for both T2D and obesity (Overton et al., 2008).

GPR119 Structure

Homology clustering analysis revealed that the closest relatives of GPR119 are the cannabinoid receptors (Overton et al., 2006). In addition, through phylogenetic analysis, Godlewski et al 2009 placed GPR119 to the MECA (melanocortin; endothelial

differentiation gene; cannabinoid; adenosine) receptor cluster and confirmed that the closest relatives of GPR119 are CB1 and CB2 cannabinoid receptors (Godlewski et al., 2009).

GPR119 Receptor Expression

GPR119 is primarily expressed in pancreatic beta-cells and enteroendocrine cells of the gastrointestinal tract (GI) (Chu et al., 2007b; Lauffer et al., 2009; Soga et al., 2005). Immunohistochemical and autoradiographic data demonstrate that GPR119 is mainly localized to a subset of cells in the pancreatic islets of Langerhans where it was found to co-localize with insulin (Chu et al., 2007b). GPR119 immunoreactivty was also found in the small intestine where it co-localizes with glucagon-like-peptide-1 (GLP-1) (Chu et al., 2007b). In addition, GPR119 has been found in the following pancreatic beta cell lines: NIT-1, MIN6, RIN5, HIT-T15 (Chu et al., 2007b; Lan et al., 2009; Ning et al., 2008; Reimann et al., 2008; Soga et al., 2005). Furthermore, GPR119 was found in enteroendocrine L-cell models such as FRIC, mGLUTag, and hNCI-H716 and in mouse L-cell primary cultures (Chu et al., 2007b; Lan et al., 2009; Ning et al., 2008; Reimann et al., 2008; Soga et al., 2005).

Although it has not been detected in the human CNS, GPR119 expression has been detected in several regions of the rat brain, including cerebellum, cerebral cortex, choroid plexus, hippocampus and hypothalamus (Jones et al., 2009).

GPR119 Receptor Signaling

GPR119-expressing cells display a constitutive increase in intracellular cAMP suggesting that this receptor is coupled to the stimulatory G-protein (Gs) (Chu et al., 2007b). It has been shown that GPR119 agonists activate adenylyl cyclase, increase

cAMP, and increase protein kinase A activity in GPR119-expressing cells (Chu et al., 2007b; Lauffer et al., 2009; Reimann et al., 2008; Semple et al., 2008; Soga et al., 2005). In addition to Gs coupling, there is evidence for GPR119-mediated activation of ATP-sensitive K+ and voltage-dependent Ca2+ channels (Ning et al., 2008).

GPR119 Endogenous Agonists

Oleoylethanolamide (OEA) was the first putative endogenous fatty acid ethanolamide ligand reported for GPR119 (Overton et al., 2006). Overton and coworkers have also tested the endogenous cannabinoid agonist AEA and the saturated fatty-acid ethanolamide palmitoylethanolamide (PEA) for GPR119 activity in a yeast-based assay. Their results showed that OEA was the most efficacious to activate GPR119, followed by PEA and then AEA (Overton et al., 2006).

In an attempt to identify novel ligands for GPR119 more than 3000 endogenously produced compounds were screened for GPR119 activity (Chu et al., 2010). Among the compounds tested, several fatty acid amides were found to be active. OEA was confirmed to be a GPR119 agonist. Oleamide, an endogenously produced free amide displayed agonist activity for GPR119. In addition, N-oleoyldopamine (OLDA) activated GPR119 with a similar potency to OEA (Chu et al., 2010).

Very recently, Hansen et al. (2011) indentified a dietary fat-derived naturally occurring 2-oleoyl glycerol (2-OG), as a GPR119 agonist. It was also shown that 2-OG administration to fasting humans led to increased glucagon-like peptide-1 (GLP-1) secretion (Hansen et al., 2011).

GPR119 Synthetic Agonists

High-throughput screening in the pharmaceutical industry resulted in the identification of PSN632408 and AR231453, two prototypical oxadizone analogues, as synthetic GPR119 agonists (Semple et al., 2008). AR231453 is notable for its nanomolar affinity for GPR119. Both of these compounds have been shown to increase intracellular cAMP, and enhance the secretion of insulin and GLP-1 (Semple et al., 2008). Currently, the one synthetic GPR119 agonist, APD668 (Arena Pharmaceuticals), has entered clinical trials.

GPR119: Diabetes and Obesity

Since GPR119 is primarily distributed in pancreatic β-cells and enterocrine L-cells, it was hypothesized that this receptor may modulate glucose homeostasis and obesity (Overton et al., 2006).

It has been shown previously that GPR119 agonists (synthetic and endogenous) stimulate insulin release by at least two mechanisms (Flock et al., 2011). The first mechanism is that the increase in cAMP signaling directly leads to an enhanced glucose-dependant insulin secretion. The second mechanism is that the increase in cAMP signaling results in increased glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) levels, which stimulates glucose-dependant insulin secretion and also inhibits glucagon secretion, appetite, and delays gastric emptying (Lauffer et al., 2008).

Recently, endogenous and small molecule synthetic GPR119 agonists have been shown to stimulate insulin release (Chu et al., 2007b; Overton et al., 2006; Soga et al.,

2005). These data suggest that orally effective GPR119 agonists may be used to improve glucose homeostasis.

It has been demonstrated that AR231453 increased secretion of insulin and GLP-1 *in vitro* (Chu et al., 2008; Semple et al., 2008). In addition, it has been shown that *in vivo* administration of AR231453 stimulated GLP-1 secretion, as well as improved glucose tolerance directly by acting on pancreatic β-cells to enhance glucose-dependant insulin release (Chu et al., 2008). Furthermore, the insulinotropic effect of AR231453 was completely lost in GPR119-deficient mice, demonstrating the involvement of GPR119 (Chu et al., 2007a) .

It has been shown that OLDA also stimulated insulin release in HIT-T15 (Hamster insulinoma cell line) cells expressing GPR119 (Chu et al., 2010). It was further shown that OLDA improves glucose handling in mice in a GPR119-dependent manner, because OLDA increased glucose tolerance in control mice and had virtually no effect on glucose tolerance in GPR119-deficient mice (Chu et al., 2010).

In addition to diabetes, GPR119 is also a potential target for the treatment of obesity (Overton et al., 2006). Both the synthetic GPR119 agonist PSN632408 and the putative endogenous GPR119 agonist OEA possess hypophagic properties (Lan et al., 2009; Overton et al., 2006). In contrast to OEA, PSN632408 displayed no activity towards Peroxisome Proliferator-Activated Receptor alpha (PPARα) (Overton et al., 2006). The hypophagic effects of OEA may not be mediated by GPR119 since the effect was the same in GPR119-defecient mice indicating that OEA and PSN632408 do not act through similar mechanisms (Lan et al., 2009).

CHAPTER 2

OPTIMIZATION, VALIDATION, AND APPLICATION OF A CELL-BASED SCREENING TECHNOLOGY FOR SEARCHING NOVEL LIGANDS FOR THE CANNABINOID RECEPTORS CB1 AND CB2

INTRODUCTION

The cannabinoid receptors and endocannbinoids have been shown to be involved in numerous pathological conditions (Miller and Devi, 2011). Thus, the discovery of new cannabinoid ligands may lead to novel therapeutic approaches for a wide range of diseases.

The purpose of this study was two-fold: 1) To develop and optimize a cell-based cAMP assay for screening novel CB1 and CB2 ligands; 2) To test a library of 60 putative endocannabinoids for their activities towards CB1 and CB2.

The first purpose of the current study was to develop and optimize an assay appropriate for searching novel ligands for CB1 and CB2. There are many cAMP assays available for screening purposes. 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 (Gabriel et al., 2003). One distinct advantage of this assay over the other technologies is HTRF's ratiometric measurement. In this assay, measurements are taken at two wavelengths (620 and 655 nm), which allows for

the ratiometric reduction of data. The donor (Eu^{3+} Cryptate) emits at 620 nm while the acceptor (d2) emits at 665 nm. The donor measurement serves as an internal reference while emissions from acceptor are indicators of biological reaction. This feature is extremely advantageous because it allows the reduction of well-to-well 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 to develop and optimize for this thesis.

The second purpose of this current study was to test a library of 60 putative endocannabinoids for activity towards CB1 or CB2. We choose this library for our initial screening experiments because all the ligands in the library are structurally related to the endocannabinoid AEA, but each compound has some distinct structural features. These ligands differ in their degree of saturation, fatty acid chain length, and head group composition. The ligands consist of 10 different fatty acids with 6 different polar head groups. Several distinct chemical classes were tested in this study, including amides, ethanolamides, lipo-amino acids, acyl-GABAs, and acyl-dopamines. By examining the activity of these 60 compounds towards CB1 and CB2, we tested our hypothesis that these ligands with structures related to AEA may activate these receptors.

MATERIALS AND METHODS

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). A cell-based cAMP HiRange kit was purchased from CisBio International (Bedford, MA).

An endocannabinoid library containing 60 compounds pre-dissolved in DMSO solutions was purchased from Enzo Life Sciences (Farmingdale, NY). CP-55, 940, WIN-55, 212-2, HU-210, 2-arachidonoyl glycerol, and anandamide were purchased from Tocris Bioscience (Ellisville, MO). Forskolin was obtained from Sigma-Aldrich (St. Louis, MO). RO 20-1724 was purchased from Enzo Life Sciences (Farmingdale, NY).

Cell Transfection and Culture

Human Embryonic Kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 2mM 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 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 CB1 and CB2 receptors, the cells were maintained in growth medium containing 400 μg/ml of geneticin until needed for experiments.

Cell-based 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 Ro20-1724 (2 μM). 5000 cells were added at 5 μl per well into 384-well, round bottom, low volume white plates (Grenier Bio One). Compounds were diluted in drug buffer (DMEM, plus 2.5 % fatty acid free bovine serum albumin and 2 μM forskolin) and added to the assay plate at 5 μl per well. Following incubation of cells with the drugs or vehicle for 7 minutes 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 hour 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.

Statistical Analysis

Data analyses were performed based on the ratio of fluorescence intensity of each well at 620nm 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] x 100. The standard curves were generated by plotting delta F% versus cAMP concentration using non-linear least squares fit (Prism software, GraphPad, San Diego, CA). Unknowns were determined from the standard curve as nanomolar concentrations of cAMP. The data from the cAMP assays were expressed as percentage of forskolin-stimulated cAMP accumulation. After the unknowns were determined, the sigmoidal concentration-response equations were used (via GraphPad Prism) to determine EC_{50} and E_{max} values of the tested compounds.

RESULTS

Z' Factor Determination

To determine the Z' value, experiments were performed in 384-well plates using many replicates of the HTRF cell-based cAMP assay with positive and negative controls. For positive controls, the HEK293 cells expressing CB1 (Fig. 2.1) or CB2 (Fig. 2.2) were treated with the potent mixed CB1/CB2 agonist CP-55,940 at a concentration of 100 nM for 7 minutes at room temperature. For negative controls, the cells were treated with vehicle for 30 minutes. The Z' value was calculated using the formula: Z' = 1-3[(standard deviation of negative control) + standard deviation of positive control)] / [(mean of negative control) – (mean of positive control)] (Zhang et al., 1999). In the current study, the Z factor was determined to be 0.77 and 0.79, respectively, for CB1 and CB2.

