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Searching for novel ligands for the cannabinoid and related receptors.

Pritesh Prakash Kumar
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SEARCHING FOR NOVEL LIGANDS FOR THE CANNABINOID AND
RELATED RECEPTORS

By

Pritesh Prakash Kumar
BS., University of Kentucky, Lexington, KY; 2009
M.S., University of Louisville, KY; 2011

A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
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for the Degree of

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Department of Pharmacology and Toxicology
University of Louisville, Louisville KY

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A Dissertation Approved on

June 26, 2015

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DEDICATION

This dissertation is dedicated to my parents

Prakash Patel

And

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

ABSTRACT

SEARCHING FOR NOVEL LIGANDS FOR THE CANNABINOID AND
RELATED RECEPTORS

Pritesh Prakash Kumar

June 10, 2015

The first purpose of the present dissertation 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. The dissertation reporting these results demonstrated that by acting on CB2,
raloxifene enhances forskolin-stimulated cAMP accumulation in a concentration-
dependent manner. Furthermore, the 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. The discovery that raloxifene is as 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.

The second 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.

The third purpose of this dissertation was to investigate the structure-activity relationships of fatty acid amides for activating and desensitizing G protein-coupled receptor 119, a promising therapeutic target for both type 2 diabetes and obesity. Using novel fatty acid amides and detailed potency and efficacy analyses, the dissertation reporting these results demonstrated that degree of saturation in acyl chain and charged head groups of fatty acid amides have profound effects on the ability of these compounds to activate G protein-coupled receptor 119. In addition, the dissertation reporting these results demonstrated for the first time that pretreatments with G protein-coupled receptor 119 agonists desensitize the receptor and the degrees of desensitization caused by fatty acid amides correlate well with their structure-activity relationships in activating the receptor.

The fourth purpose of this dissertation was to use a fragment-based approach, categorical-SAR (cat-SAR), to model ligands for GPR119. Using compounds that are known GPR119 agonists and compounds that were confirmed experimentally that are not GPR119 agonists, four distinct cat-SAR models were developed. Using a leave-one out validation routine, the best GPR119 model had an overall concordance of 99 %, a sensitivity of 99 %, and a specificity of 100 %. The dissertation reporting these results from the in-depth fragment analysis of several known GPR119 agonists was consistent with previously reported GPR119 structure-activity relationship (SAR) analyses. Overall, while the dissertation reporting these results indicates the development a highly predictive cat-SAR model that can be potentially used to rapidly screen for prospective

GPR119 ligands the applicability domain must be taken into consideration. Moreover, the dissertation demonstrating these results was the first report that the cat-SAR expert system can be used to model G protein-coupled receptor ligands, many of which are important therapeutic agents.

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CHAPTER 1

INTRODUCTION

Marijuana (*Cannabis sativa*) is one of the oldest and most widely abused drugs has also been used for medicinal purposes by various cultures. The primary psychoactive constituent of marijuana is Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni & 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 & 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 & Breivogel, 1998; Childers & Deadwyler, 1996; Devane, Dysarz, Johnson, Melvin, & Howlett, 1988; Howlett, 1995). Several cDNAs and genes encoding cannabinoid (CB) receptors have been cloned, including CB1 and CB2 (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990; Munro, Thomas, & Abu-Shaar, 1993). Endogenous cannabinoid ligands have been isolated from the brain (Devane, Breuer, et al., 1992); high affinity cannabinoid mimetics with a variety of chemical structures have been synthesized, and subtype-selectiveligands for cannabinoid receptors are becoming available (Huffman, 2000; Palmer, Thakur, & Makriyannis, 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 (R. G. 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 (R. G. Pertwee, 2005). CB2 receptors have also been detected in the spleen and tonsils (R. G. 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 & Felder, 1997; Maneuf & Brotchie, 1997). It has been reported that activation of the CB2 receptor can produce stimulation of cAMP formation, as well (Rhee, Bayewitch, Avidor-Reiss, Levy, & Vogel, 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 & Brown, 1992; Felder et al., 1995;

Gebremedhin, Lange, Campbell, Hillard, & Harder, 1999; Henry & Chavkin, 1995;

Mackie & Hille, 1992; Mackie, Lai, Westenbroek, & Mitchell, 1995; Pan, Ikeda, &

Lewis, 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 & 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 & 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,

Compton, Mechoulam, & Martin, 1989; Mechoulam et al., 1988), the non-classical

cannabinoids typified by CP-55,940 (D'Ambra et al., 1992; Melvin, Milne, Johnson,

Wilken, & Howlett, 1995), the aminoalkylindoles (AAIs) typified by WIN-55,212-2

(Compton, Gold, Ward, Balster, & Martin, 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, Panu, & Miles, 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, Hanus, et al., 1992). 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, Schweitzer, & Piomelli, 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 & Makriyannis, 2002; Hollister, 1986; R. G. Pertwee, 2000, 2001a, 2001b; Piomelli, Giuffrida, Calignano, & Rodriguez de Fonseca, 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 & 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 intraperitoneal or oral administration (Barth & Rinaldi-Carmona, 1999). Other CB_1 antagonists have been reported, including AM-630

(K. Hosohata et al., 1997; Y. Hosohata et al., 1997; R. 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 & Rinaldi-Carmona, 1999; Rinaldi-Carmona et al., 1998). SR144528 displays sub-nanomolar affinity for both the rat spleen and cloned human CB2 receptors ($K_i = 0.60\pm0.13$ 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, Desnoyer, Carayon, Combes, & Casellas, 1999; Bouaboula et al., 1997). This constitutive activity can be blocked by the CB1-selective SR141716A and CB2-selective SR144528 antagonists, 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, Fyfe, & Reynet, 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, Offertaler, Wagner, & Kunos, 2009).

GPR119 Receptor Expression

GPR119 is primarily expressed in pancreatic beta-cells and enteroendocrine cells of the gastrointestinal tract (GI) (Z. L. Chu et al., 2007; L. M. Lauffer, Iakoubov, & Brubaker, 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 (Z. L. Chu et al., 2007). GPR119 immuno-reactivity was also found in the small intestine where it co-localizes with glucagon-like-peptide-1 (GLP-1) (Z. L. Chu et al., 2007). In addition, GPR119 has been found in the following pancreatic beta cell lines: NIT-1, MIN6, RIN5, HIT-T15 (Z. L. Chu et al., 2007; 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 (Z. L. Chu et al., 2007; 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 (R. M. Jones, Leonard, Buzard, & Lehmann, 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) (Z. L. Chu et al., 2007). It has been shown that GPR119 agonists activate adenylyl cyclase, increase cAMP, and increase protein kinase A activity in GPR119-expressing cells (Z. L. Chu et al., 2007; L. M. 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).

Recently, Hansen et al. (2011) identified 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, Holland, Seino, & Drucker, 2011). The first mechanism is that the increase in cAMP signaling directly leads to an enhanced glucose-dependent 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-dependent insulin secretion and also inhibits glucagon secretion, appetite, and delays gastric emptying (L. Lauffer, Iakoubov, & Brubaker, 2008).

Recently, endogenous and small molecule synthetic GPR119 agonists have been shown to stimulate insulin release (Z. L. Chu et al., 2007; 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-dependent insulin release (Chu et al., 2008). Furthermore, the insulinotropic effect of AR231453 was completely lost in GPR119-deficient mice, demonstrating the involvement of GPR119 (Z. Chu et al., 2007) .

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-deficient mice indicating that OEA and PSN632408 do not act

through similar mechanisms (Lan et al., 2009).

CHAPTER 2

IDENTIFICATION OF RALOXIFENE AS A NOVEL CB2 INVERSE AGONIST

INTRODUCTION

Two cannabinoid receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), have been identified and cloned (Matsuda et al., 1990; Munro et al., 1993). 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 (Howlett, 2005; Pertwee, 2005).

CB1 receptors are distributed in the central nervous system as well as several peripheral tissues (Howlett, 2005; R. G. Pertwee, 2005). CB2 receptors are primarily located in immune cells, including neutrophils, monocytes, natural killer cells, T cells, B cells, macrophages, mast cells, and microglia cells (Howlett, 2005; Pertwee, 2005). This distribution suggests an important role for the CB2 receptor in mediating many of the immumnomodulatory, 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 (Marriott & Huffman, 2008; Riether, 2012). However, it is estimated that pharmaceutical product development requires at least 10 to 15 years and costs between $500 million and $2 billion (Adams & Brantner, 2006; DiMasi, Hansen, & Grabowski, 2003).

Virtually all clinically used drugs exhibit effects on biological targets other than those for which they were designed. 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 (Carley, 2005a, 2005b). 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 (Carley, 2005a, 2005b).

In the dissertation reporting these results, a high throughput cAMP assay appropriate for testing novel ligands for CB2 receptor was validated. There are many cAMP assays available for screening purposes (Gabriel et al., 2003; Williams, 2004). 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 (Degorce et al., 2009; Gabriel et al., 2003). 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 variation and it eliminates the interference of compound auto-fluorescence. 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 these purposes (Degorce et al., 2009; Gabriel et al., 2003).

In an attempt to rapidly and efficiently identify drugs that may act as agonist or inverse agonist for CB2, a library of compounds consisting 640 FDA-approved drugs was screened using the validated high throughput cAMP assay. All of the compounds in the library have well-characterized bioactivity, bioavailability, and safety profiles that could enhance drug repurposing. The rationale 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 (Muchmore, 2000; Riggs & Hartmann, 2003). As a result, not only does raloxifene decrease the risk of vertebral fractures, it is also reduces the prevalence of hormone-positive breast cancer (Muchmore, 2000; Riggs & Hartmann, 2003). In the dissertation reporting these results, the 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, the pharmacological profile of raloxifene was investigated for CB2 by conducting cell-based cAMP accumulation assays, as well as competitive radioligand binding assays. This dissertation provides the first evidence that raloxifene is a novel CB2 inverse

agonist. The 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.

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). 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).

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 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 2000g. The cells were resuspended in cell buffer (DMEM plus 0.2 % fatty acid free bovine serum albumin) and centrifuged a second time at 2000g 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 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. To assess receptor antagonism, HEK293 cells stably expressing CB2 were pre-incubated for 20 minutes with vehicle or raloxifene at a concentration of 1 or 10 μM before subject to stimulation with cannabinoid agonists.

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.

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.

Data Analysis

Data analyses for cell-based HTRF cAMP assays were performed based on the ratio of fluorescence intensity of each well at 620 nm and 665 nm. Data are expressed as

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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 Prism program, GraphPad Software, San Diego, CA) to generate the curves of the tested compounds. Data from ligand binding assays were analyzed, and competition binding curves were generated with the non-linear regression analyses using the Prism program.

RESULTS

Z´Factor Determination

To determine the Z´ value, experiments were performed in 384-well plates using many replicates of the cell-based HTRF cAMP assay with positive and negative controls (See Fig. 2.1). For positive controls, the HEK293 cells expressing CB2 were treated with the 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 7 minutes. The Z´ value was calculated using the formula: Z´ = 1 - (3 x [(standard deviation of negative control) + (standard deviation of positive control)] / [(mean of positive control) – (mean of negative control)] (Zhang, Chung, & Oldenburg, 1999). In the dissertation reporting these results, the Z´ factor was determined to be 0.79.

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, the effect of DMSO was tested at concentrations ranging from 0.01% to 100 %. As shown in Fig. 2.2, the cell-based HTRF cAMP assay for CB2 can tolerate DMSO up to 1 % without any loss of signal.

Pharmacological Testing of Known Cannabinoid Agonists

The ability of cannabinoid agonists to activate CB2 was verified using the HTRF cAMP assay. As shown in Fig. 2.3, in HEK293 cells stably expressing CB2, HU-210, CP-55,940, and WIN55,212-2 inhibited forskolin-stimulated cAMP accumulation in a concentration-dependent manner, with a rank order of potency of HU-210 > CP-55,940 >

WIN55,212-2. In addition, these three compounds failed to elicit any response in HEK293 cells transfected with an empty vector (data not shown).

Effects of Raloxifene on Forskolin-stimulated cAMP Accumulation

In an attempt to find novel ligands for CB2, each compound from a chemical library containing 640 FDA-approved drugs was tested for its ability to activate CB2. The screening of this library resulted in the identification of raloxifene as a potential CB2 inverse agonist.

As shown in Fig. 2.4, in HEK293 cells stably expressing CB2, the cannabinoid agonist CP-55,940 concentration-dependently inhibited forskolin-stimulated cAMP production. Most importantly, the dissertation reporting these results was the first report that raloxifene behaved as an inverse agonist for CB2 by enhancing forskolin-stimulated cAMP accumulation in a concentration-dependent manner. Furthermore, neither CP-55,940 nor raloxifene had any effects on forskolin-stimulated cAMP accumulation in empty vector- transfected HEK293 cells (data not shown).

Competition of [³H]CP-55,940 Binding by Raloxifene

In order to investigate whether raloxifene binds to the CB2 receptor, competition ligand binding experiments using membranes prepared from HEK293 cells stably transfected with CB2 were performed. As shown in Fig. 2.5, the cannabinoid agonist CP-55,940 competed for specific [³H]CP-55,940 binding. Furthermore, Raloxifene was also able to compete for specific [³H]CP-55,940 binding in a concentration-dependent manner. In addition, there was no detectable level of specific [³H]CP-55,940 binding in membranes prepared from HEK293 cells transfected with an empty vector (data not shown).