Tolerance to Dimethyl Sulfoxide (DMSO)

One important condition to define is the concentration of dimethyl sulfoxide (DMSO) that the HTRF cAMP assay is able to tolerate without any loss in signal. For this purpose, we tested the effect of DMSO at concentrations ranging from 0.001% to 100 %. In the present study, we have shown that the HTRF cAMP assay can tolerate DMSO up to 1.0 % for both CB1 (Fig. 2.3) and CB2 (Fig. 2.4) without any loss of signal.

Forskolin Optimization

In order to determine the optimal forskolin concentration to be used to stimulate cAMP production, forskolin concentration-response experiments were performed for both HEK293 cells stably expressing CB1 and HEK293 cells stably expressing CB2. With concentrations ranging from 0.01 nM to 100 μM, forskolin increased cAMP accumulation in both CB1-expressing cells (Fig. 2.5) and CB2-expressing cells (Fig. 2.6).

The forskolin EC_{50} values for CB1-expressing cells and CB2-expressing cells were determined to be 1.39 (0.60 to 3.00) μM and 0.76 (0.52 to 1.10) μM, respectively.

Pharmacological Testing of Known Cannabinoid Agonists

The ability of known cannabinoid agonists to activate cannabinoid receptors was tested in a functional cAMP accumulation assay (HTRF) via HEK293 cells stably expressing either CB1 or CB2. As shown in Fig. 2.7, in HEK293 cells stably expressing CB1, all five previously reported cannabinoid ligands, HU-210, CP-55,940, WIN-55,212-2, AEA, and 2-AG, inhibited forskolin-stimulated cAMP accumulation in a concentration-dependant manner, with a rank order of potency of HU-210 > CP-55,940 > WIN-55,212-2 > AEA = 2-AG (Table 2.1). In HEK293 cells stably expressing CB2, HU-210, CP-55,940, WIN-55,212-2, AEA, and 2-AG also inhibited forskolin-stimulated cAMP accumulation in a concentration-dependant manner (Fig. 2.8), with a rank order of potency of HU-210 = CP-55,940 > WIN-55,212-2 > AEA = 2-AG (Table 2.1).

Screen of a Library of Putative Endocannabinoids and The Structure-Activity Relationships of Fatty Acid Ethanolamides in Activating CB1 and CB2.

In an attempt to discover novel ligands for either CB1 or CB2, and to examine the structure-activity relationship of putative endocannabinoids towards these two receptors, each compound from a chemical library containing 60 putative endocannabinoids was tested for its ability to activate these receptors. Several distinct classes of ligands were tested in this screen, including amides, ethanolamides, lipo-amino acids, acyl-GABAs, and acyl-dopamines. None of the amides, lipo-amino acids, acyl-GABAs, and acyl-dopamines was able to activate either CB1 or CB2 (Data not shown). In contrast, several

compounds in the fatty acid ethanolamide series exhibited agonist activity towards CB1 and CB2 (Table 2.2).

Among the fatty acid ethanolamide series, AEA, Docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA) and Dihomo-γ-linolenoyl ethanolamide (DLEA) activated both CB1 and CB2 in a concentration-dependent manner. For CB1 (Fig. 2.9), the potency of DLEA was similar to AEA whereas the potency DTEA was weaker than AEA. For CB2 (Fig. 2.10), the rank order of potency is DLEA > DTEA > AEA. In addition, the rank order of efficacy is AEA > DLEA = DTEA for CB1 and AEA > DLEA > DTEA for CB2. In contrast, OEA and Linoleoyl ethanolamide (LEA) displayed no agonist activity towards either CB1 (Fig. 2.9) or CB2 (Fig. 2.10).

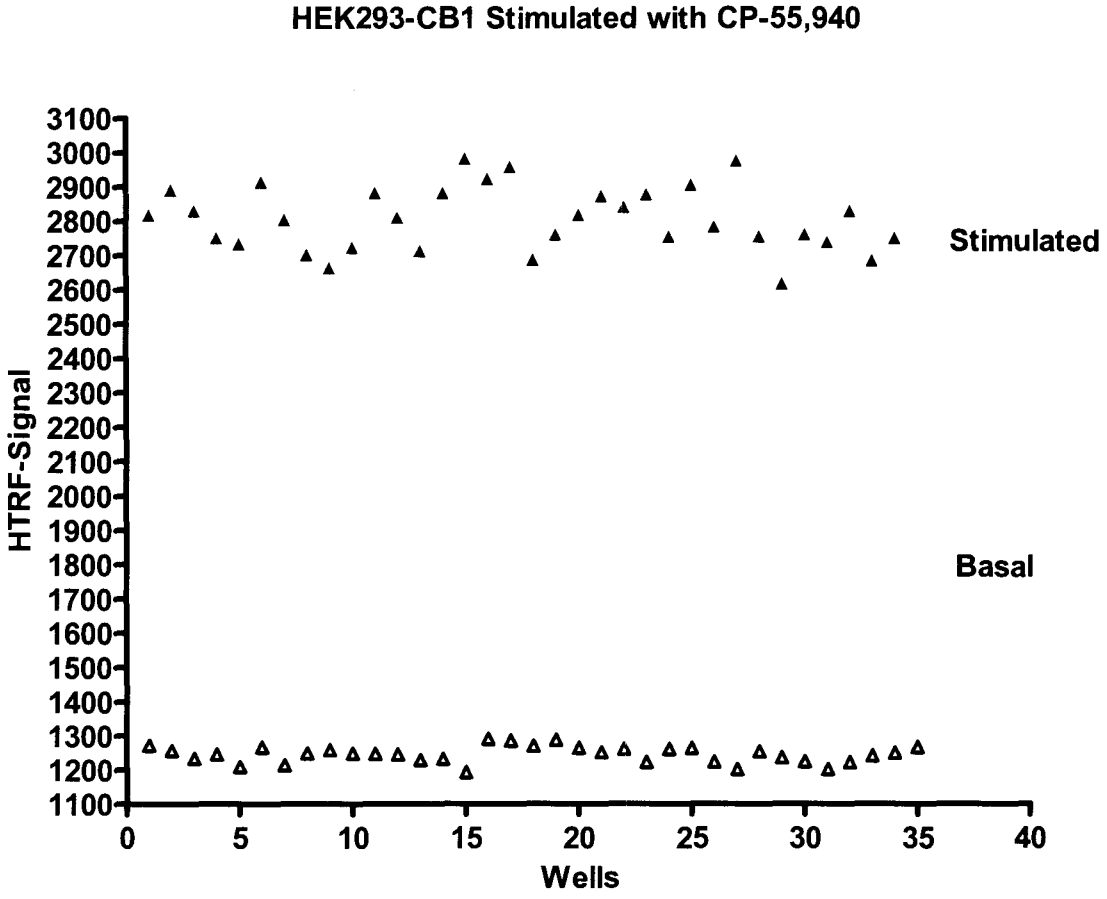

Figure 2.1. Z' factor determination. The solid symbols represent positive controls (cells stimulated with 100 nM CP-55,940), while the open symbols represent negative controls (basal level). The Z' factor was calculated to be 0.77 for CB1.

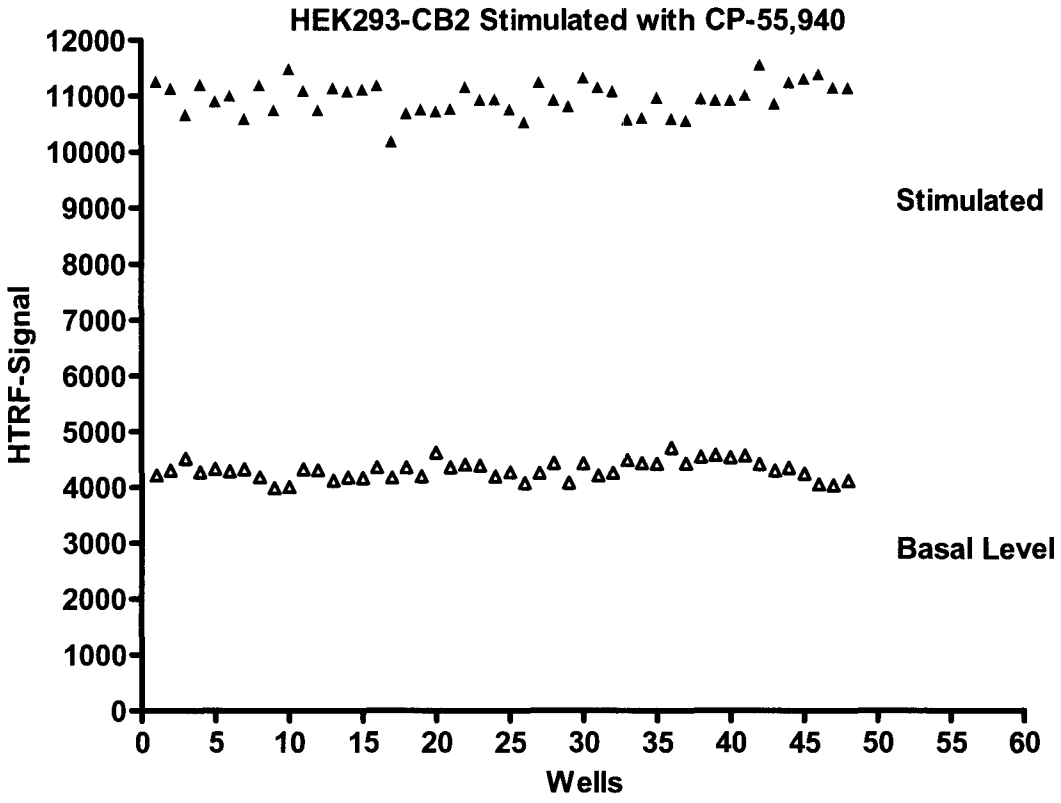

Figure 2.2. Z' factor determination. The solid symbols represent positive controls (cells stimulated with 100 nM CP-55,940), while the open symbols represent negative controls (basal level). The Z'factor was calculated to be 0.79 for CB2.