Antagonism of Cannabinoid Agonist-induced Inhibition of Forskolin-stimulated cAMP Accumulation by Raloxifene

As shown in Fig. 2.6, in HEK293 cells stably expressing CB2, the cannabinoid agonists CP-55, 940, HU-210, and WIN55212-2 concentration-dependently inhibited forskolin-stimulated cAMP production. Most importantly, in a concentration-dependent manner, 1 μM and 10 μM raloxifene pretreatments resulted in a rightward, parallel shift of the concentration-response curves for the three cannabinoid agonists (Fig. 2.6A-C).
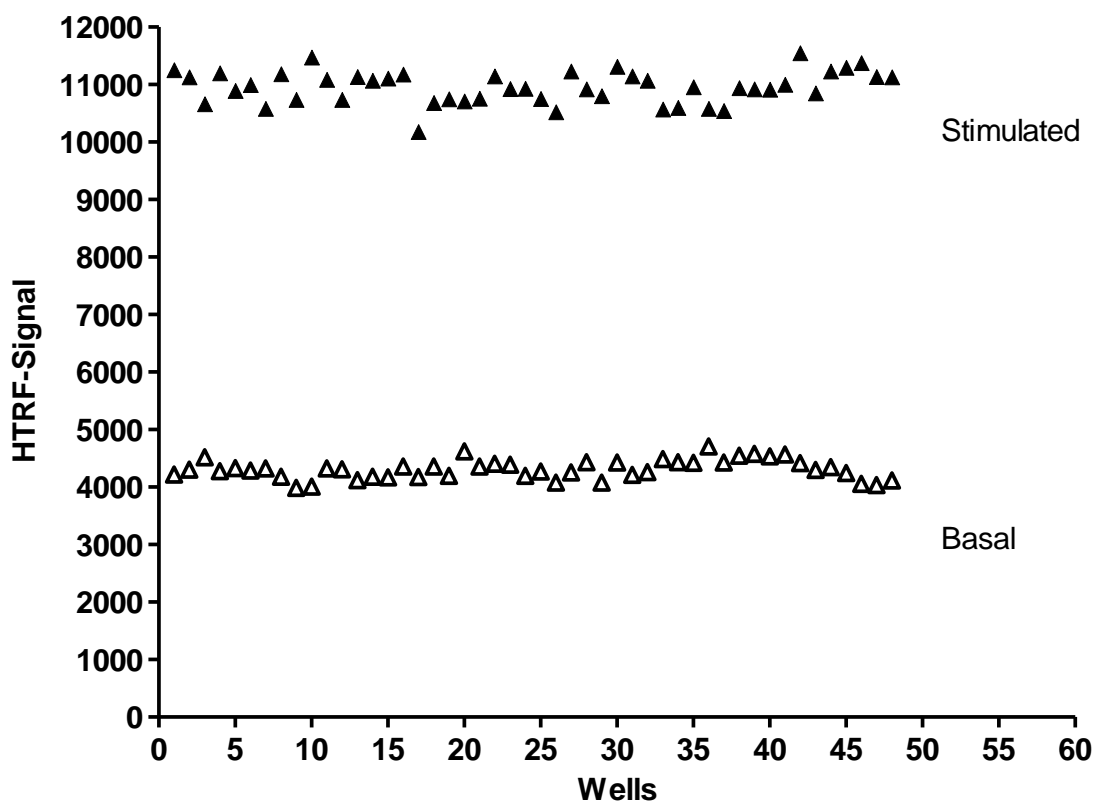
Figure 2.1. Z´ factor determination. The solid symbols represent positive controls (cells stimulated with 100 nM CP-55,940), while the open symbols represent negative controls (cells stimulated with vehicle). The Z´ factor was calculated to be 0.79 using 48 positive and 48 negative control points.
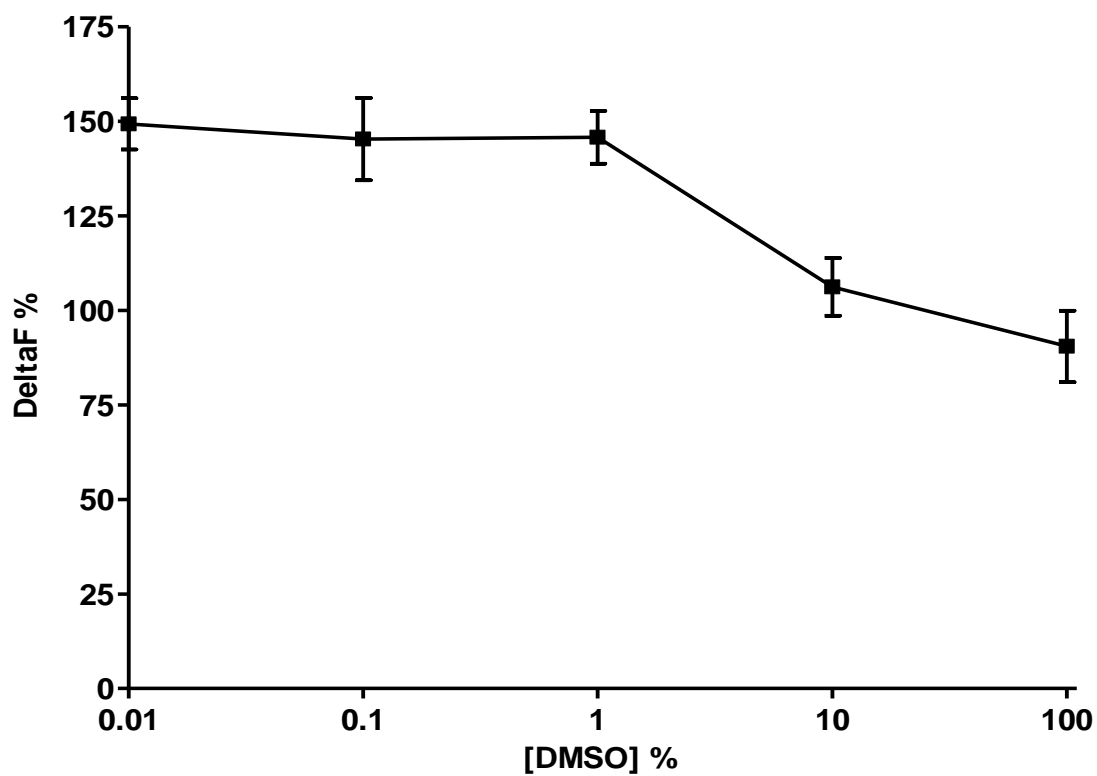
Figure 2.2. DMSO tolerance. HEK293 cells stably expressing CB2 were treated with different concentrations of DMSO. Delta F % was calculated using the following formula: Delta F % = [(standard or sample ratio − ratio of the negative control) / ratio of the negative control] x 100. Data shown represent the mean ± S.E.M. of three experiments each performed in duplicate.
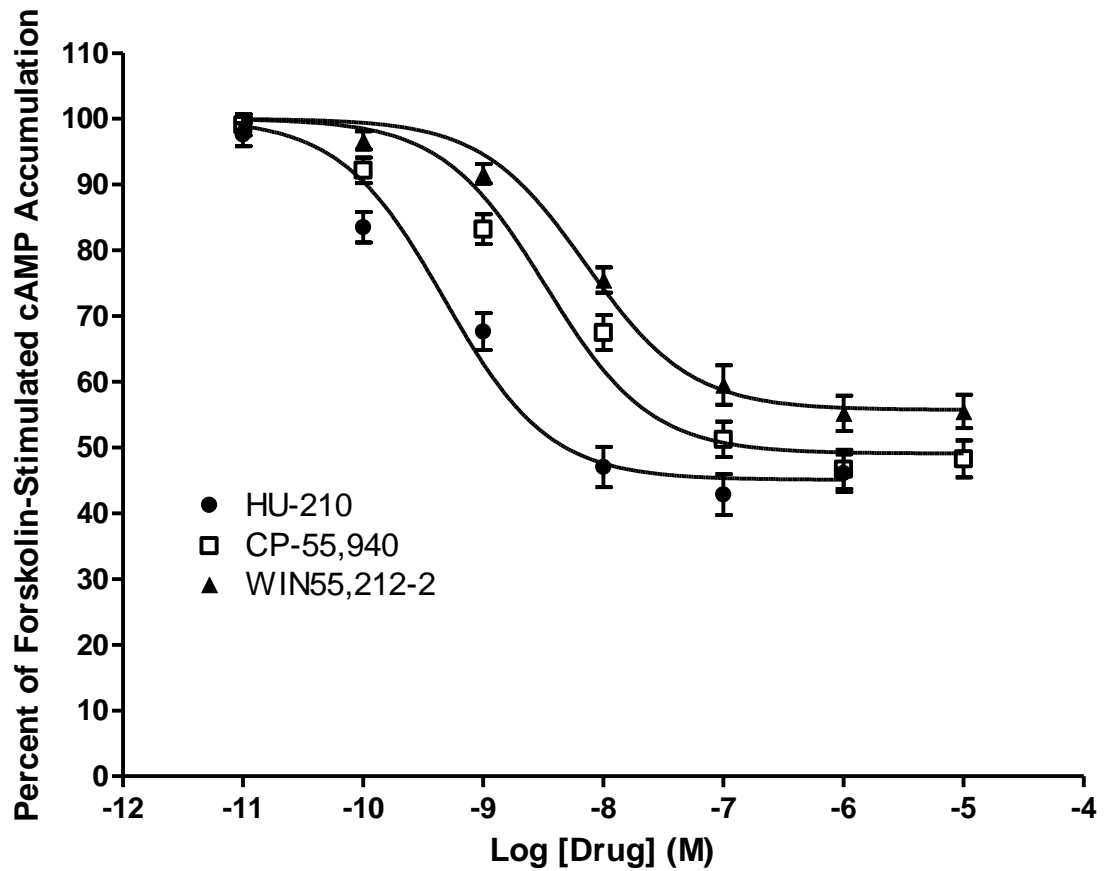
Figure 2.3. Pharmacological testing of known cannabinoid agonists. HEK293 cells stably expressing CB2 were treated with different concentrations of cannabinoid agonists HU-210, CP-55,940, and WIN55,212-2 for 7 minutes. Results are expressed as percent forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of five independent experiments.
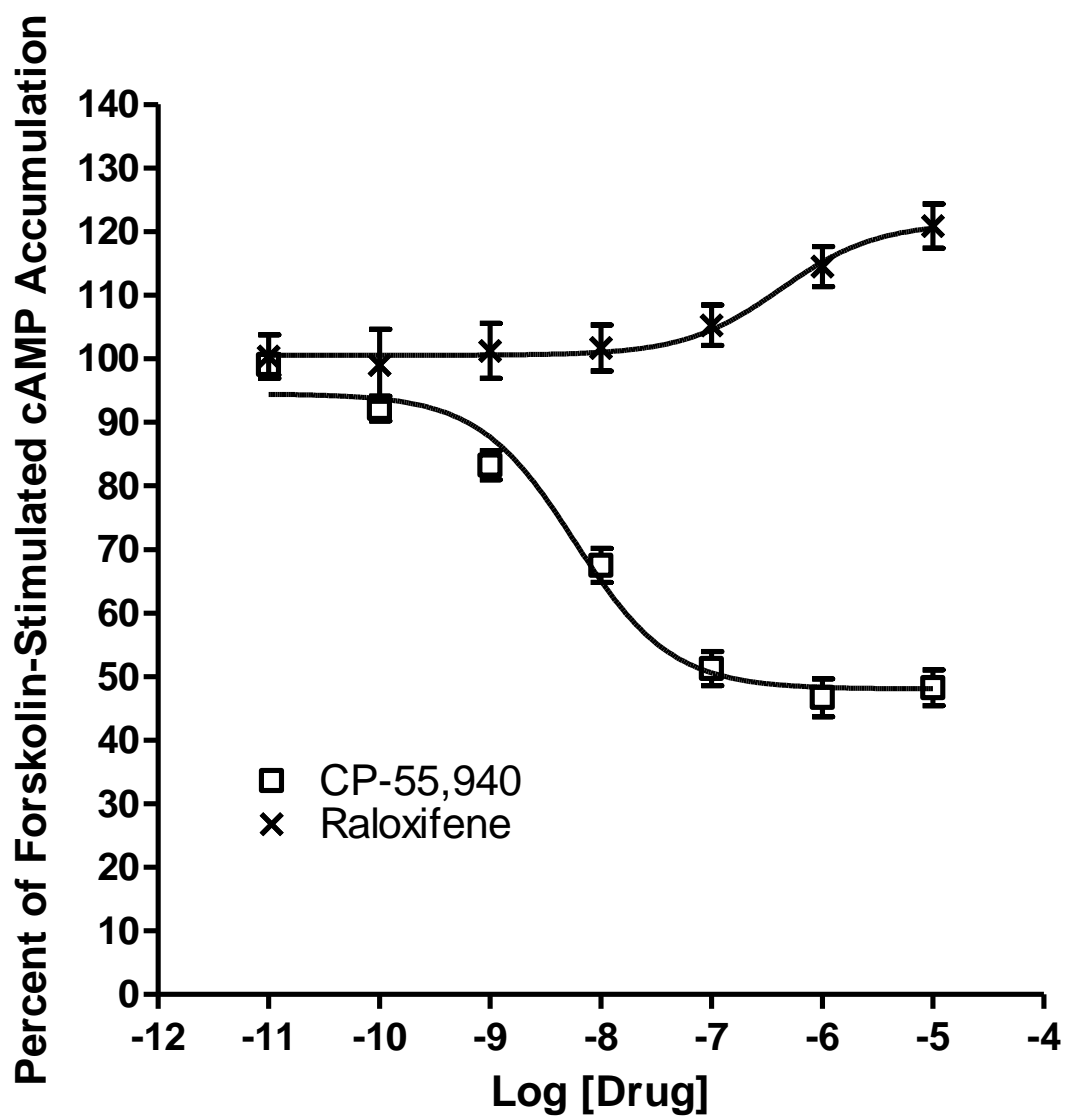
Figure 2.4. Effects of raloxifene on forskolin-stimulated cAMP accumulation.

HEK293 cells stably expressing CB2 were treated with different concentrations of CP-55,940 and raloxifene for 7 minutes. Results are expressed as percent forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of five independent experiments.
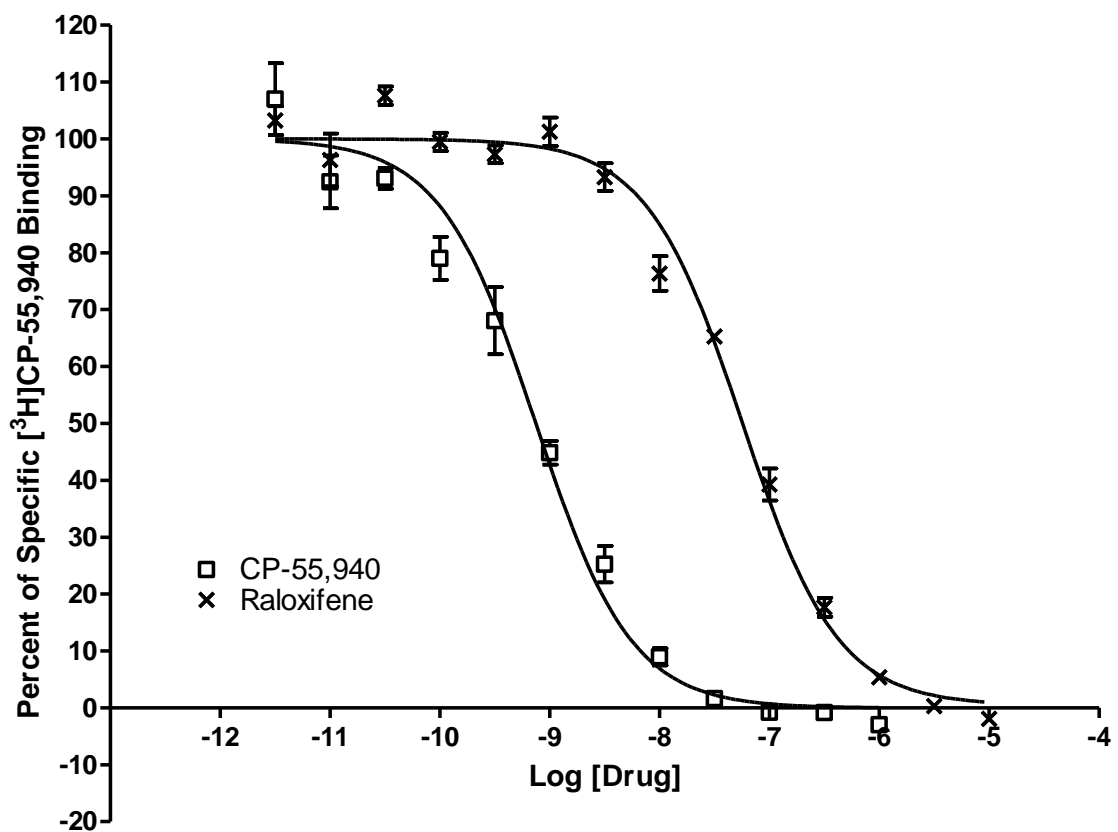
Figure 2.5. Competition of [³H]CP-55,940 binding by raloxifene. CP-55,950 and

raloxifene were used to compete for specific [³H] CP-55,940 binding to membranes

prepared from HEK293 cells stably expressing CB2. Data shown represent the mean ±

SEM of three experiments performed in duplicate.
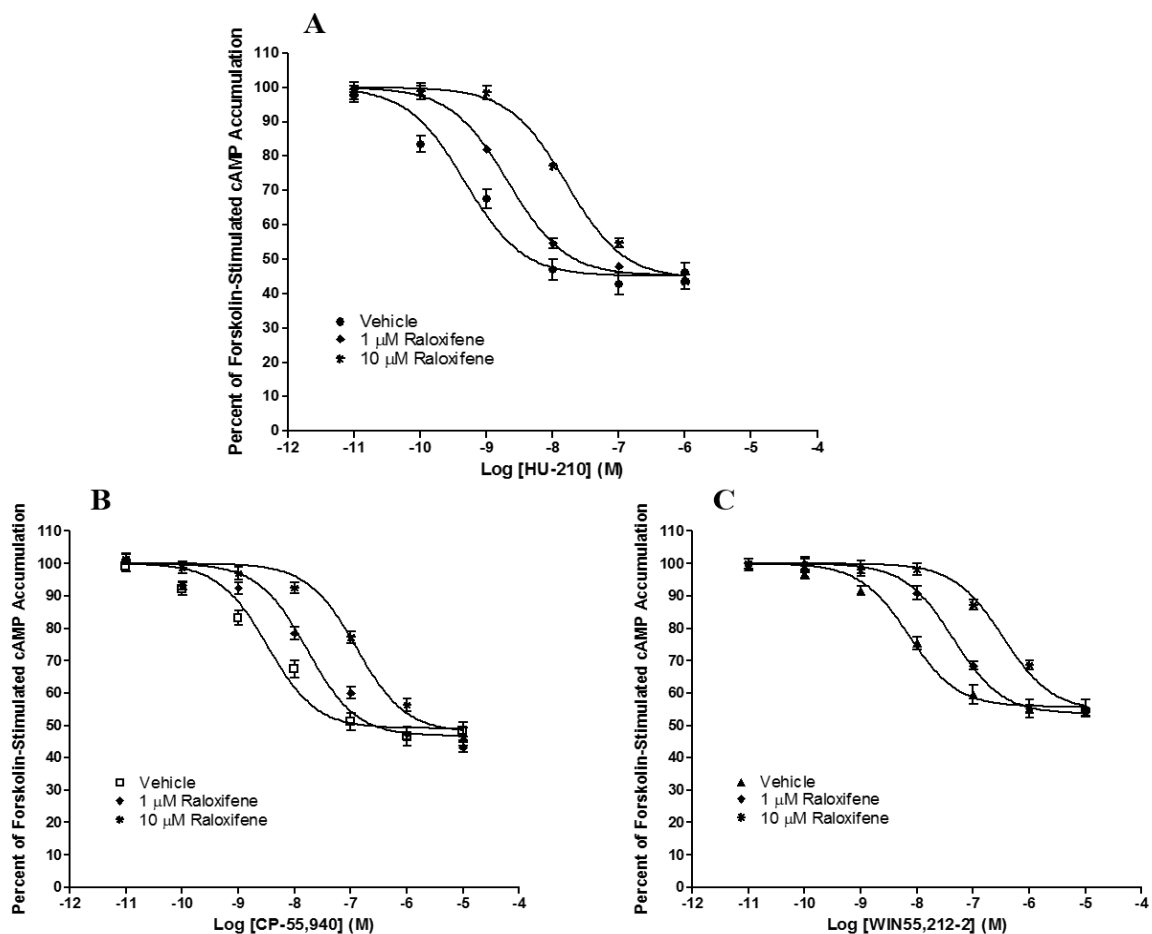
Figure 2.6. Antagonism of cannabinoid agonist-induced inhibition of forskolin-stimulated cAMP accumulation by raloxifene. HEK293 cells stably expressing CB2 were pre-incubated for 20 minutes with vehicle or raloxifene at a concentration of 1 or 10 µM before subject to stimulation with cannabinoid agonists HU-210, (B) CP-55,940, and (C) WIN55,212-2 for 7 minutes. Results are expressed as percent forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of five independent experiments.

DISCUSSION

Agonist binding to CB2 leads to G_i coupling and inhibition of adenylate cyclase (Howlett, 2005; Pertwee, 2005). As a result, there is a decrease in intracellular cAMP levels that was measured as an increase in HTRF signal. The dissertation reporting these results was the first report of a validated cell-based, HTRF cAMP assay for screening novel ligands for CB2.

The Z´ factor is a standard statistical parameter used to evaluate the robustness of a high throughput assay (Zhang et al., 1999). The Z´ factor can range between 0 and 1, with values approaching 1 indicating excellent assay robustness. In this dissertation reporting these results the calculated Z´ factor for the assay was 0.79. Since a Z´ factor greater than 0.5 indicates a suitable difference between signal and background values with low variability, the results demonstrate that the cell-based, HTRF cAMP assay is robust and suitable for screening ligands that activate CB2.

Since most chemical compound libraries come pre-dissolved in dimethylsulfoxide (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 (Williams, 2004). Therefore, the effect of DMSO on the cell-based, HTRF cAMP assay was tested. DMSO was tested 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 CB2 at a DMSO concentration of less than 1 %.

To validate further that the cell-based, HTRF cAMP assay is suitable for assaying ligands that may activate CB2, concentration-response studies for three known cannabinoid agonists was performed. The rank order of potency of these agonists in

inhibiting cAMP levels in HEK293 cells expressing CB2 was HU-210 > CP-55,940 > WIN55,212-2. These data are consistent with previous reports regarding the potency of these CB2 agonists (Howlett, 2005; Pertwee, 2005). These results also confirmed the suitability of this cell-based, HTRF cAMP assay for testing ligands for CB2.

In an attempt to discover novel ligands for CB2, each compound from a chemical library containing 640 FDA-approved drugs was tested for its ability to activate CB2 using the validated HTRF cAMP assay. If a compound is an agonist, it will inhibit the forskolin-stimulated cAMP response, which is shown as an increase in HTRF signal. In contrast, if a compound is an inverse agonist, it will further increase the forskolin-stimulated cAMP response, which is characterized as a decrease in HTRF signal. The screening of the 640 FDA-approved drug library at 1 μM resulted in the identification of raloxifene as a potential inverse agonist for CB2, since it caused a decrease in HTRF signal.

In previous reports, it has been demonstrated that CB2 receptors expressed in HEK293 cells exhibit constitutive activity, since the expression of CB2 caused a decrease of cellular cAMP levels compared with vector transfected HEK293 cells (Feng & Song, 2003). In addition, previously it has been shown that SR144528, a known inverse agonist for CB2, is able to enhance forskolin-stimulated cAMP accumulation in HEK293 cells stably expressing CB2 (Feng & Song, 2003). Similar to SR144528, raloxifene was able to enhance cAMP accumulation concentration-dependently in HEK293 cells stably expressing human CB2. Since raloxifene did not have any effect on forskolin-stimulated cAMP accumulation in empty vector-transfected HEK293 cells, this suggests that the effect of raloxifene on cAMP accumulation was mediated through CB2 receptor.

Consistent with previous reports (Song, Slowey, Hurst, & Reggio, 1999), the cannabinoid agonist CP-55,940 competed concentration-dependently the specific binding of [^{3}H]CP-55,940 to CB2. Similarly, raloxifene was able to compete, in a concentration-dependent manner, for specific [^{3}H]CP-55,940 binding to CB2. These data further demonstrate that raloxifene acted on the same receptor as the cannabinoid agonist CP-55,940.

To further characterize the pharmacological properties of raloxifene, the dissertation reporting these results evaluated its ability to antagonize the effects of the synthetic cannabinoid agonists CP-55,940, HU-210, and WIN55,212-2. Raloxifene concentration-dependently caused rightward shifts of the CP-55,940, HU-210, and WIN55,212-2 concentration-response curves. These data indicate that the raloxifene antagonism is most likely competitive in nature, as these rightward shifts were parallel and were not associated with any change in the efficacy of these agonists.

Raloxifene belongs to the class of selective estrogen receptor modulators (SERMs), which exhibit estrogen agonist activity in some target tissues while exert estrogen antagonist activity in other tissues (Muchmore, 2000; Riggs & Hartmann, 2003). Raloxifene has been approved for the treatment and prevention of post-menopausal osteoporosis and is currently under study for other therapeutic indications such as breast cancer (Muchmore, 2000; Riggs & Hartmann, 2003).

Estrogen deficiency is the main cause of post-menopausal osteoporosis (Muchmore, 2000; Riggs & Hartmann, 2003). When estrogen is deficient, bone turnover increases, and bone resorption increases more than bone formation, leading to bone loss. The effects of raloxifene on bone have been investigated in great detail and are well

established. A large clinical trial, the Multiple Outcomes of Raloxifene Evaluation (MORE), was conducted in post-menopausal women with osteoporosis. The results from the MORE trial demonstrated that raloxifene reduced the incidence of new vertebral fractures by 30% and 50% (in women with and without prevalent vertebral fractures, respectively) compared to placebo (Muchmore, 2000; Riggs & Hartmann, 2003).

The biological actions of raloxifene are well known to be mediated through binding to estrogen receptors (Muchmore, 2000; Riggs & Hartmann, 2003). However, to our knowledge, the dissertation reporting these results is the first to demonstrate that raloxifene is an inverse agonist for CB2. Recently, there is accumulating evidence to suggest that CB2 inverse agonists are effective for controlling inflammatory cell migration, thus is useful for a variety of inflammatory diseases, such as arthritis and multiple sclerosis [19]. Thus, the identification of raloxifene as a novel CB2 inverse agonist suggests that this FDA-approved drug for post-menopausal osteoporosis has great potential to be repurposed for other therapeutic indications.

Cannabinoids and their receptors play important roles in bone metabolism by regulating bone cell function (Idris, Sophocleous, Landao-Bassonga, van't Hof, & Ralston, 2008). It has been found that CB2 inverse agonists such as SR144528 are able to inhibit osteoclast formation and bone resorption, thus reducing bone loss (Idris et al., 2008). Therefore, the discovery that raloxifene is a CB2 inverse agonist implicates a possible novel mechanism for the anti-osteoporosis activity of raloxifene--it might be partially mediated through the CB2 cannabinoid receptor in the bone.

In summary, the present dissertation validated a cell based, HTRF cAMP assay for testing ligands for CB2, and using this assay, a library of FDA-approved drugs against

CB2 was screened. The dissertation reporting these results was the first report that raloxifene binds to CB2 and is an inverse agonist for CB2. The discovery that raloxifene 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 raloxifene.