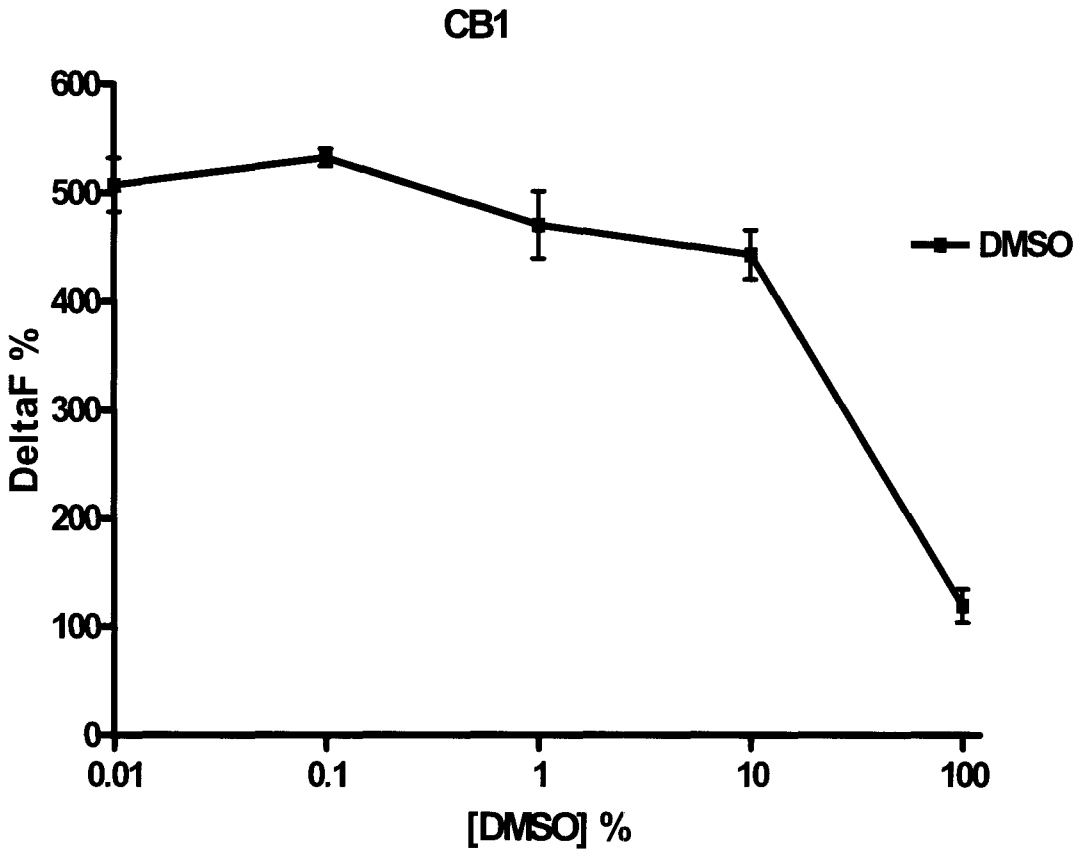

Figure 2.3. DMSO tolerance. HEK293 cells stably expressing either CB1 were treated with different concentrations of DMSO. Delta F % was calculated using the following formula: Delta F % = [(standard or sample ratio – ratio of the negative control) / ratio of the negative control] x 100. Data shown represent the mean ± S.E.M. of three experiments each performed in duplicate.

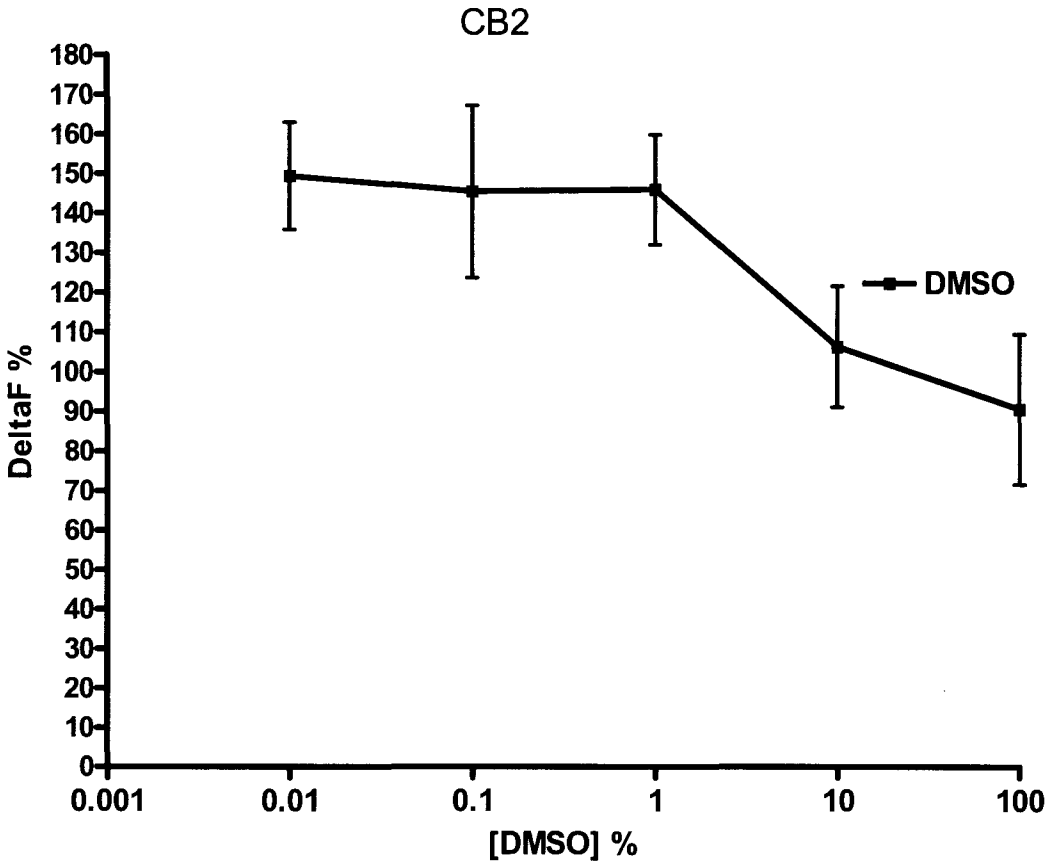

Figure 2.4. DMSO tolerance. HEK293 cells stably expressing CB2 were treated with different concentrations of DMSO. Delta F % was calculated using the following formula: Delta F % = [(standard or sample ratio – ratio of the negative control) / ratio of the negative control] x 100. Data shown represent the mean ± S.E.M. of three experiments each performed in duplicate.

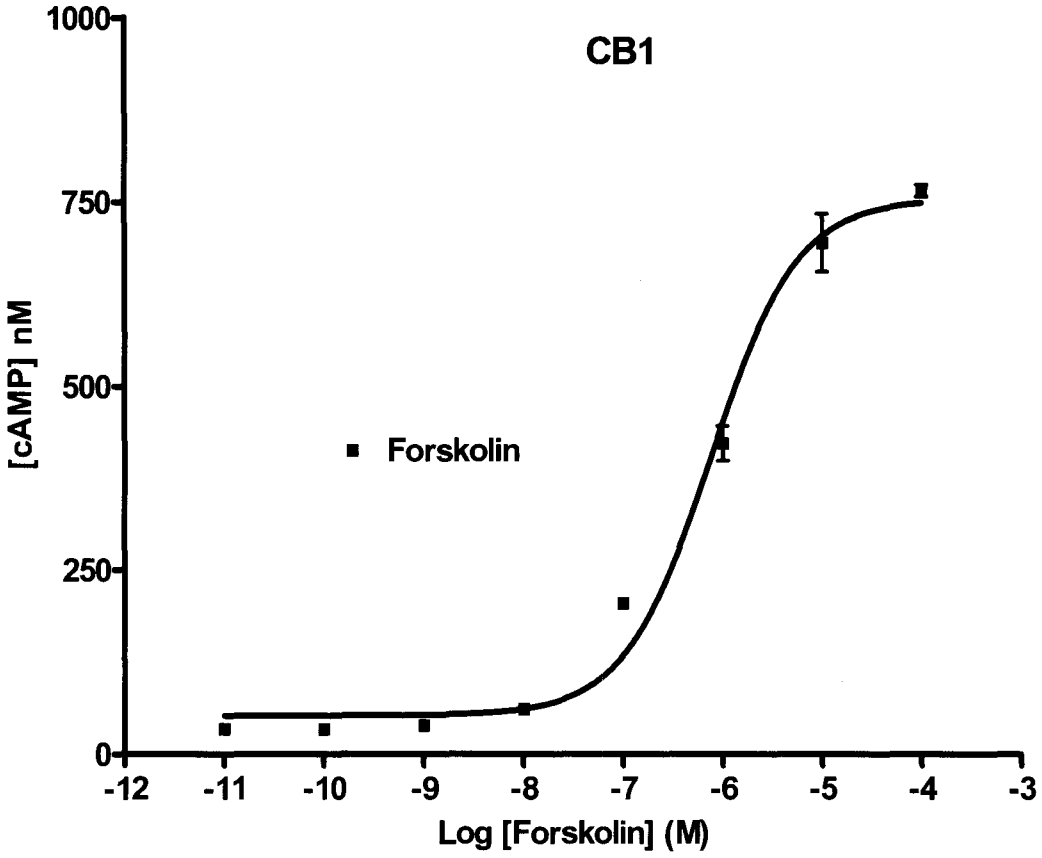

Figure 2.5. Forskolin concentration optimization. HEK293 cells stably expressing CB1 were treated with different concentrations of forskolin. Data shown represent the mean ± S.E.M. of three experiments each performed in duplicate.

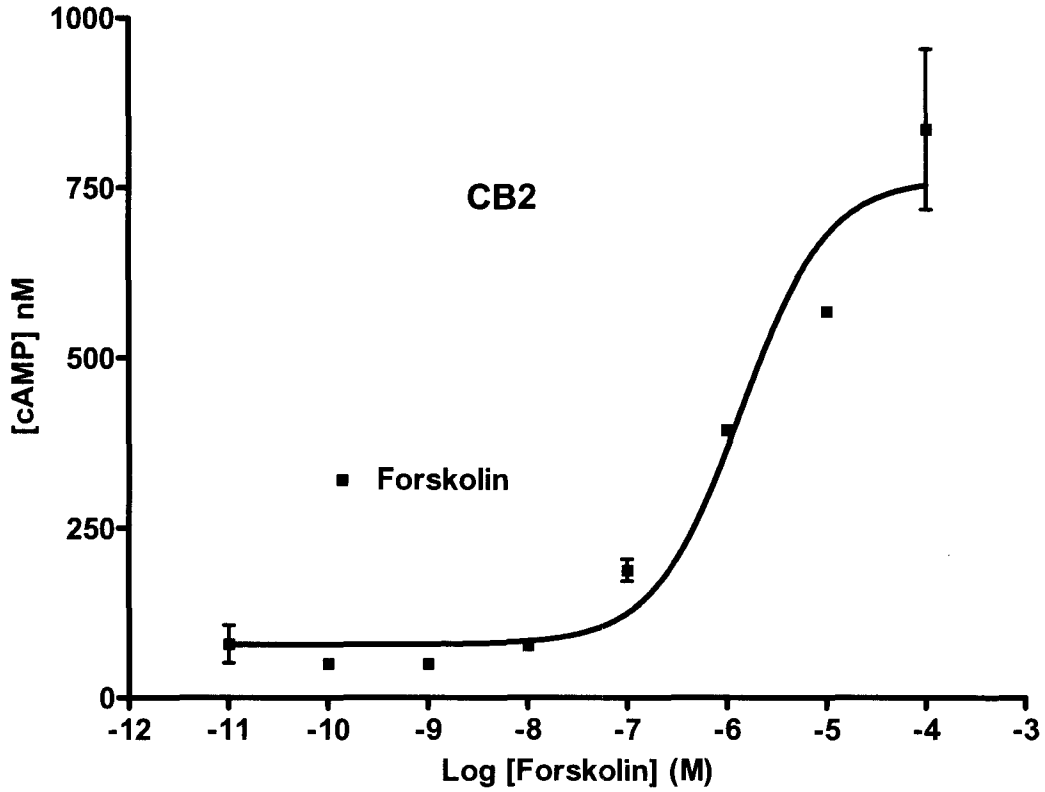

Figure 2.6. Forskolin concentration optimization. HEK293 cells stably expressing CB2 were treated with different concentrations of forskolin. Data shown represent the mean ± S.E.M. of three experiments each performed in duplicate.