CHAPTER 3

CB2 CANNABINOID RECEPTOR IS A NOVEL TARGET FOR THIRD-GENERATION SELECTIVE ESTROGEN RECEPTOR MODULATORS BAZEDOXIFENE AND LASOFOXIFENE

INTRODUCTION

Selective estrogen receptor modulators (SERMs) exhibit a unique pharmacological profile (Maximov, Lee, & Jordan, 2013; Riggs & Hartmann, 2003). 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 (Maximov et al., 2013; Riggs & Hartmann, 2003).

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 (Maximov et al., 2013; Riggs & Hartmann, 2003), 2) Raloxifene, a benzothiophene, is a member of second generation SERMs (Maximov et al., 2013; Riggs & Hartmann, 2003), 3) Third generation SERMs are typified by indole-based bazeoxifene (Maximov et al., 2013; Riggs & Hartmann, 2003; Stump, Kelley, & Wensel, 2007) and

napthalene derivative lasofoxifene (Gennari, Merlotti, Martini, & Nuti, 2006; Maximov et al., 2013; Riggs & Hartmann, 2003).

Both first generation SERM tamoxifen and second generation SERM raloxifene have been approved by FDA to be used in the United States (Maximov et al., 2013; Riggs & Hartmann, 2003). 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 (Maximov et al., 2013; Riggs & Hartmann, 2003). In 2009, third generation SERMs bazedoxifene and lasofoxifene were approved for use in the Europe to prevent and treat post-menopausal osteoporosis under the trade names Conbriza and Fablyn, respectively (Gennari et al., 2006; Maximov et al., 2013; Riggs & Hartmann, 2003; Stump et al., 2007).

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 (Howlett, 2005; Matsuda et al., 1990; Munro et al., 1993; R. G. Pertwee, 2005). 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 (Howlett, 2005; Matsuda et al., 1990; Munro et al., 1993; R. G. Pertwee, 2005). 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 (Howlett, 2005; R. G. Pertwee, 2005).

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 (Marriott & Huffman, 2008;

Riether, 2012). 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 to 15 years (Adams & Brantner, 2006; DiMasi et al., 2003). 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 (Carley, 2005a, 2005b). The advantages of drug repurposing include: 1) Existing approval by regulatory agencies for human use, and 2) Existing human pharmacokinetic and safety data (Carley, 2005a, 2005b).

Previously, in an attempt to rapidly and efficiently identify drugs that may act as agonists or inverse agonists for CB2, a library of 640 FDA-approved drugs was screened using a validated high throughput cAMP assay (Kumar & Song, 2013). These previous efforts resulted in the identification of raloxifene (Evista), a second generation SERM, as a novel CB2 inverse agonist (Kumar & Song, 2013).

The previous finding that raloxifene is an inverse agonist for the CB2 cannabinoid receptor prompted the hypothesis that third-generation SERMs bazedoxifene and lasofoxifene may also act as inverse agonists for CB2. To test this hypothesis, the dissertation reporting these results 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, the dissertation reporting these results was the first report to demonstrate that bazedoxifene and lasofoxifene are inverse agonists for the CB2 cannabinoid receptor. The findings indicate that these two marketed drugs can potentially

be repurposed for novel therapeutic indications for which CB2 is a target. The discovery that CB2 is a novel target for bazedoxifene and lasofoxifene suggests novel mechanisms of actions for these third-generation SERMs.

MATERIALS AND METHODS

Materials

Refer to Chapter 2 – Section Materials and Methods.

Cell Transfection and Culture

Refer to Chapter 2 – Section Materials and Methods.

Cell-based HTRF cAMP assay

Refer to Chapter 2 – Section Materials and Methods.

Note: 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.

Cell harvesting and membrane preparation

Refer to Chapter 2 – Section Materials and Methods.

Ligand binding assays

Refer to Chapter 2 – Section Materials and Methods.

Statistical Analysis

Refer to Chapter 2 – Section Materials and Methods.

RESULTS

Competition of specific [³H]CP-55,940 binding by bazedoxifene and lasofoxifene

In order to investigate whether bazedoxifene or lasofoxifene binds to the CB2 receptor, competition ligand binding experiments using membranes prepared from HEK293 cells stably expressing CB2 were performed. As shown in Fig. 3.1, bazedoxifene and lasofoxifene were able to compete for specific [³H]CP-55,940 binding in a concentration-dependent manner. In addition, there was no detectable level of specific [³H]CP-55,940 binding in membranes prepared from HEK293 cells transfected with an empty vector (data not shown).

Effects of bazedoxifene and lasofoxifene on forskolin-stimulated cAMP accumulation

In order to investigate whether bazedoxifene and lasofoxifene act on the CB2 receptor, cAMP accumulation assays using HEK293 cells stably expressing CB2 were perrformed. The dissertation reporting these results was the first report that bazedoxifene and lasofoxifene behaved as inverse agonists for CB2 by enhancing forskolin-stimulated cAMP accumulation in a concentration-dependent manner (Fig. 3.2). Furthermore, bazedoxifene and lasofoxifene did not have any effects on forskolin-stimulated cAMP accumulation in empty vector- transfected HEK293 cells (data not shown).

Antagonism of cannabinoid agonist-induced inhibition forskolin-stimulated cAMP accumulation by bazedoxifene

As shown in Fig. 3.3A–C, in HEK293 cells stably expressing CB2, the cannabinoid agonists CP-55,940, HU-210, and WIN55,212–2 concentration-dependently inhibited forskolin-stimulated cAMP production. Most importantly, in a concentration-

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dependent manner, 1 and 10 μM bazedoxifene pretreatments resulted in a rightward, parallel shift of the concentration-response curves for the three cannabinoid agonists (Fig. 3.3A–C).

Antagonism of cannabinoid agonist-induced inhibition forskolin-stimulated cAMP accumulation by lasofoxifene

As shown in Fig. 3.4A–C, in HEK293 cells stably expressing CB2, in a concentration-dependent manner, pretreatment with lasofoxifene at a concentration of 1 and 10 μM resulted in a rightward, parallel shift of the concentration-response curves for three cannabinoid agonists CP-55, 940, HU-210, and WIN55,212–2.
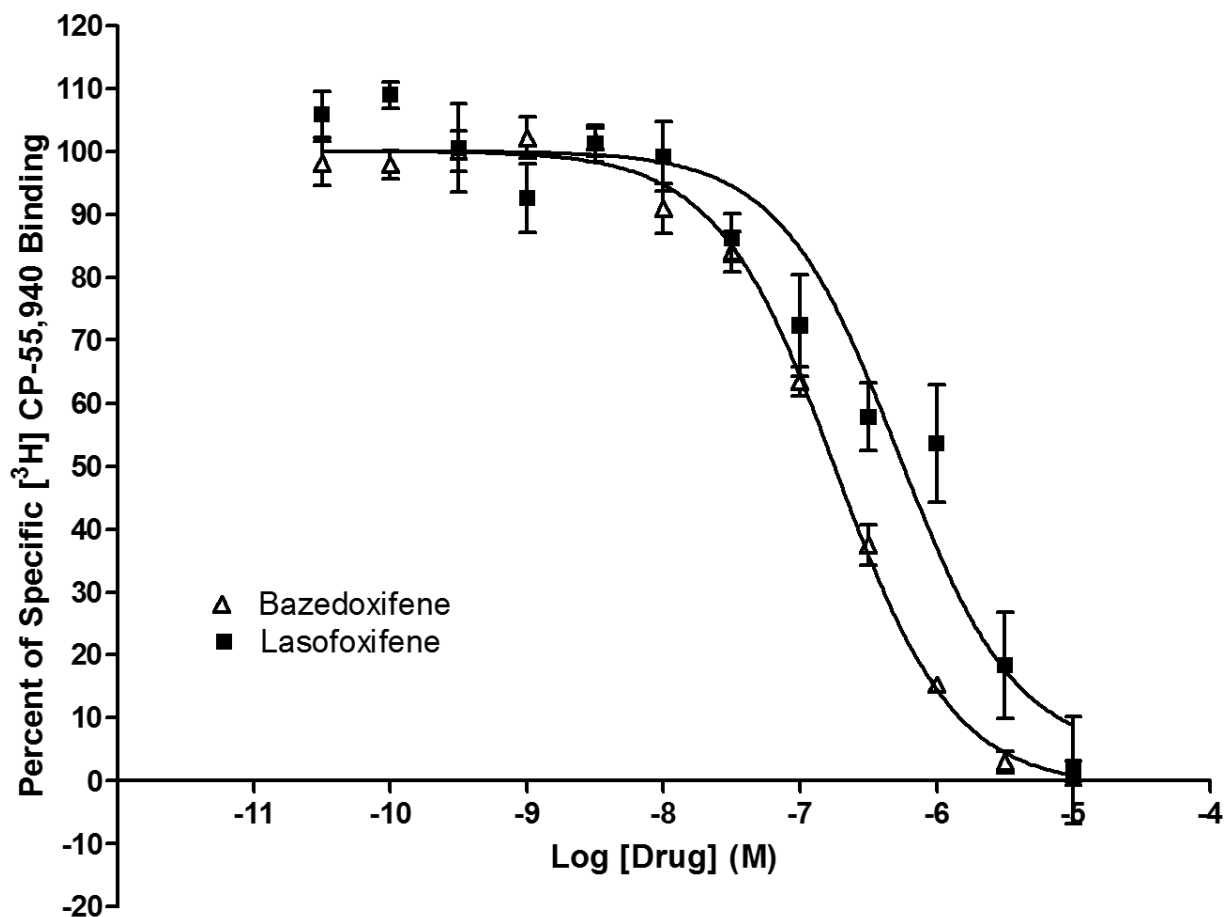
Fig. 3.1 Bazedoxifene and lasofoxifene bind to the CB2 cannabinoid receptor. Competition of specific [^{3}H]CP-55,940 binding by bazedoxifene and lasofoxifene. Bazedoxifene and lasofoxifene were used to compete for specific [^{3}H] CP-55,940 binding to membranes prepared from HEK293 cells transfected with CB2. Data shown represent the mean ± SEM of three experiments performed in duplicate.
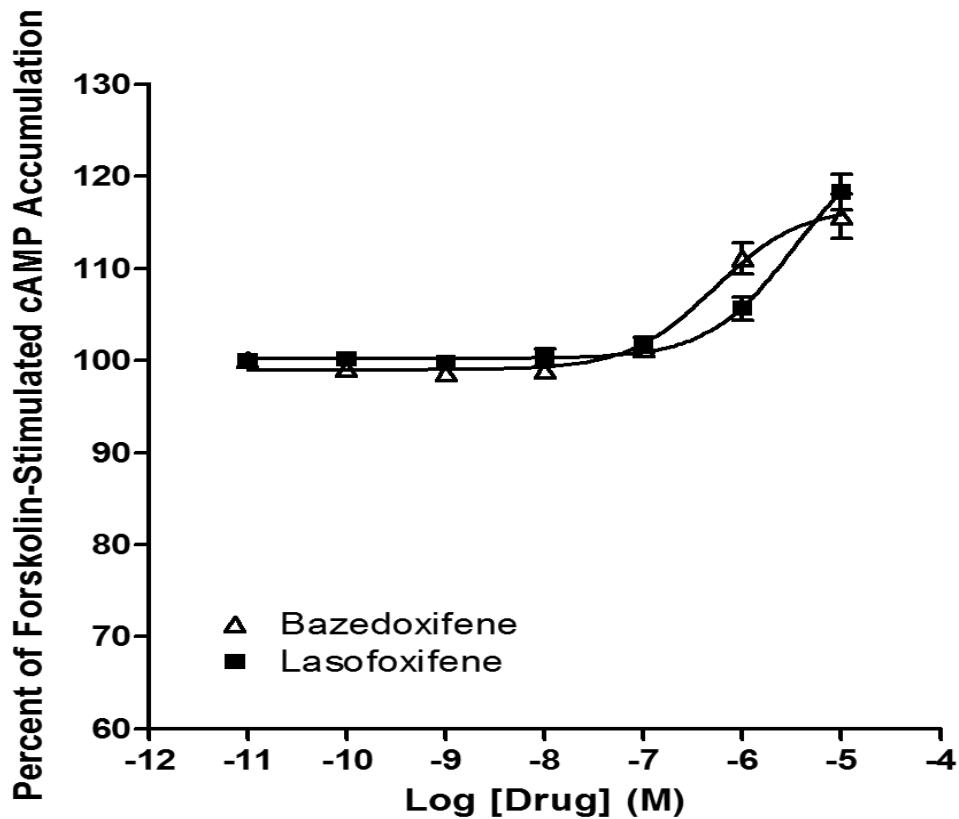
Figure 3.2. Bazedoxifene and lasofoxifene act on the CB2 cannabinoid receptor.
Effects of bazedoxifene and lasofoxifene on forskolin-stimulated cAMP accumulation.
HEK293 cells stably expressing CB2 were treated with different concentrations of
bazedoxifene and lasofoxifene for 7 minutes. Results are expressed as percent forskolin-
stimulated cAMP accumulation. Data shown represent the mean $\pm$ SEM of five
experiments.
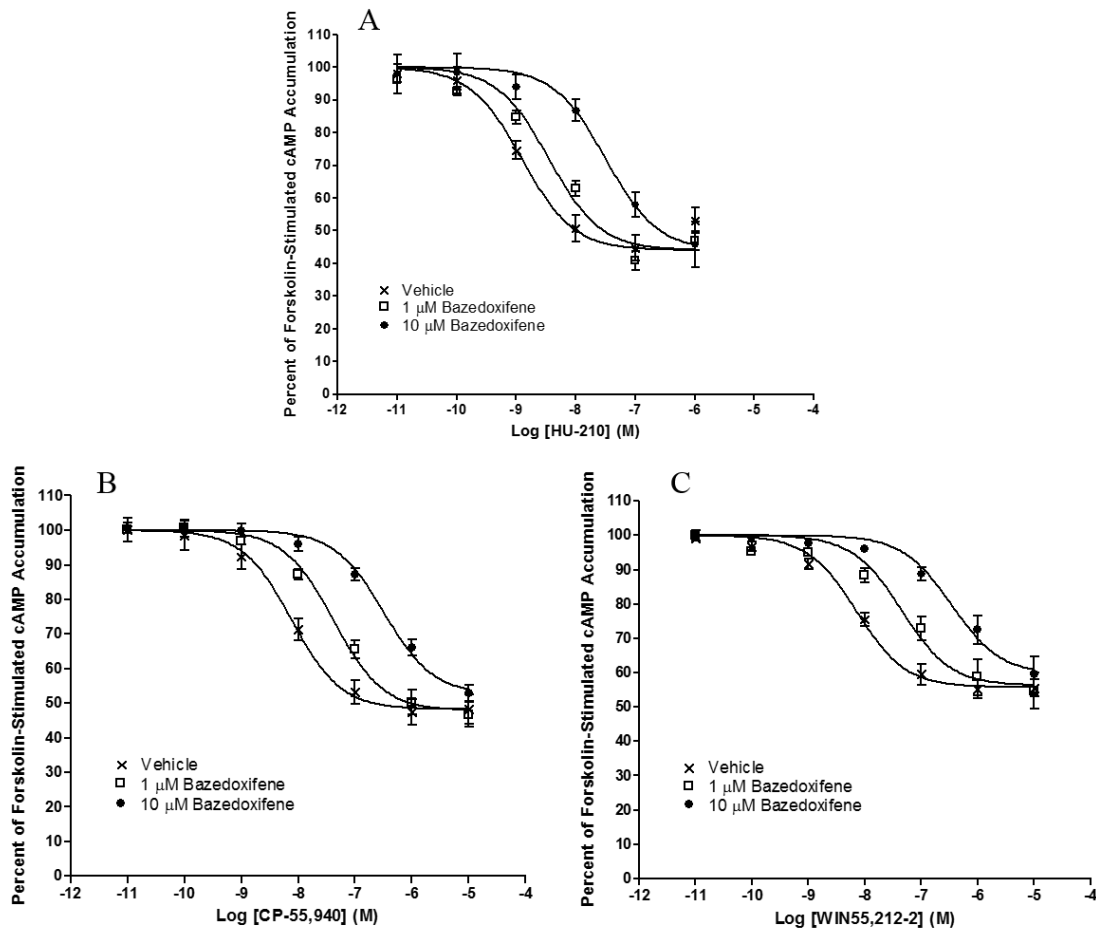
Fig. 3.3 Antagonism of cannabinoid agonist-induced inhibition of forskolin-stimulated cAMP accumulation by bazedoxifene. HEK293 cells stably expressing CB2 were pre-incubated for 20 min with vehicle or bazedoxifene at a concentration of 1 or 10 μM before subject to stimulation with cannabinoid agonists (A) HU-210, (B) CP-55,940, and (C) WIN55,212–2 for 7 min. Results are expressed as percent forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of five experiments.
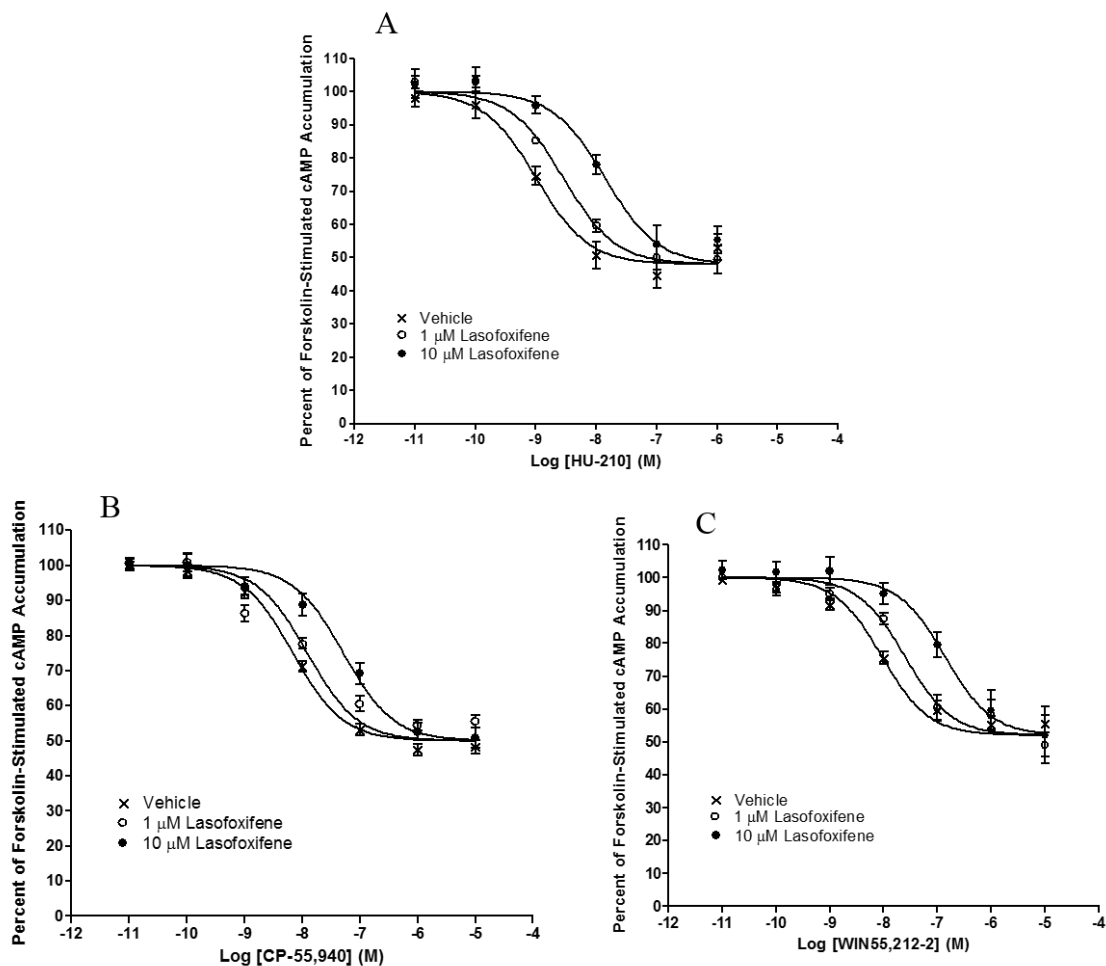
Fig. 3.4 Antagonism of cannabinoid agonist-induced inhibition of forskolin-stimulated cAMP accumulation by Lasofoxifene. HEK293 cells stably expressing CB2 were pre-incubated for 20 min with vehicle or Lasofoxifene at a concentration of 1 or 10 µM before subject to stimulation with cannabinoid agonists (A) HU-210, (B) CP-55,940, and (C) WIN55,212–2 for 7 min. Results are expressed as percent forskolin-stimulated cAMP accumulation. Data shown represent the mean ± SEM of five experiments.

DISCUSSION

Previously, in an attempt to identify novel ligands for CB2, a library of 640 FDA-approved drugs was screened using a cell-based HTRF assay for measuring changes in intracellular cAMP (Kumar & Song, 2013). The efforts resulted in the identification of raloxifene, a second generation SERM used to treat/prevent post-menopausal osteoporosis, as a novel CB2 inverse agonist (Kumar & Song, 2013). The dissertation reporting these results is the first report that CB2 is a novel target for third-generation SERMs bazedoxifene and lasofoxifene.

In the dissertation reporting these results, bazedoxifene and lasofoxifene were able to compete, in a concentration-dependent manner, for specific [^{3}H]CP-55,940 binding to CB2. Analysis of the competition curves revealed that the rank order of affinity of these SERMs for CB2 was bazedoxifene > lasofoxifene. These data demonstrate that these two drugs were able to bind specifically to the CB2 cannabinoid receptor.

In the dissertation reporting these results, bazedoxifene and lasofoxifene enhanced cAMP accumulation concentration-dependently in HEK293 cells stably expressing CB2. The rank order of potency of these two drugs in enhancing cAMP accumulation was found to be bazedoxifene > lasofoxifene. Since these two drugs did not have any effect on forskolin-stimulated cAMP accumulation in empty vector-transfected HEK293 cells, the data show that the effect of bazedoxifene and lasofoxifene on cAMP accumulation was mediated through CB2 receptor.

To further characterize the pharmacological properties of these two drugs on CB2, the dissertation reporting these results evaluated its ability to antagonize the effects of

three cannabinoid agonists. Bazedoxifene and lasofoxifene concentration-dependently caused rightward shifts of the CP-55,940, HU-210, and WIN55,212-2 concentration-response curves. The data indicate that the mode of CB2 antagonism induced by bazedoxifene and lasofoxifene is most likely competitive in nature, as these rightward shifts were parallel and were not associated with any change in the E_{max} of cannabinoid agonists.

Estrogen deficiency is the main cause of post-menopausal osteoporosis. When estrogen is deficient, bone turnover increases, and bone resorption increases more than bone formation, leading to bone loss (Gennari et al., 2006; Maximov et al., 2013; Riggs & Hartmann, 2003; Stump et al., 2007). Bazedoxifene and lasofoxifene belong to the classes of SERMs, which exhibit estrogen agonist activity in some target tissues while exert estrogen antagonist activity in other tissues (Gennari et al., 2006; Maximov et al., 2013; Riggs & Hartmann, 2003; Stump et al., 2007). Both are estrogen agonists in the bone and have been approved for the treatment and prevention of post-menopausal osteoporosis in Europe (Gennari et al., 2006; Maximov et al., 2013; Riggs & Hartmann, 2003; Stump et al., 2007).

The effects of bazedoxifene on bone have been investigated in post-menopausal women with osteoporosis in a large phase III clinical trial (Silverman et al., 2008). Compared to placebo, bazedoxifene at a dose 20 mg or 40 mg per day significantly reduced the risk of new vertebral fractures (Silverman et al., 2008). Furthermore, compared to placebo, bazedoxifene significantly improved bone mineral density (Silverman et al., 2008).

The effects of lasofoxifene on bone have been investigated in great detail and are well established (Cummings et al., 2010). A large clinical trial, the Postmenopausal Evaluation and Risk-Reduction with Lasofoxifene (PEARL), was conducted in post-menopausal women with osteoporosis. Lasofoxifene at a dose of 0.5 mg per day was associated with reduced risks of nonvertebral and vertebral fractures (Cummings et al., 2010). Furthermore, lasofoxifene improved bone mineral density compared to placebo group (Cummings et al., 2010).

The pharmacological actions of bazedoxifene and lasofoxifene are known to be mediated through binding to estrogen receptors (Gennari et al., 2006; Maximov et al., 2013; Riggs & Hartmann, 2003; Stump et al., 2007). This binding results in activation of estrogenic pathways in certain tissues such as bone, and blockade of estrogen pathways in other tissues such as breast (Gennari et al., 2006; Maximov et al., 2013; Riggs & Hartmann, 2003; Stump et al., 2007). Bazedoxifene and lasofoxifene are well known for their SERM properties. However, to our knowledge, this report is the first time that bazedoxifene and lasofoxifene have been demonstrated to behave as inverse agonists for CB2. Cannabinoids and their receptors play important roles in bone metabolism by regulating bone cell function (Idris et al., 2008). It has been shown that the CB2 inverse agonist SR144528 can reduce bone loss by inhibiting osteoclast formation and bone resorption (Idris et al., 2008). Therefore, the new discovery that bazedoxifene and lasofoxifene are CB2 inverse agonists implicates a novel mechanism for the anti-osteoporosis activity of these third-generation SERMs—the effects might be partially mediated through the CB2 cannabinoid receptor in the bone.

Recently, there is accumulating evidence to suggest that CB2 inverse agonists are effective for controlling inflammatory cell migration, thus are useful for a variety of inflammatory diseases, such as arthritis and multiple sclerosis (Lunn et al., 2008). Therefore, the identification of bazedoxifene and lasofoxifene as a novel CB2 inverse agonist suggests that these third-generation SERMs have great potential to be repurposed for other therapeutic indications for which CB2 is a target. In summary, the dissertation reporting these results identified bazedoxifene and lasofoxifene, two third-generation SERMs, as novel CB2 inverse agonists. The discovery also suggests that bazedoxifene and lasofoxifene can potentially be repurposed for novel therapeutic indications.

CHAPTER 4

STRUCTURE-ACTIVITY RELATIONSHIPS OF

FATTY ACID AMIDE LIGANDS IN ACTIVATING AND DESENSITIZING

G PROTEIN-COUPLED RECEPTOR 119

INTRODUCTION

Type 2 diabetes (T2D) and associated obesity are growing public health concerns.
As a result, many pharmaceutical companies have focused their efforts to discover novel,
orally effective agents that can modulate glucose homeostasis and concurrently reduce
body weight. G protein-coupled receptor 119 is a member of the rhodopsin family of G
protein-coupled receptors (GPCRs). Recently G protein-coupled receptor 119 has
emerged as a promising therapeutic target for both T2D and obesity (Dhayal & Morgan,
2010; R. M. Jones et al., 2009; Overton et al., 2008; Shah & Kowalski, 2010).