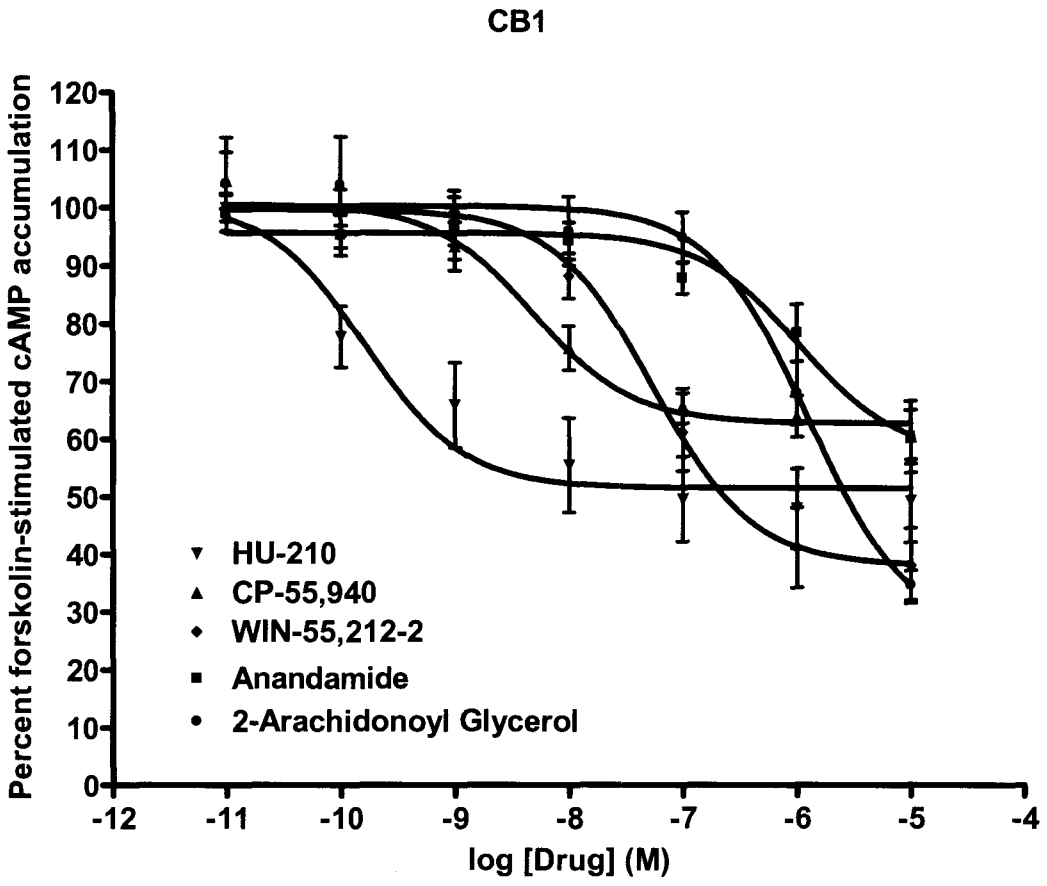

Figure 2.7. Pharmacological testing of known cannabinoid agonists. HEK293 cells stably expressing CB1 were treated with different concentrations of cannabinoid agonists HU-210, CP-55,940, WIN-55,212-2, 2-arachidonoyl glycerol, and anandamide for 7 minutes. Results are expressed as percent forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of three experiments performed in duplicate.

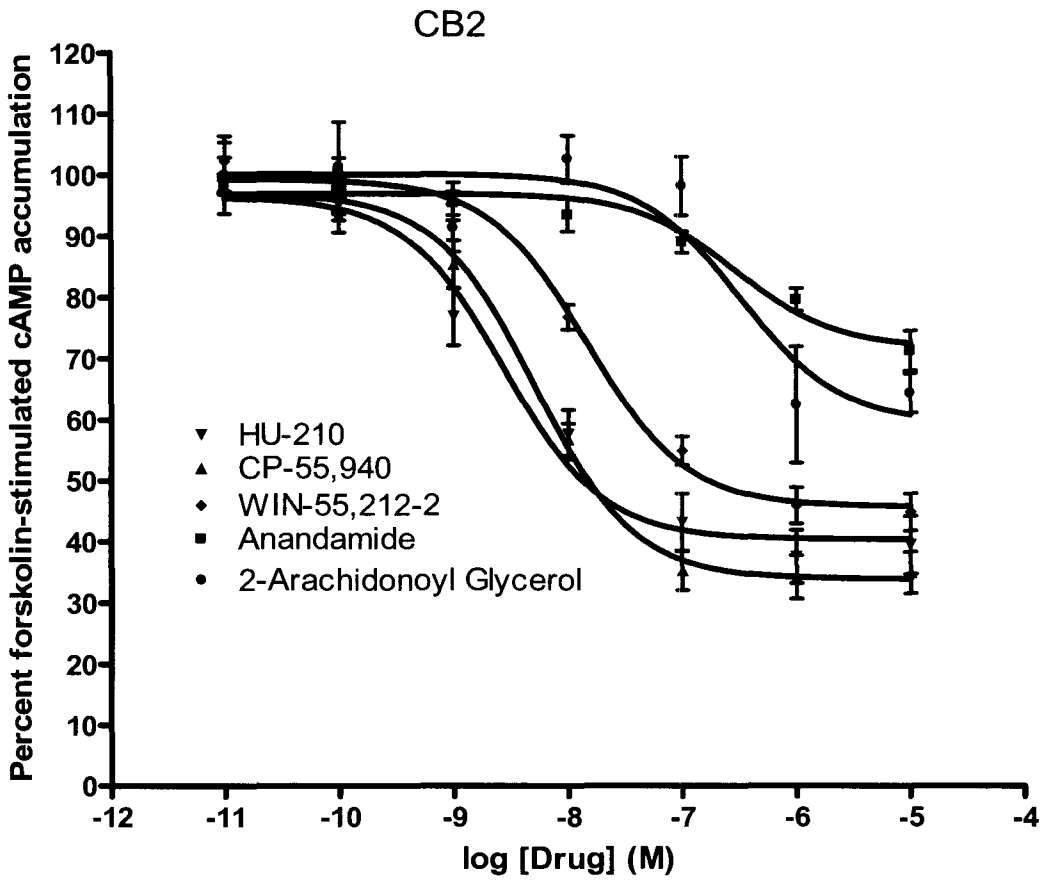

Figure 2.8. Pharmacological testing of known cannabinoid agonists. HEK293 cells stably expressing CB2 were treated with different concentrations of cannabinoid agonists HU-210, CP-55,940, WIN-55,212-2, 2-arachidonoyl glycerol, and anandamide for 7 minutes. Results are expressed as percent forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of three experiments performed in duplicate.

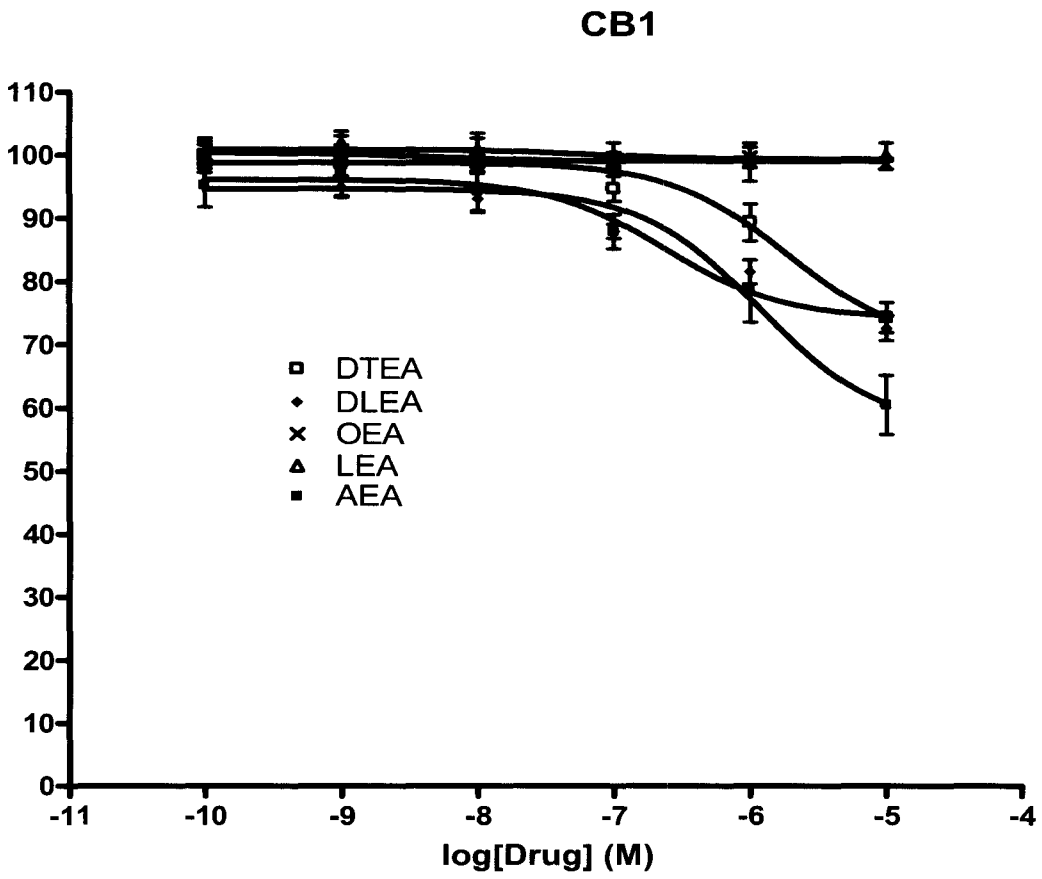

Figure 2.9. The structure-activity relationships of fatty acid ethanolamides in activating CB1. HEK293 cells stably expressing CB1were treated with different concentrations of oleoyl ethanolamide (OEA), linolenoyl ethanolamide (LEA), docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA), arachidonoyl ethanolamide (AEA), and dihomo-γ-linolenoyl ethanolamide (DLEA) for 7 minutes. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of three independent experiments performed in duplicate.

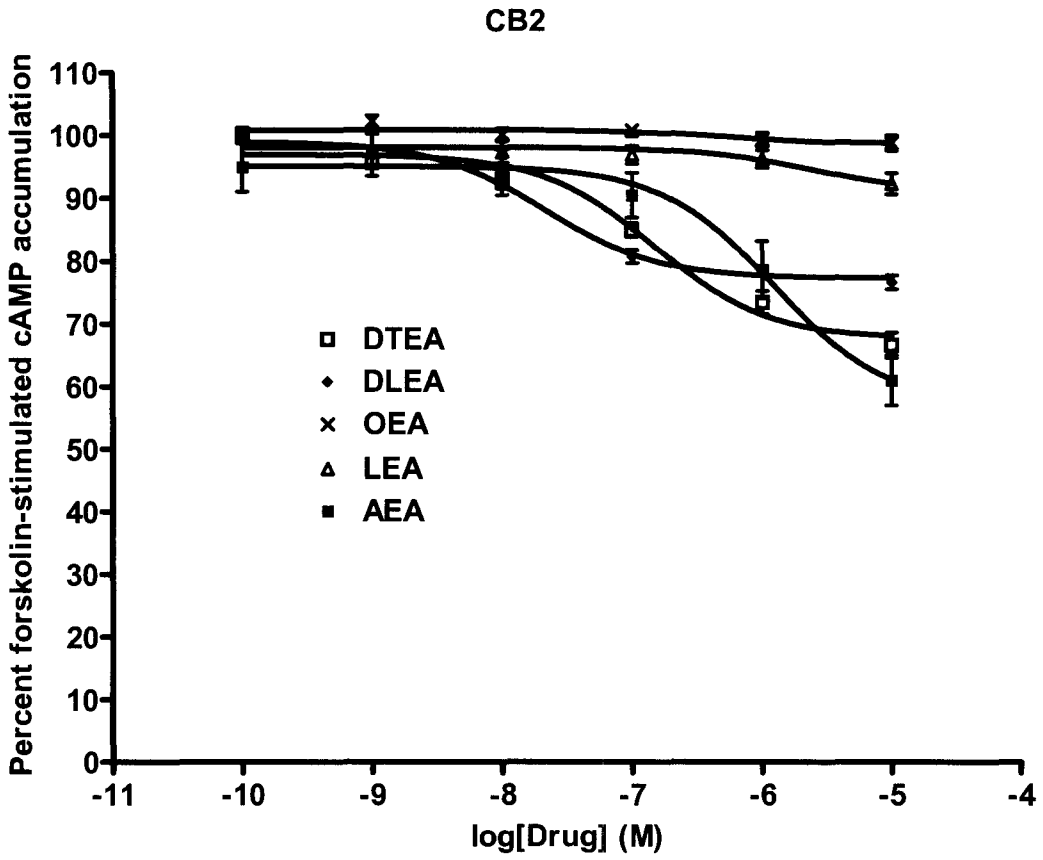

Figure 2.10. The structure-activity relationships of fatty acid ethanolamides in activating CB2. HEK293 cells stably expressing CB2 were treated with different concentrations of oleoyl ethanolamide (OEA), linolenoyl ethanolamide (LEA), docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA), arachidonoyl ethanolamide (AEA), and dihomo-γ-linolenoyl ethanolamide (DLEA) for 7 minutes. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of three independent experiments performed in duplicate.