G protein-coupled receptor 119 is predominantly expressed in the beta cells of the
pancreas and enteroendocrine cells of the gastrointestinal tract (Z. L. Chu et al., 2007; L.
M. Lauffer et al., 2009). G protein-coupled receptor 119 is coupled to Gs, so upon its
activation, there is an enhancement of cAMP levels within the cell (Z. L. Chu et al.,
2007). It has been shown previously that G protein-coupled receptor 119 agonists
stimulate insulin release by at least two mechanisms (Flock et al., 2011; L. Lauffer et al.,
2008). The first mechanism is that the increase in cAMP signaling directly leads to an

enhanced glucose-dependent insulin secretion. The second mechanism is that the increase in cAMP signaling results in an increased glucagon-like peptide 1 (GLP-1) level. GLP-1 is an anti-diabetic hormone which stimulates glucose-dependent insulin secretion and also inhibits glucagon secretion, appetite, and delays gastric emptying (Baggio & Drucker, 2007; L. Lauffer et al., 2008). It has been shown that administration of G protein-coupled receptor 119 agonists improves glucose tolerance in rodents (Z. Chu et al., 2007; Chu et al., 2010; Semple et al., 2008). In addition, it has been demonstrated that G protein-coupled receptor 119 agonists decrease feeding, body weight gain and adiposity in rats (Overton et al., 2006). Thus, G protein-coupled receptor 119 is a highly attractive potential therapeutic target for both diabetes and obesity.

Previously, several studies have demonstrated through phylogenetic analysis that the closest relatives of G protein-coupled receptor 119 are the cannabinoid receptors and placed G protein-coupled receptor 119 to the MECA (melanocortin; endothelial differentiation gene; cannabinoid; adenosine) receptor cluster (Fredriksson, Hoglund, Gloriam, Lagerstrom, & Schioth, 2003; Godlewski et al., 2009; Oh, Kim, Kwon, & Seong, 2006). Since homology clustering analysis revealed that the closest relatives of G protein-coupled receptor 119 are the cannabinoid receptors, it has been hypothesized that fatty acid amides related to the endocannabinoid anandamide, also named arachidonoyl ethanolamide (AEA), may be potential ligands for G protein-coupled receptor 119 (Overton et al., 2006).

A number of cannabinoid ligands and fatty-acid amides have been tested as potential agonists for G protein-coupled receptor 119 (Chu et al., 2010; Overton et al., 2006). However, the data from different research groups have not always been consistent.

For example, Overton and coworkers identified oleoyl ethanolamide (OEA) as an endogenous G protein-coupled receptor 119 ligand (Overton et al., 2006). However, not all groups have observed OEA agonism on G protein-coupled receptor 119 (Brown, 2007). Also, detailed pharmacological analyses comparing the potency and efficacy of various fatty acid amides have not been reported. Therefore, the aim of this dissertation is to examine and compare the potency and efficacy of a variety of fatty acid amides, including several novel compounds that have never been tested, towards G protein-coupled receptor 119 and to investigate the structure-activity relationships of the acyl side chains as well as the charged head groups in fatty acid amides for activating G protein-coupled receptor 119.

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). The cell-based HTRF cAMP HiRange assay kits were purchased from CisBio International (Bedford, MA).

Forskolin was obtained from Sigma (St. Louis, MO). AR231453, Ro 20-1724 and palmitoyl ethanolamide were purchased from Enzo Life Sciences (Farmingdale, NY).

PSN632408, oleoyl ethanolamide, linoleoyl ethanolamide, dihomo-gamma-linolenoyl ethanolamide, docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide, eicosapentaenoyl ethanolamide, docosahexaenoyl ethanolamide, anandamide, N-oleoyl glycine, and N-oleoyl dopamine were purchased from Cayman Chemical Company (Ann Arbor, Michigan). Oleamide and N-oleoyl GABA were purchased from Tocris Bioscience (Ellisville, MO).

Cell Transfection and Culture

Human Embryonic Kidney 293 (HEK293) cells (purchased from ATCC, Manassas, VA) were maintained in 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 plasmid containing the human GPR119 receptor was 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 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 as described previously (Kumar & Song, 2013) using reagents supplied by Cisbio International (HTRF HiRange cAMP kit). 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 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 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. To assess receptor desensitization, HEK293 cells stably expressing G protein-coupled receptor 119 were pre-incubated for 20 min with vehicle or drugs at a concentration of 10 µM before subject to stimulation with OEA.

Statistical Analysis

Data analyses were performed based on the ratio of fluorescence intensity of each well at 620 nm 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 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 cell-based HTRF cAMP assay with positive and negative controls (Fig. 4.1). For positive controls, the HEK293 cells stably expressing G protein-coupled receptor 119 were treated with the potent G protein-coupled receptor 119 agonist AR231453 at a concentration of 10 µM for 30 min at room temperature. For negative controls, the cells were treated with vehicle for 30 min. 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 dissertation reporting these results, the Z factor was determined to be 0.71.

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, DMSO was tested at concentrations ranging from 0.001% to 100%. As shown in Fig. 4.2, the cell-based HTRF cAMP assay for G protein-coupled receptor 119 can tolerate DMSO up to 1% without any loss of signal.

Pharmacological Testing of Known G protein-coupled receptor 119 Agonists

The ability of known agonists to activate G protein-coupled receptor 119 was tested using the HTRF cAMP assay in HEK293 cells stably expressing G protein-coupled receptor 119. As shown in Fig. 4.3 and Table 1, all three previously reported G protein-coupled receptor 119 ligands, AR231453 (Semple et al., 2008), OEA (Overton et al., 2006), and PSN632408 (Overton et al., 2006), increased 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. In addition, these three compounds failed to elicit any response in HEK293 cells transfected with an empty vector (data not shown).

The Effects of Acyl Chain Degree of Saturation on the Ability of Fatty Acid Ethanolamides to Activate G protein-coupled receptor 119

Three endogenous fatty acids, oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA) and arachidonoyl ethanolamide (AEA) were tested for their activity

on G protein-coupled receptor 119 (Fig. 4.4 and Table 2). All three compounds significantly increased cAMP levels in a concentration-dependent manner, with rank orders of both potency and efficacy of OEA > PEA > AEA.

Furthermore, the dissertation reporting these results examined the structure-activity relationship on a subset of novel fatty acid ethanolamides, whose potency (EC_{50} values) and efficacy (E_{max} values) towards G protein-coupled receptor 119 has not been previously analyzed in detail (Fig. 4.5 and Table 2). Among fatty acid ethanolamides that were tested, the rank order of potency was OEA = linoleoyl ethanolamide (LEA) > dihomo-γ-linolenoyl ethanolamide (DLEA) > docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA) > eicosapentaenoyl ethanolamide (EPEA) = docosahexaenoyl ethanolamide (DHEA). The rank of order of efficacy was OEA > LEA > DLEA = DTEA > EPEA = DHEA. In addition, none of the above compounds elicited any response in HEK293 cells transfected with an empty vector (data not shown).

The Effects of Different Head Groups on the Ability of Oleoyl Amides to Activate G protein-coupled receptor 119

The hypothesis of the dissertation reporting these results was that different head groups on the oleoyl amides may impact the ability of oleoyl amides to activate G protein-coupled receptor 119. 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 G protein-coupled receptor 119. Fig. 4.6 and Table 3 demonstrate the agonist activity of different oleoyl amides as compared to OEA. In HEK293 cells stably expressing G protein-coupled

receptor 119, both OLDA and oleamide increased cAMP levels in a concentration-dependent manner, with a rank order of potency of OEA > OLDA = oleamide, and a rank order of efficacy of OEA > OLDA > oleamide. On the contrary, oleoyl alanine, oleoyl glycine, and oleoyl GABA failed to activate G protein-coupled receptor 119 with concentrations up to 100 μM. In addition, none of the above compounds elicited any response in HEK293 cells transfected with an empty vector (data not shown).

Receptor Desensitization Produced by Pretreatment with G protein-coupled receptor 119 Agonists

To study receptor desensitization, HEK293 cells stably expressing G protein-coupled receptor 119 were pretreated for 20 min with 10 μM of various G protein-coupled receptor 119 agonists. Subsequently, OEA-induced enhancement of cAMP was measured as an indicator of receptor activation. As shown in Fig. 4.7, AR231453 pretreatment completely abolished OEA-induced activation of G protein-coupled receptor 119, whereas OEA and PSN632408 pretreatments significantly desensitized the OEA-induced activation of G protein-coupled receptor 119. As shown in Fig. 4.8, pretreatments with fatty acid amides OEA, PEA, and AEA caused a desensitization of OEA-induced G protein-coupled receptor 119 activation, and the degree of desensitization follows the order of OEA > PEA > AEA. Fig. 4.9 demonstrates that pretreatments with OEA, LEA, DLEA, and DTEA caused a desensitization of OEA-induced activation of G protein-coupled receptor 119 and the degree of desensitization follows the order of OEA > LEA > DLEA > DTEA. In contrast, at a concentration of 10 μM, neither EPEA nor DHEA caused G protein-coupled receptor 119 receptor desensitization. Furthermore, as shown in Fig. 4.10, pretreatments with OEA, OLDA,

and oleamide led to a desensitization of OEA-induced activation of G protein-coupled

receptor 119 and the degree of desensitization follows the order of OEA > OLDA >

oleamide. On the contrary, pretreatments with oleoyl alanine, oleoyl glycine, and oleoyl

GABA did not result in a desensitization of the G protein-coupled receptor 119 receptor.
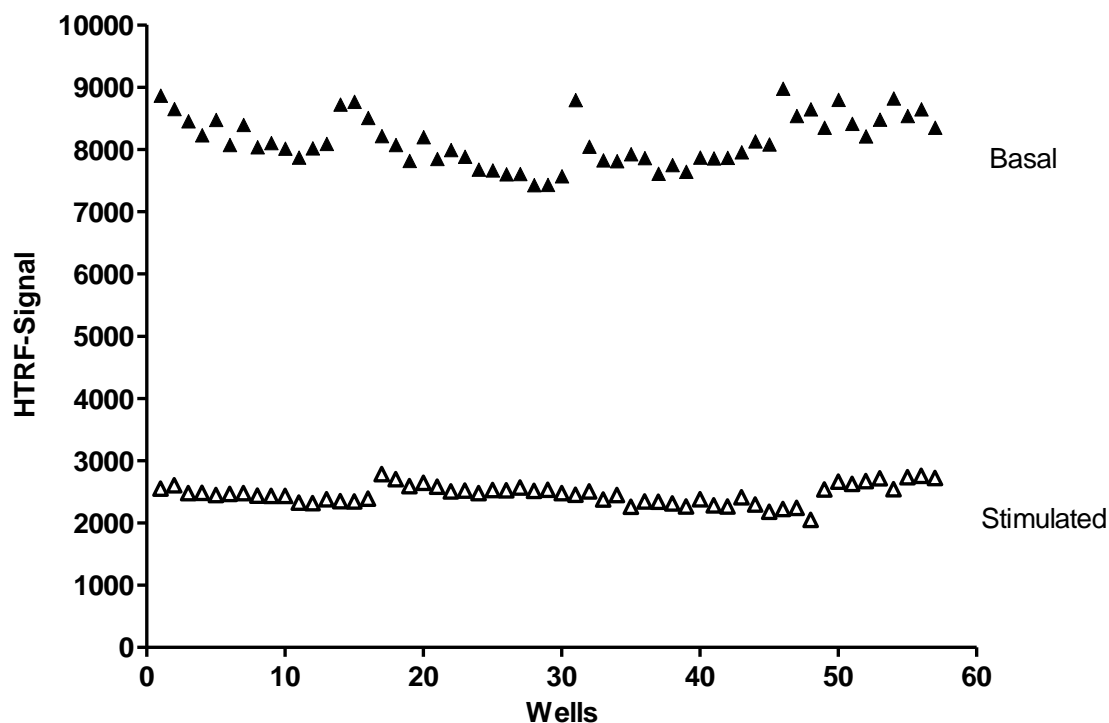
Fig. 4.1. Validation of the cell-based, HTRF cAMP assay for G protein-coupled receptor 119.   (A) Z′ factor determination. Open symbols represent positive controls (cells stimulated with 10 μM AR231453), while solid symbols represent negative controls (cells stimulated with vehicle). The Z′ factor was calculated to be 0.71 using 57 positive and 57 negative control points.

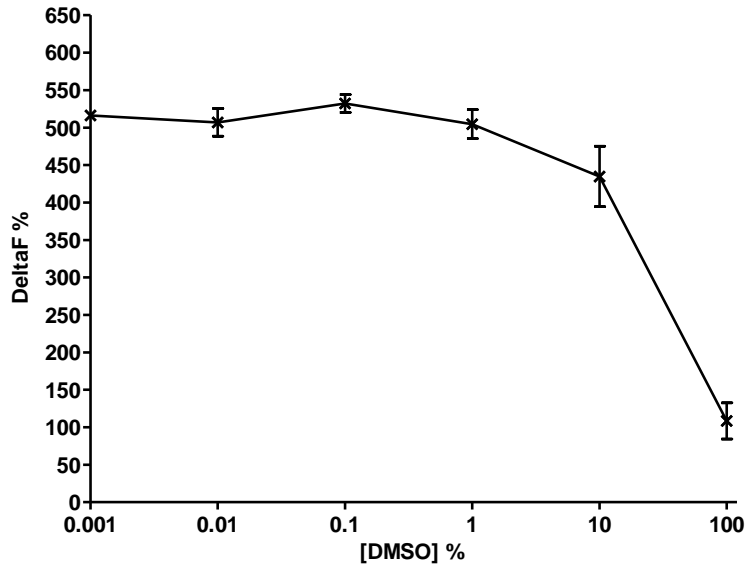**Fig. 4.2. DMSO tolerance.** HEK293 cells stably expressing G protein-coupled receptor 119 was treated with different concentrations of DMSO. Delta F % was calculated using the following formula: Delta F % = [(standard or sample ratio − ratio of the negative control) / ratio of the negative control] x 100. Values represent the mean ± S.E.M. of three independent experiments, each performed in duplicate.

Fig. 4.3. The effects of known agonists to activate G protein-coupled receptor 119.
HEK293 stably expressing G protein-coupled receptor 119 were treated with G protein-coupled receptor 119 agonists AR231453, oleoyl ethanolamide (OEA), and PSN632408 for 30 min. Results are expressed as percent of maximum OEA-induced cAMP accumulation. Values represent the mean ± S.E.M. of five independent experiments.

Fig. 4.4. The effects of acyl chain degree of saturation on the ability of fatty acid ethanolamides to activate G protein-coupled receptor 119. (A) HEK293 stably expressing G protein-coupled receptor 119 were treated with oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA), or AEA for 30 min.

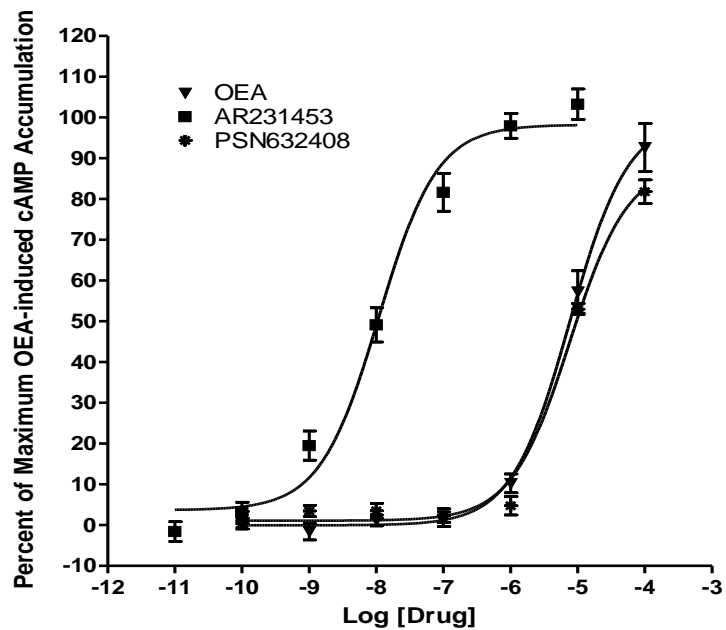**Fig. 4.5. The effects of acyl chain degree of saturation on the ability of fatty acid ethanolamides to activate G protein-coupled receptor 119.** HEK293 stably expressing G protein-coupled receptor 119 were treated with oleoyl ethanolamide (OEA), linoleoyl ethanolamide (LEA), dihomo-gamma-linolenoyl ethanolamide (DLEA), docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA), eicosapentaenoyl ethanolamide (EPEA) or docosahexaenoyl ethanolamide (DHEA) for 30 min. Results are expressed as percent of maximum OEA-induced cAMP accumulation. Values represent the mean ± S.E.M. of five independent experiments.

Fig. 4.6. The effects of different head groups on the ability of oleoyl amides to activate G protein-coupled receptor 119. HEK293 stably expressing G protein-coupled receptor 119 were treated with oleoyl ethanolamide (OEA), oleoyl dopamine (OLDA), oleamide, oleoyl alanine, oleoyl glycine, or oleoyl GABA for 30 min. Results are expressed as percent of maximum OEA-induced cAMP accumulation. Values represent the mean ± S.E.M. of five independent experiments.
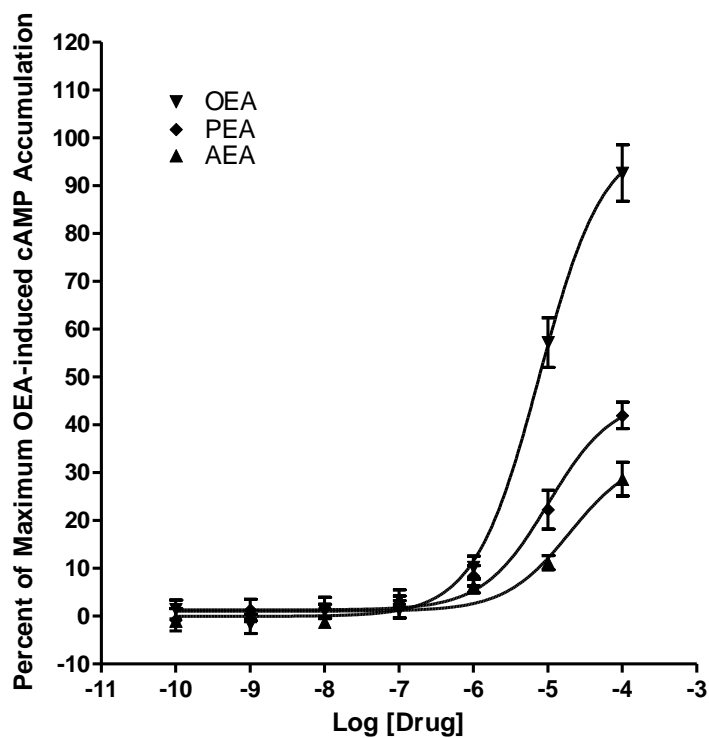
Fig. 4.7. The effects of known agonists to desensitize G protein-coupled receptor 119.
HEK293 cells stably expressing G protein-coupled receptor 119 were pretreated with

known G protein-coupled receptor 119 agonists AR231453, oleoyl ethanolamide (OEA),

or PSN632408 for 20 min, followed by stimulation with OEA for 30 min. Results are

expressed as percent of maximum OEA-induced cAMP accumulation. Values represent

the mean ± S.E.M. of five independent experiments.