Table 2.1. The effects of known cannabinoid agonists on inhibiting forskolin-stimulated cAMP accumulation in HEK293 cells stably expressing CB1 or CB2.

Drugs	CB1- EC_{50} (95% CI) (nM)	CB2- EC_{50} (95% CI) (nM)
HU-210	0.16 (0.03 to 0.695)	3.92 (1.68 to 9.15)
CP-55,940	4.91 (1.72 to 14.04)	5.15 (3.21 to 8.25)
WIN-55,212-2	55.36 (25.71 to 119.2)	14.69 (9.11 to 23.69)
Anandamide	1032 (356 to 3000)	290 (112 to 751)
2-Arachidonoyl Glycerol	910 (400 to 2100)	320 (64 to 1600)

CI, confidence interval. Data shown are from three experiments, each performed in duplicate.

Table 2.2. The structure-activity relationships of fatty acid ethanolamides in inhibiting forskolin-stimulated cAMP accumulation in HEK293 cells expressing CB1 or CB2.

Drug	CB1 EC_{50} (95% CI)(nM)	CB2 EC_{50} (95% CI) (nM)
Oleoyl ethanolamide (OEA)	NR	NR
Linoleoyl ethanolamide (LEA)	NR	NR
Dihomo-gamma-linolenoyl ethanolamide (DLEA)	238 (86.1 to 658)	21.4 (13.8 to 33.2)
Arachidonoyl ethanolamide (AEA)	1165 (373 to 3643)	1239 (384 to 3994)
Docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA)	1870 (599 to 5837)	149 (86.2 to 258.1)

CI, confidence interval. NR, No response. Data shown are from three experiments, each performed in duplicates.

DISCUSSION

The purpose of this study was two-fold: 1) To optimize a cell-based, HTRF cAMP assay for screening novel ligands for the cannabinoid receptors; and 2) To conduct a pilot study on a library of 60 putative endocannabinoids to search for novel ligands and to investigate the structure-activity relationships of fatty acid ethanolamides for activating cannabinoid receptors.

Agonist binding to CB1 and CB2 leads to Gi coupling and inhibition of adenylate cyclase (Felder et al., 1995). As a result, there is a decrease in intracellular cAMP levels which was measured as an increase in HTRF signal in this study. In this study, we have shown that the HTRF HiRange cell-based cAMP assay is a suitable technology for screening ligands that may act on CB1 and CB2.

The Z'factor is a standard statistical parameter used to evaluate the robustness of a screening assay (Zhang et al., 1999). The Z'factor value can range between 0 and 1, with values approaching 1 indicating excellent assay robustness. A value of greater than 0.5 indicates a suitable difference between signal and background values with low variability. In this study we determined Z'factor according to the methods published previously (Zhang et al., 1999). The calculated Z'factors for the cell-based cAMP assay for CB1 and CB2 receptors were 0.77 and 0.79, respectively (Figs. 2.1, 2.2). These results demonstrated that this assay is robust and suitable for screening ligands that activate both CB1 and CB2 since the determined Z'factor is greater than 0.5.

Since most chemical compound libraries come pre-dissolved in dimethyl sulfoxide (DMSO), it is critical to determine the maximum concentration that a

compound can be screened before DMSO reaches a concentration that is too high to be tolerated by the assay. Therefore, we determined the effect of DMSO on the HTRF HiRange cAMP cell-based assay. We tested DMSO at a variety of concentrations and the results showed that the assay can tolerate DMSO up to 1 % (Figs. 2.3, 2.4). These data indicate that the assay is suitable for screening ligands that may act on CB1 or CB2 at a DMSO concentration of less than 1 %.

Since the cannabinoid receptors are negatively coupled to adenylate cyclase (Felder et al., 1995), it is necessary to first raise the cellular cAMP levels in order to observe a robust decrease in cAMP upon ligand binding. Concentration-response studies were performed in HEK293 stably expressing CB1 and HEK293 cells stably expressing CB2 to determine the optimal forskolin concentration for our screening assays (Figs. 2.5, 2.6). Based on our data, for compound screening and testing, 1 μM forskolin was used to stimulate cAMP production in both the CB1-expressing and the CB2-expressing HEK293 cells.

To validate that the HTRF HiRange cell-based cAMP assay is suitable for screening ligands that may activate CB1 and CB2, we performed concentration-response studies for five previously reported CB1 and CB2 agonists (Figs. 2.7, 2.8). The rank order of potencies of these known agonists in inhibiting cAMP levels in both CB1- and CB2-expressing HEK293 cells HU-210 > CP-55,940 > WIN-55,212-2 > AEA = 2-AG and HU-210 = CP-55,940 > WIN-55,212-2 > AEA = 2-AG , respectively (Table 2.1). These data are consistent with previous reports regarding the potency of these CB1 and CB2 agonists. These results also confirmed the suitability of HTRF HiRange cell-based cAMP assay for screening potential novel ligands for both CB1 and CB2.

In 1992, Devane et al. reported AEA, a prototypical N-acyl ethanolamide (Di Marzo et al., 1994), as the first endogenous ligand for CB1 (Devane et al., 1992b). Recently, several fatty acid amide derivatives have been identified in mammals in which the ethanolamide moiety is replaced by amino acids (glycine and alanine), dopamine or GABA to form N-acyl-amino acids (Lipo-amino acids), N-acyl-dopamines, and N-acyl-GABAs (Connor et al., 2010). Since these compounds are structurally related to AEA, it has been hypothesized that they may activate cannabinoid receptors. However, to our knowledge, this hypothesis has not been tested thoroughly by experimental studies. In order to test this hypothesis in a systemic and comprehensive manner, in the current study we examined 60 putative endocannabinoids from a compound library for their ability to activate either CB1 or CB2. This library is designed as an array of 10 different fatty acids and 6 different polar head groups and includes the following classes of ligands: Amides, Ethanolamides, Lipo-amino acids, Acyl-dopamines, and Acyl-GABAs.

Our data demonstrate that none of the Amides, Lipo-amino acids (glycine and alanine), Acyl-dopamines, and Acyl-GABAs were able to activate either CB1 or CB2 in cAMP accumulation assays. Therefore, our data disapprove the hypothesis that these compounds are endogenous agonists for either CB1 or CB2 in cAMP accumulation assays. In contrast to our findings using cAMP assays, it has been reported previously that N-arachidonoyl dopamine (NADA), one of the acyl-dopamines, was able to bind CB1 receptor and activates calcium mobilization in neuroblastoma cells in a fashion consistent with CB1 recptor activation (Bisogno et al., 2000). Taken together, this suggests that NADA is an agonist on CB1 for causing calcium mobilization, but not an agonist on CB1 to inhibit adenylate cyclase. This type of ligand-biased signaling has been

reported for other ligands of G-protein coupled receptors (GPCRs) (Kenakin, 2007). It has been suggested that GPCRs can adopt multiple conformations, leading to different signaling events. Furthermore, it has been postulated that these different conformations can be stabilized by different ligands, causing ligand-biased signaling (Kenakin, 2007).

In addition to AEA, the first identified endogenous agonist for cannabinoid receptors, several other endogenous ethanolamides have been isolated and identified in mammals (Hanus et al., 1993). Among these endogenous ethanolamides, DLEA and DTEA have been shown to bind CB1 (Hanus et al., 1993). In the current study we have shown that DLEA and DTEA display similar potencies for CB1 in cAMP accumulation assays which is consistent with a previous report (Hanus et al., 1993). In addition, we have demonstrated that both of these compounds have similar efficacy as AEA to activate CB1 in the cAMP accumulation assays. Furthermore, we have demonstrated that the putative endocannabinoids OEA and LEA do not activate either CB1 or CB2.

Using HEK293 cells stably expressing CB2, we have found that the rank order of potency is DLEA > DTEA > AEA, and both DLEA and DTEA have significantly lower efficacy than AEA in inhibiting cAMP accumulation. DLEA has previously been reported to be essentially equipotent at the CB1 and CB2 receptors (Felder et al., 1995). To the best of our knowledge, the agonist activity of DTEA on CB2 has not been reported. In the current study, we have demonstrated that both DLEA and DTEA have higher potency on CB2 than on CB1, thus exhibiting a significant level of CB2 selectivity. In terms of efficacy, AEA was the most efficacious followed by DTEA then DLEA. In addition, our results showed that the putative endocannabinoids OEA and LEA do not activate CB2.

It has previously been shown that endocannabinoids with increased saturation in the acyl chain have decreased affinity for CB1 (Khanolkar and Makriyannis, 1999). In addition it has been suggested that AEA analogs with greater than 3 double bonds exhibit significant CB1 affinity and that the presence of 4 cis bonds is optimal (Khanolkar and Makriyannis, 1999). OEA and LEA contain one and two double bonds, respectively, in the acyl chain, which could be the reason why both OEA and LEA displayed no agonist activity on cannabinoid receptors in cAMP accumulation assays. In contrast, DLEA and DTEA have three and four double bonds respectively, which could be the reason that both of these ligands exhibited agonist activity at CB1 and CB2 receptors in the current study.

CHAPTER 3

OPTIMIZATION, VALIDATION, AND APPLICATION OF A CELL-BASED SCREENING TECHNOLOGY FOR SEARCHING FOR NOVEL LIGANDS FOR GPR119

INTRODUCTION

GPR119 is a G protein-coupled receptor (GPCR) predominantly expressed in the beta cells of the pancreas and enteroendocrine cells of the gastrointestinal tract (Chu et al., 2007b; Lauffer et al., 2009; Soga et al., 2005). GPR119 is coupled to Gs, so upon its activation, there is an enhancement of cAMP levels within the cell (Chu et al., 2007b). It has been shown previously that GPR119 agonists stimulate insulin release by at least two mechanisms (Flock et al., 2011). The first mechanism is that the increase in cAMP signaling directly leads to an enhanced glucose-dependant insulin secretion. The second mechanism is that the increase in cAMP signaling results in an increased glucagon-like peptide 1 (GLP-1) levels. GLP-1 is an anti-diabetic hormone which stimulates glucose-dependant insulin secretion and also inhibits glucagon secretion, appetite, and delays gastric emptying (Lauffer et al., 2008). It has been shown that administration of GPR119 agonists improves glucose tolerance in rodents (Chu et al., 2007b; Overton et al., 2006; Soga et al., 2005). In

addition, it has been demonstrated that GPR119 agonists decrease feeding, body weight gain and adiposity in rats (Overton et al., 2006) Thus, GPR119 is a highly attractive potential therapeutic target for both diabetes and obesity.

The first purpose of the current study is to develop an assay appropriate for searching compounds to discover novel ligands for GPR119. There are many cAMP assays available for screening purposes. 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 (Gabriel et al., 2003). One distinct advantage of this assay over the other technologies is HTRF's ratiometric measurement. In this assay, measurements are taken at two wavelengths (620 and 655 nm), which allows for the ratiometric reduction of data. The donor (Eu^{3+} Cryptate) emits at 620 nm while the acceptor (d2) emits at 665 nm. The donor measurement serves as an internal reference while emissions from acceptor are indicators of biological reaction.

Homology clustering analysis revealed that the closest relatives of GPR119 are the cannabinoid receptors (Overton et al., 2006). A separate group confirmed, through phylogenetic analysis, that the closest relatives of GPR119 are the cannabinoid receptors and placed GPR119 to the MECA (melanocortin; endothelial differentiation gene; cannabinoid; adenosine) receptor cluster (Godlewski et al., 2009). Based on these observations, it has been hypothesized that synthetic cannabinoid ligands and fatty acid amides related to endocannabinoid AEA may be potential ligands for GPR119.

Previously, a number of cannabinoid ligands and fatty-acid amides have been tested as potential agonists for GPR119 (Overton et al., 2006). However, the data from different research groups have not always been consistent. For example, Overton and

coworkers identified OEA as an endogenous GPR119 ligand (Overton et al., 2006). However, others found that OEA does not activate GPR119 (Brown, 2007).

It has been proposed that 1) unsaturation in the fatty acid acyl chain might be important for activating GPR119; and 2) there is a broad permissiveness in the amine-derived moieties (the head groups) of lipid amides for being an agonist for GPR119 (Chu et al., 2010). However, these hypotheses either have not been examined by different research groups or have not been tested comprehensively using novel ligands. Therefore, the second purpose of this study is to re-examine the activity of a number of cannabinoid ligands and fatty acid amides towards GPR119, and to investigate, using novel compounds that have never been tested on GPR119, the structure-activity relationships of the acyl side chains as well as the charged head groups in fatty acid amides for activating GPR119.

MATERIALS AND METHODS

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). A cell-based cAMP HiRange kit was purchased from CisBio International (Bedford, MA).

CP-55,940, WIN-55,212-2, HU-210, 2-arachidonoyl glycerol, virodhamine, and anandamide were purchased from Tocris Bioscience (Ellisville, MO). Forskolin was obtained from Sigma (St. Louis, MO). AR231453, Ro20-1724 and palmitoyl ethanolamide were purchased from Enzo Life Sciences (Farmingdale, NY). PSN632408, Oleoylethanolamide, linoleoyl ethanolamide, dihomo-gamma-linolenoyl ethanolamide, docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide, eicosapentaenoyl ethanolamide, docosahexaenoyl ethanolamidel, abnormal cannabidiol, JWH200, JWH015, arachidonoyl glycine, O-1602 and O-1918 were purchased from Cayman Chemical Company (Ann Arbor, Michigan). N-oleoyl glycine and N-oleoyl dopamine were purchased from Cayman Chemical Company (Ann Arbor, Michigan). Oleamide was purchased from Tocris Bioscience (Ellisville, MO).

Cell Transfection and Culture

Human Embryonic Kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 2mM glutamine, 100units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 at 37°C. Expression plasmid containing the GPR119 receptor was stably transfected into HEK293 cells using lipofectamine, according to manufacturer's instructions. Stably transfected cells were selected in culture medium containing 800μg/ml geneticin and maintained in growth medium containing 400 μg/ml of geneticin (G418) until needed for experiments.

Cell-based 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 Ro20-1724 (2 μM). 5000 cells were added at 5μl 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 30 minutes 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 hour 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.

Statistical Analysis

Data analyses were performed based on the ratio of fluorescence intensity of each well at 620nm 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] x 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). Unknowns are determined from the standard curve as nanomolar concentrations of cAMP. After the unknowns are determined, the sigmoidal concentration-response equations were used (via GraphPad Prism) to determine EC50 and Emax values of the tested compounds.

RESULTS

Z' factor Determination

To determine the Z' value, experiments were performed in 384-well plates using many replicates of the HTRF cell-based cAMP assay with positive and negative controls (Fig. 3.1). For positive controls, the HEK293 cells expressing GPR119 were treated with the potent GPR119 agonist AR231453 at a concentration of 10 μM for 30 minutes at room temperature. For negative controls, the cells were treated with vehicle for 30 minutes. The Z' value was calculated using the formula: Z' = 1-3[(standard deviation of negative control) + standard deviation of positive control)]/ [(mean of negative control) – (mean of positive control)] (Zhang et al., 1999). In the current study, the Z factor was determined to be 0.68.

Tolerance to Dimethyl Sulfoxide (DMSO)

One important condition to define is the concentration of dimethyl sulfoxide (DMSO) that the HTRF cAMP assay is able to tolerate without any loss in signal. For this purpose, we tested the effect of DMSO at concentrations ranging from 0.001% to 100 %. As shown in Fig. 3.2, the HTRF cAMP assay can tolerate DMSO up to 1% without any loss of signal.

Pharmacological Testing of Known GPR119 Agonists

The ability of known agonists to activate GPR119 was tested in a functional HTRF cAMP accumulation assay via HEK293 cells stably expressing GPR119. As shown in Fig. 3.3 and Table 3.1, all three previously reported GPR119 ligands, AR231453 (Semple et al., 2008), OEA (Overton et al., 2006), and PSN632408 (Overton et al., 2006), increase the cellular cAMP levels in a concentration-dependent

manner, with a rank order of potency of AR231453 > OEA > PSN632408, and a rank order of efficacy of AR231453 > OEA = PSN632408.

The Effects of Synthetic Cannabinoid Ligands on GPR119

Three synthetic cannabinoids, CP-55,940, HU-210, and WIN-55,212-2, were tested for their activity towards GPR119. As shown in Table 3.2, HU-210 and CP-55,940, and WIN-55,212-2 did not activate GPR119.

The Effects of Acyl Chain Unsaturation on the Ability of Fatty Acid Ethanolamides to Activate GPR119

Three endogenous fatty acid ethanolamides, OEA, PEA and AEA, were tested for their activity on GPR119 (Fig. 3.4 and Table 3.3). All three compounds increased cAMP levels, with EC_{50} values not significantly different from each other. However, our results demonstrated that OEA was the most efficacious, followed by PEA and then AEA.

In this study, we examined the structure activity relationship on a subset of fatty acid ethanolamides (Fig. 3.5 and Table 3). Among fatty acid ethanolamides that we tested, LEA, and DLEA were found to be the most efficacious towards GPR119 (Fig. 3.5). In terms of acyl chain unsaturation, LEA and DLEA contain two and three double bonds, respectively. These two compounds had efficacy similar to those of OEA, a putative endogenous GPR119 ligand which contains one double bond.

On the contrary, Docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA), Eicosapentaenoyl ethanolamide (EPEA) and Docosahexaenoyl ethanolamide (DHEA) were found to have significantly reduced efficacy towards GPR119 than OEA (Fig.

3.5). DTEA, EPEA and DHEA contain four, five, and six double bonds in their fatty acid acyl chain, respectively.

The effects of different head groups on the ability of oleoyl amides to activate GPR119.

We hypothesized that different head groups on the oleoyl amides may impact the ability of oleoyl amides to activate GPR119. To test this hypothesis, N-oleoyldopamine (OLDA), oleamide, OEA, oleoyl alanine, oleoyl glycine, and oleoyl GABA were tested for their ability to increase cAMP levels in HEK293 cells stably expressing GPR119.

Fig. 3.6 and Table 3.4 demonstrate the agonist activity of different oleoyl amides as compared to OEA. In HEK293 cells stably expressing GPR119, OLDA, Oleamide, and OEA increased cAMP levels, with similar EC_{50} and E_{max} values. On the contrary, oleoyl alanine, oleoyl glycine, and oleoyl GABA failed to activate GPR119 with concentrations up to 10 μM.

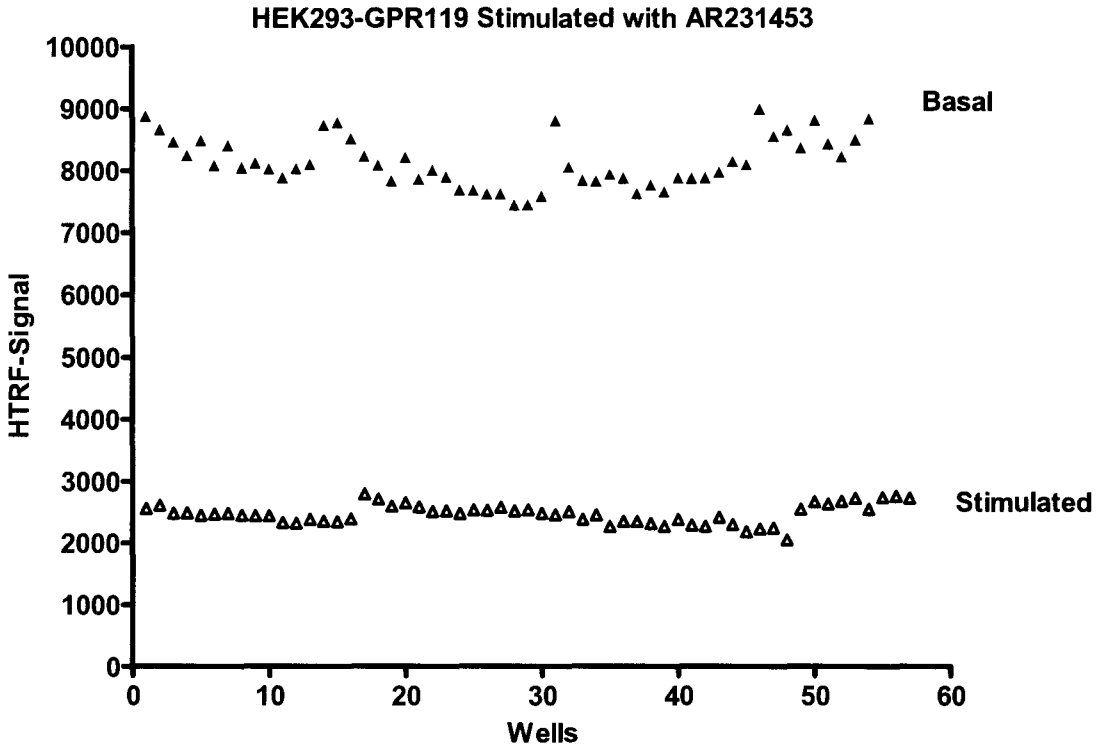

Figure 3.1. Z' factor determination. Open symbols represent positive controls (cells stimulated with 10 μM AR231453), while solid symbols represent negative controls (basal level). The Z' factor was calculated to be 0.68 using 55 basal and stimulated signal points.

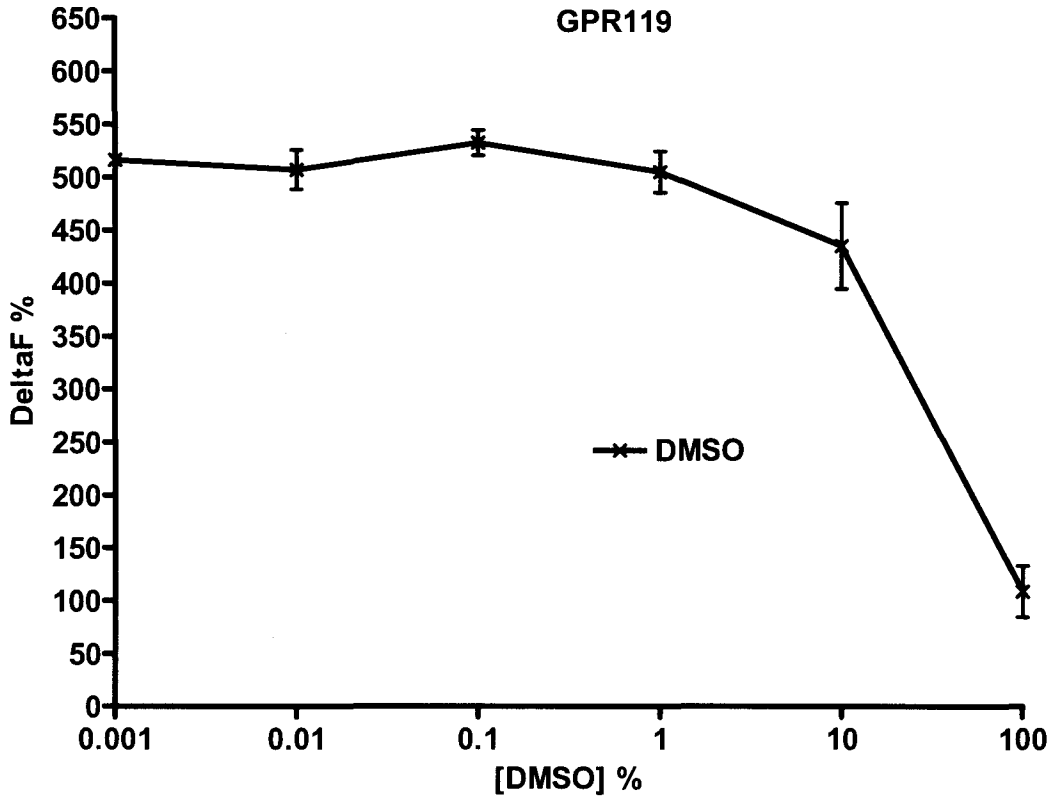

Figure 3.2. DMSO tolerance. HEK293 cells stably expressing GPR119 was treated with different concentrations of DMSO. Delta F % was calculated using the following formula: Delta F % = [(standard or sample ratio – ratio of the negative control) / ratio of the negative control] x 100. Values represent the mean ± S.E.M. of three independent experiments, each performed in duplicate.

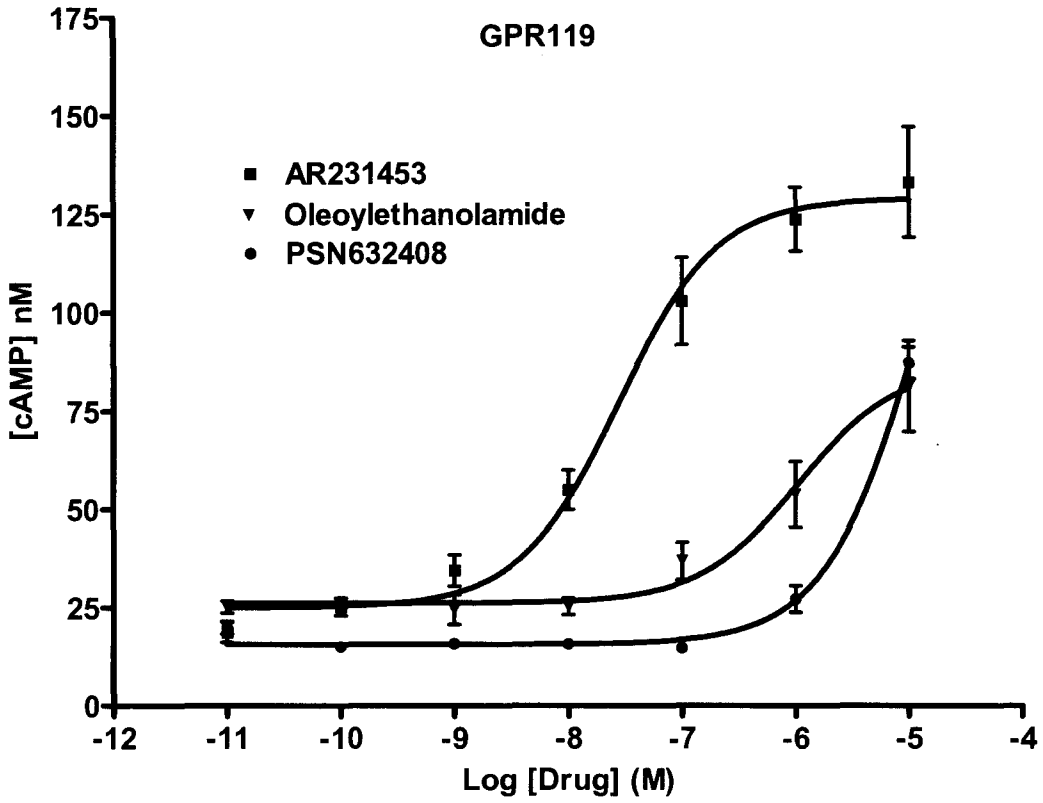

Figure 3.3. Pharmacological testing of known GPR119 agonists. HEK293 stably expressing GPR119 were treated with GPR119 agonists AR231453, oleoylethanolamide, and PSN632408 for 30 minutes. Results are expressed as actual cAMP levels determined from the standard curve. Values represent the mean ± S.E.M. of three independent experiments, each performed in duplicate.

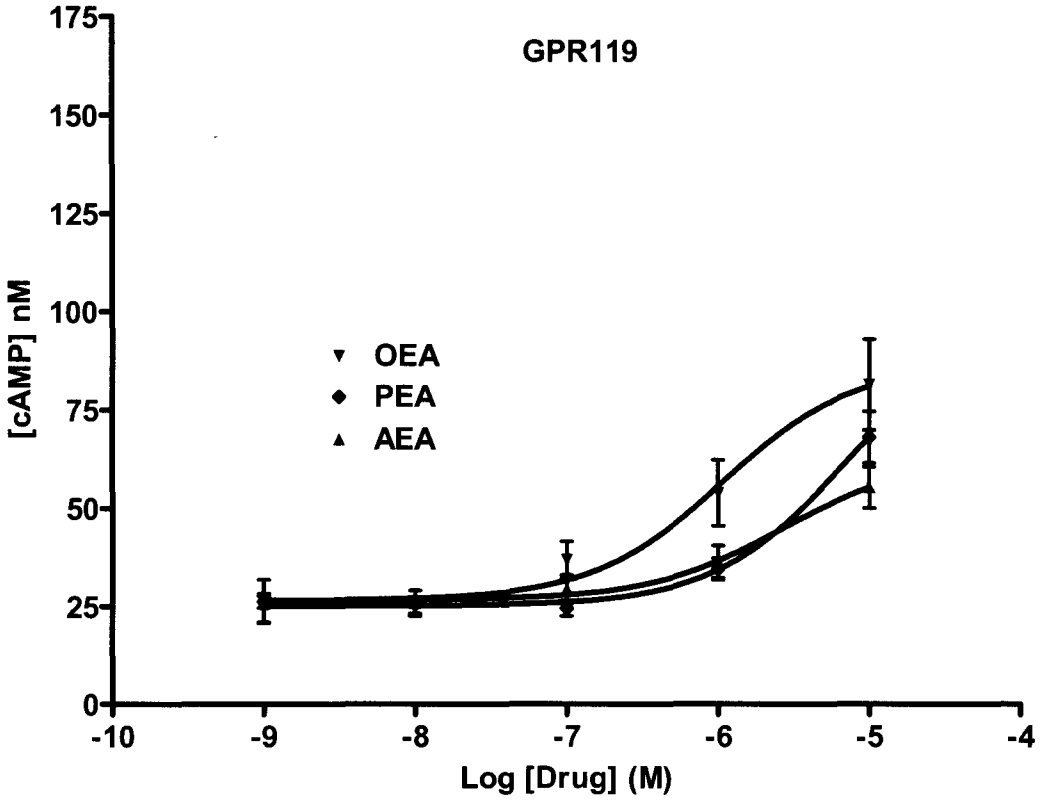

Figure 3.4. The effects of acyl chain unsaturation on the ability of fatty acid ethanolamides to activate GPR119. HEK293 stably expressing GPR119 were treated with OEA, PEA, and AEA for 30 minutes. Results are expressed as actual cAMP levels determined from the standard curve. Values represent the mean ± S.E.M. of three independent experiments, each performed in duplicate.

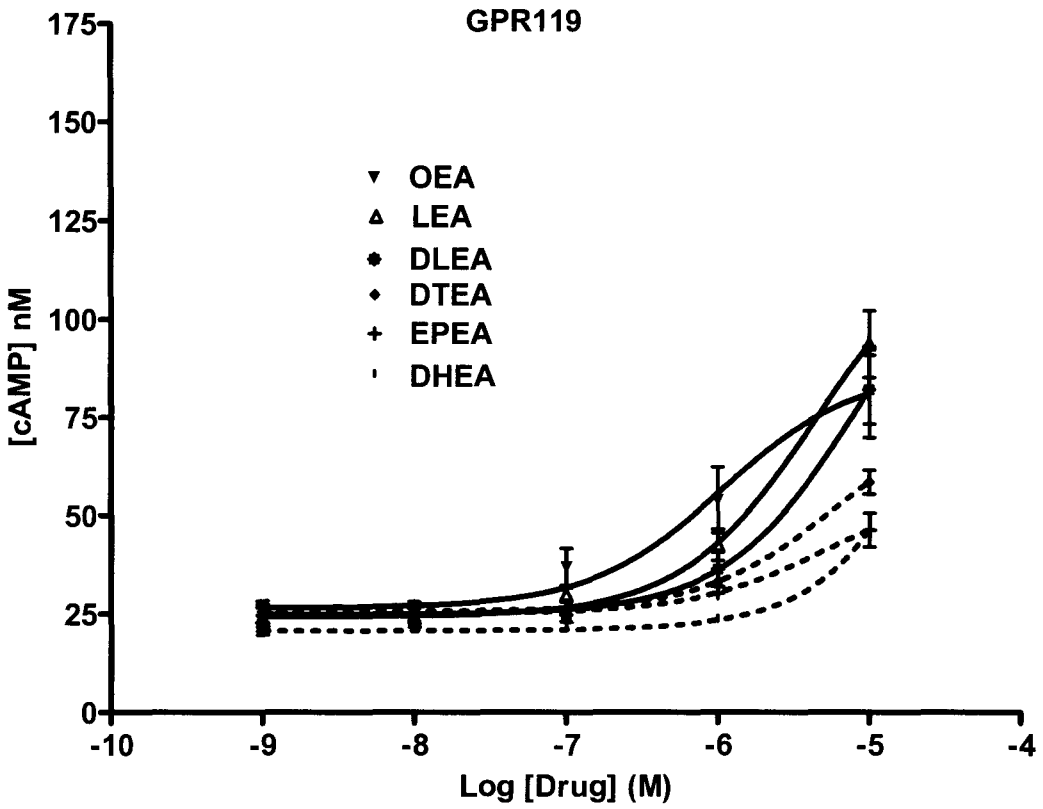

Figure 3.5. The effects of acyl chain unsaturation on the ability of fatty acid ethanolamides to activate GPR119. HEK293 stably expressing GPR119 were treated with Oleoyl ethanolamide (OEA), Linoleoyl ethanolamide (LEA), Dihomo-gamma-linolenoyl ethanolamide (DLEA), Docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA), Eicosapentaenoyl ethanolamide (EPEA) and Docosahexaenoyl ethanolamide (DHEA) for 30 minutes. Results are expressed as actual cAMP levels determined from the standard curve. Values represent the mean ± S.E.M. of three independent experiments each performed in duplicate.

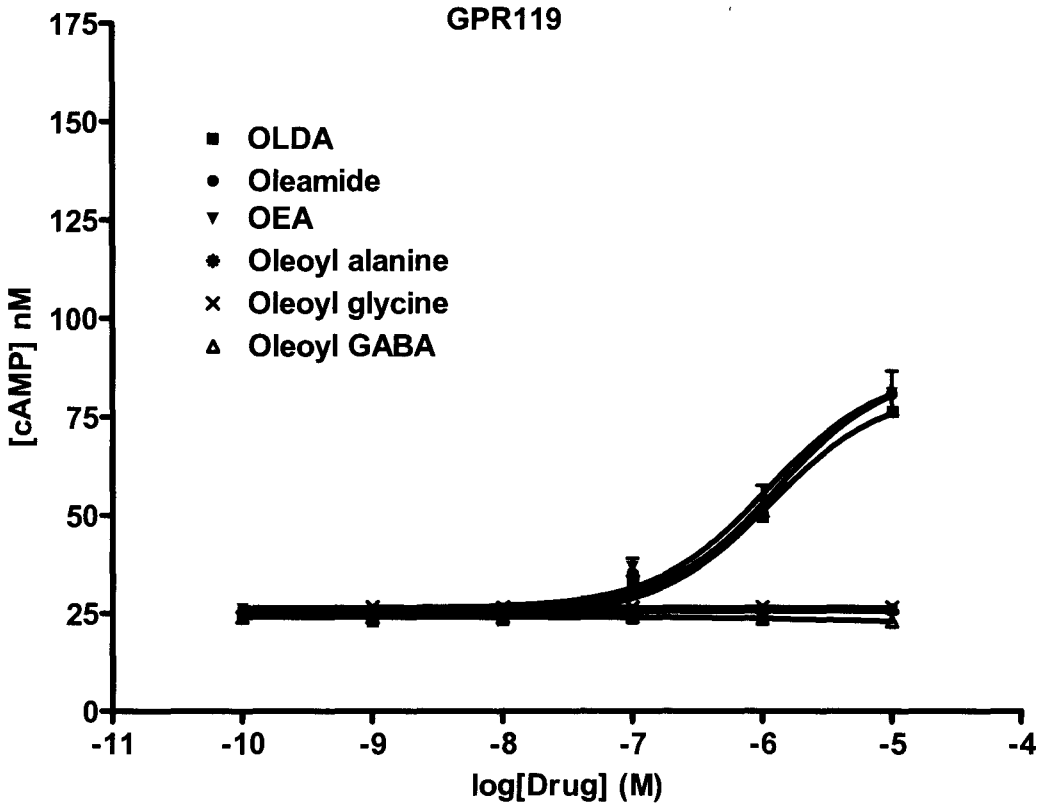

Figure 3.6. The effects of different head groups on the ability of oleoyl amides to activate GPR119. HEK293 stably expressing GPR119 were treated with OLDA, oleamide, OEA, oleoyl alanine, oleoyl glycine, and oleoyl GABA for 30 minutes. Results are expressed as actual cAMP levels determined from the standard curve. Values represent the mean ± S.E.M. of three independent experiments each performed in duplicate.

Table 3.1. Effect of known GPR119 agonists on increasing cAMP in HEK293 cells stably expressing GPR119.

Drug	EC_{50} (95% CI) (μM)
AR231453	.027 (0.013 to 0.058)
PSN632-408	1.28 (1.10 to 1.49)
Oleoylethanolamide	1.08 (0.59 to 1.98)

CI, confidence interval. Data shown are from three experiments each performed in duplicate.

Table 3.2. The effects of synthetic cannabinoids on increasing cAMP levels in HEK293 cells stably expressing GPR119.

Drug	EC_{50} (95% CI) (μM)
CP-55,950	NR
HU-210	NR
WIN55,212-2	NR

CI, confidence interval. NR, No response. Data shown are from experiments each performed in duplicate.

Table 3.3. The effects of acyl chain unsaturation on the ability of fatty acid ethanolamides to increase cAMP levels in HEK293 cells stably expressing GPR119.

Drug	**EC_{50} (95% CI) (μM)**
Linoleoyl ethanolamide	4.33 (1.48 to 12.7)
Docosahexaenoyl ethanolamide	NR
Dihomo-γ-linolenoyl ethanolamide	8.38 (0.91 to 77.2)
Docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide	6.02 (1.93 to 18.8)
Eicosapentaenoyl ethanolamide	5.09 (0.59 to 44.0)
Oleoylethanolamide	1.08 (0.59 to 1.98)
Anandamide	2.62 (0.36 to 19.3)
Palmitoylethanolamide	6.82 (1.10 to 42.5)

CI, confidence interval. NR, No response. Data shown are from at least three experiments each performed in duplicate.

Table 3.4. The effects of different head groups on the ability of oleoyl amides to increase cAMP levels in HEK293 cells stably expressing GPR119.

Drugs	**EC_{50} (95% CI) (μM)**
OLDA	1.17 (0.98 to 1.38)
Oleamide	1.28 (1.10 to 1.49)
OEA	1.08 (0.59 to 1.98)
Oleoyl alanine	NR
Oleoyl glycine	NR
Oleoyl GABA	NR

CI, confidence interval. NR, No response. Data shown are from experiments each performed in duplicate.

DISCUSSION

Agonist binding to GPR119 leads to Gs coupling and activation of adenylate cyclase (Chu et al., 2007b). As a result, there is an increase in intracellular cAMP levels which was measured as a decrease in HTRF signal in this study. We have shown that the HTRF HiRange cell-based cAMP assay is a suitable technology for screening ligands that may act on GPR119.

The Z'factor is a standard statistical parameter used to evaluate the robustness of a screening assay (Zhang et al., 1999). The Z'factor value can range between 0 and 1, with values approaching 1 indicates excellent assay robustness. A value of greater than 0.5 indicates a suitable difference between signal and background values with low variability. In this study we determined Z'factor according to the methods published previously (Zhang et al., 1999). The calculated Z'factor for the HTRF HiRange cell-based cAMP assay was 0.68. These results demonstrated that this assay is robust and suitable for screening ligands that activate GPR119 since the determined Z'factor is greater than 0.5.

Since most chemical compound libraries come pre-dissolved in dimethyl sulfoxide (DMSO), it is critical to determine the maximum concentration that a compound can be screened before DMSO reaches a concentration that is too high to be tolerated by the assay. Therefore, we determined the effect of DMSO on the HTRF HiRange cAMP cell-based assay. We tested DMSO at a variety of concentrations and the results showed that the assay can tolerate DMSO up to 1 %. These data indicate that the assay is suitable for screening ligands that may act on GPR119 at a DMSO concentration of less than 1 %.

To validate that the HTRF HiRange cell-based cAMP assay is suitable for screening ligands that may activate GPR119, we performed concentration-response studies for three previously reported GPR119 agonists. The rank order of potencies of these three known GPR119 agonists in enhancing cAMP levels in GPR119-expressing HEK293 cells was AR231453 > OEA = PSN632408. These data are consistent with previous reports regarding the potency of these GPR119 agonists (Overton et al., 2006; Semple et al., 2008). These results also confirmed the suitability of HTRF HiRange cell-based cAMP assay for screening potential novel ligands for GPR119.

Since the closest relatives of GPR119 are the cannabinoid receptors (Godlewski et al., 2009), in this study we tested the three prototypic synthetic cannabinoid agonists CP-55,940, HU-210, and WIN-55,212-2 for their potential activity towards GPR119 using the HTRF HiRange cAMP cell-based assay. Similar to the data reported by Overton et al. (2006), our study showed that the classical cannabinoid agonist HU-210, the non-classical, the bicyclic cannabinoid agonist CP-55,940, and the aminoalkylindole WIN-55,212-2 do not activate GPR119.

Recently, the fatty acid ethanolamide OEA has been reported to be a putative endogenous ligand for GPR119 (Overton et al., 2006). Overton and coworkers have also tested the endogenous cannabinoid agonist AEA and the saturated fatty-acid ethanolamide PEA for GPR119 activity in a yeast-based assay. Their results showed that OEA was the most efficacious, followed by PEA and then AEA. As shown by the concentration-response curves in this study, our results on OEA, PEA, and AEA are very much consistent with those reported by Overton et al. (2006).

Previously, based on the data with OEA, PEA, and AEA, Overton and co-workers have proposed that unsaturation in fatty acid aryl chain might be important for activating GPR119 (Overton et al., 2006) .

In this study, we report for the first time the structure activity relationship for GPR119 activation on a subset of fatty acid ethanolamides, including linoleoyl ethanolamide (LEA), dihomo-gamma-linolenoyl ethanolamide (DLEA), docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA), eicosapentaenoyl ethanolamide (EPEA), and docosahexaenoyl ethanolamide (DHEA). Overall, our new data in the present study provide direct evidence to further support the hypothesis that unsaturation in the acyl chain of fatty acid ethanolamides affects the ability of these compounds to activate GPR119.

Our results indicate that increasing unsaturation reduces the ability of these ligands to activate GPR119; with compounds containing 1-3 double bonds have significantly higher efficacy and potency than those compounds containing 4-6 double bonds.

Chu et al. (2010) reported that a diverse set of lipid amides, including OLDA and oleamide, activate GPR119. Thus, they suggested that there is a broad permissiveness in the amine-derived moieties (the head groups) of lipid amides for being an agonist for GPR119 (Chu et al., 2010). In the present study, we demonstrated that both OLDA and oleamide activate GPR119, with similar potency and efficacy as OEA. These data are consistent with the findings of Chu et al. (2010) and confirm the notion that there is a considerable level of permissiveness in the head group of oleoyl amides.

However, in the current study, we also demonstrated that oleoyl alanine, oleoyl glycine, and oleoyl GABA were unable to activate GPR119. These data suggest that although there are certain levels of permissiveness, in order to activate GPR119, there are also certain structural requirements for the head groups of oleoyl amides. An interesting observation is that all three compounds (oleoyl alanine, oleoyl glycine, and oleoyl GABA) that failed to activate GPR119 have a carboxcylic group. This suggests that the reason that these ligands failed to activate GPR119 might be due to either the spatial hindrance or the acidic nature of the carboxylate group.

Among the three oleoyl amides that activated GPR119, both OLDA and OEA contain hydrogen donating hydroxyl groups in their structure for potential hydrogen bonding interactions with the receptor. This indicates that these hydrogen donating groups might be important for their ability to interact and activate GPR119.

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