Fig. 4.8. The effects of acyl chain degree of saturation on the ability of fatty acid ethanolamides to desensitize G protein-coupled receptor 119. (A) HEK293 cells stably .expressing G protein-coupled receptor 119 were pretreated with oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA), or AEA for 20 min, followed by stimulation with OEA for 30 min.
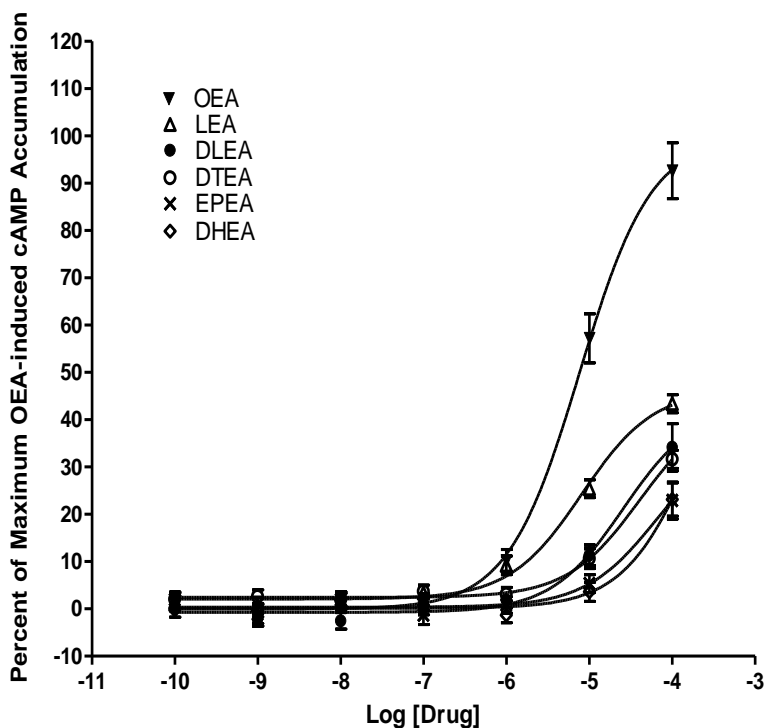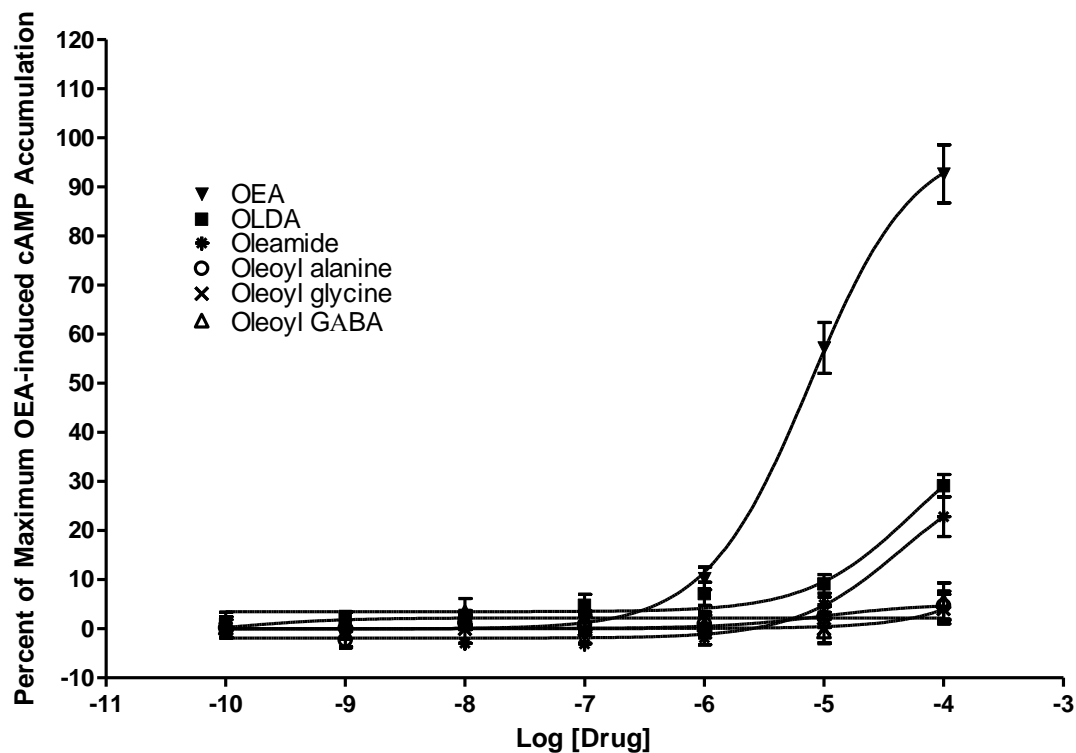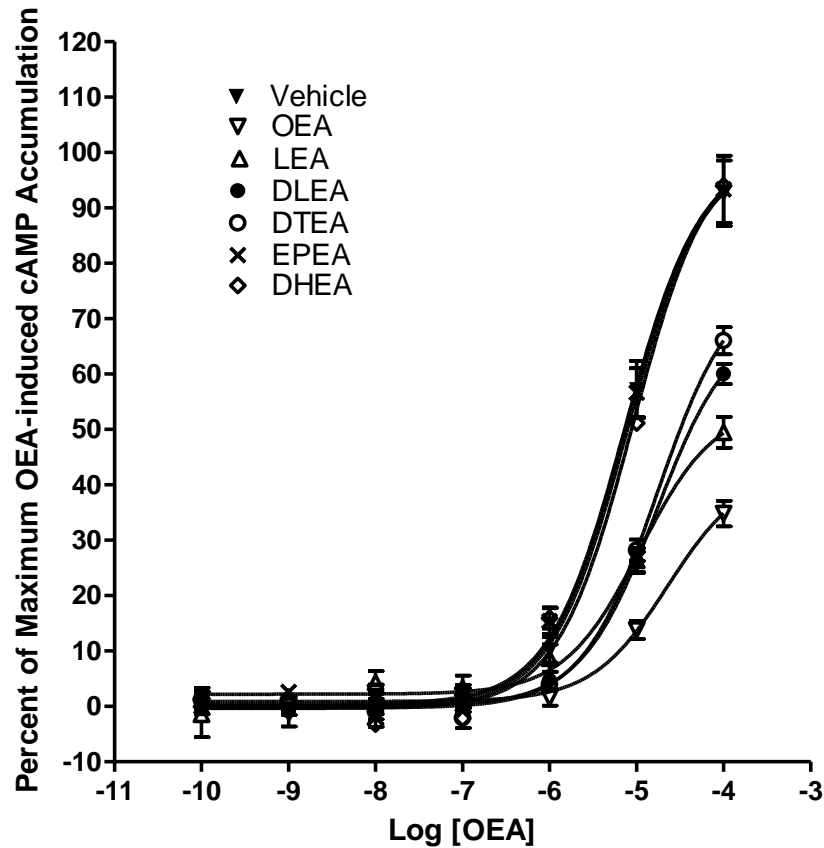
Fig. 4.9. The effects of acyl chain degree of saturation on the ability of fatty acid ethanolamides to desensitize G protein-coupled receptor 119. HEK293 cells stably expressing G protein-coupled receptor 119 were pretreated with oleoyl ethanolamide (OEA), linoleoyl ethanolamide (LEA), dihomo-gamma-linolenoyl ethanolamide (DLEA), docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA), eicosapentaenoyl ethanolamide (EPEA) or docosahexaenoyl ethanolamide (DHEA) for 20 min, followed by stimulation with OEA for 30 min. Results are expressed as percent of maximum OEA-induced cAMP accumulation. Values represent the mean ± S.E.M. of five independent experiments.
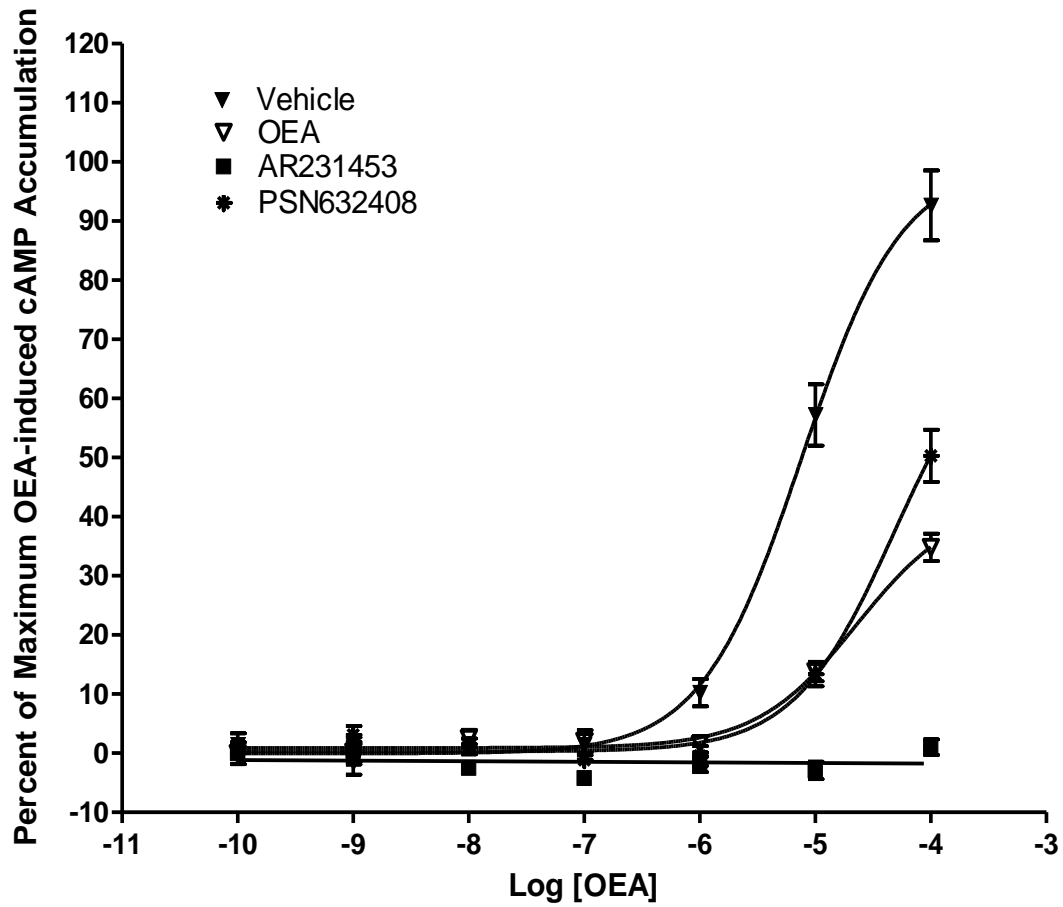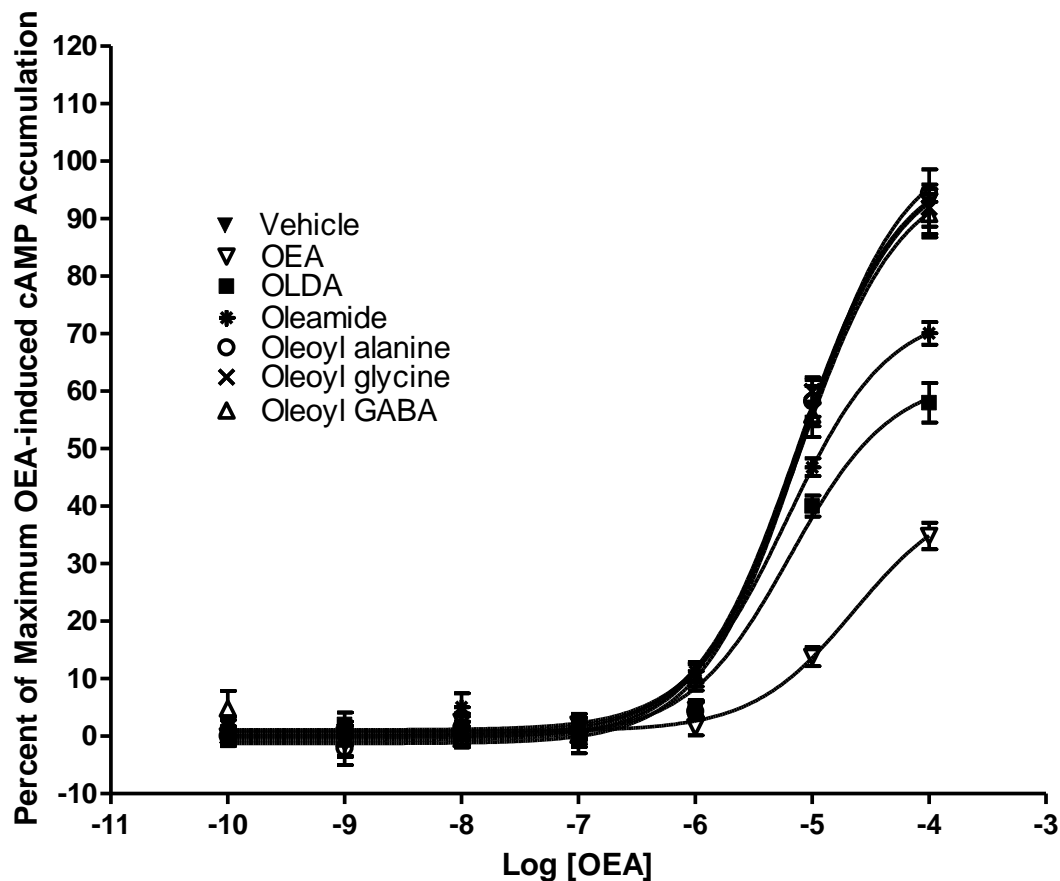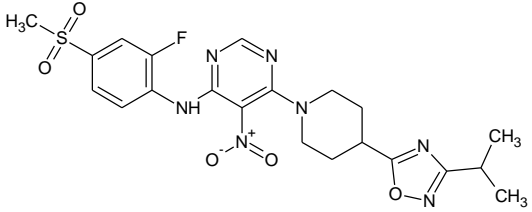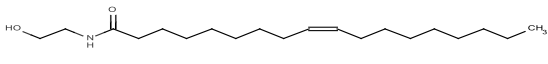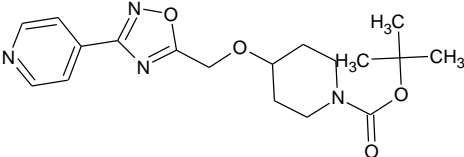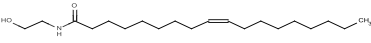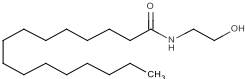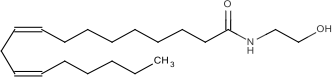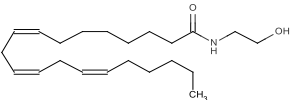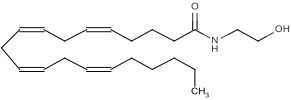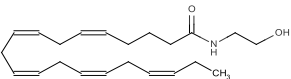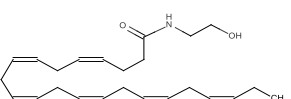
Fig. 4.10. The effects of different head groups on the ability of oleoyl amides to desensitize G protein-coupled receptor 119. HEK293 cells stably expressing G protein-coupled receptor 119 were pretreated with oleoyl ethanolamide (OEA), oleoyl dopamine (OLDA), oleamide, oleoyl alanine, oleoyl glycine, or oleoyl GABA for 20 min, followed by stimulation with OEA for 30 min. Results are expressed as percent of maximum OEA-induced cAMP accumulation. Values represent the mean ± S.E.M. of five independent experiments.
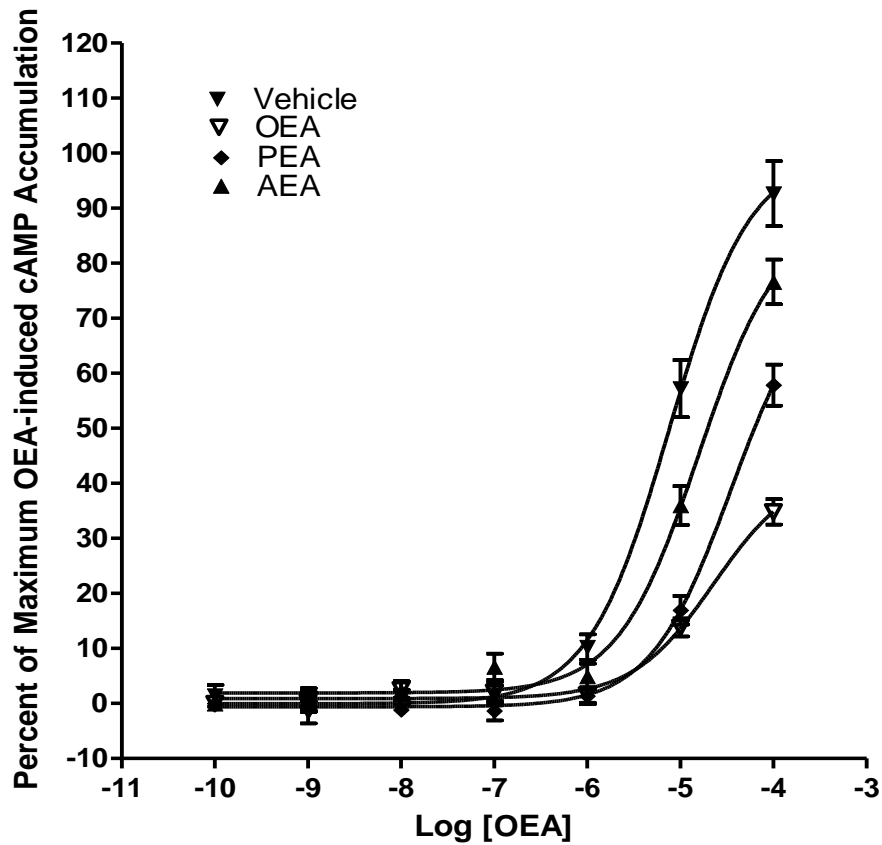
Table 1. The effects of known G protein-coupled receptor 119 agonists on increasing cAMP in HEK293 cells stably expressing G protein-coupled receptor 119.

Drug	Structure	EC$_{50}$ (95% CI) (μM)	E$_{max}$ (95% CI) (% OEA response)
AR231453		0.011 (0.0090 - 0.0131)[a]	98.23 (95.71 - 100.80)
Oleoyl ethanolamide (OEA)		7.65 (7.56 - 7.74)	100.00 (99.70 - 100.30)
PSN632408		7.61 (7.01 - 8.26)	88.72 (87.04 - 90.40)[a]
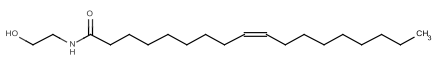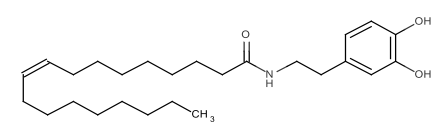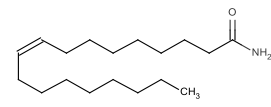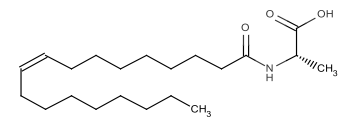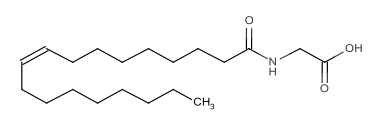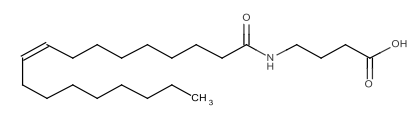
[a] Significantly different (P < 0.05) from OEA.

Table 2. The effects of acyl chain degree of saturation on the ability of fatty acid ethanolamides to increase cAMP levels in HEK293 cells stably expressing G protein-coupled receptor 119.

Drug	Structure	EC$_{50}$ (95% CI) (μM)	E$_{max}$ (95% CI) (% OEA response)
Oleoyl ethanolamide (OEA)		7.65 (7.56 - 7.74)	100.00 (99.70 - 100.30)
Palmitoyl ethanolamide (PEA)		10.12 (8.72 - 11.73)[a]	45.76 (44.08 - 47.44)[a]
Linoleoyl ethanolamide (LEA)		8.11 (7.55 - 8.70)	46.41 (45.69 - 47.13)[a]
Dihomo-γ-linolenoyl ethanolamide (DLEA)		25.64 (22.93 - 28.67)[a]	43.07 (41.63 - 44.51)[a]
Docosatetra-7Z,10Z,13Z,16Z-enoyl ethanolamide (DTEA)		40.16 (37.41 - 43.11)[a]	43.35 (42.38 - 44.33)[a]
Arachidonoyl ethanolamide (AEA)		19.67 (16.35 - 23.67)[a].	33.79 (31.72 - 35.87)[a]
Eicosapentaenoyl ethanolamide (EPEA)		65.60 (43.56 – 98.78)[a]	33.96 (31.44 – 36.47)[a]
Docosahexxaenoyl ethanolamide (DHEA)		63.79 (47.50 – 85.68)[a]	34.87 (30.97 – 38.77)[a]
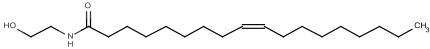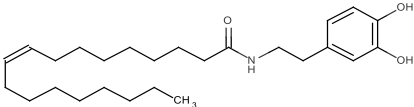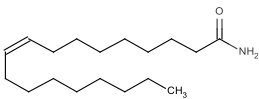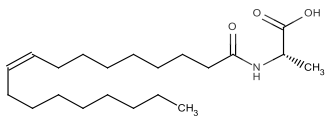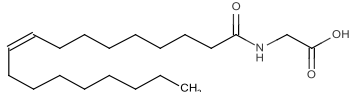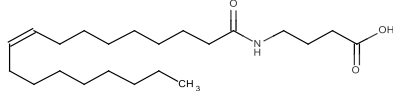
[a] Significantly different (P < 0.05) from OEA.

Table 3. The effects of different head groups on the ability of oleoyl amides to increase cAMP levels in HEK293 cells stably expressing G protein-coupled receptor 119.

Drug	Structure	EC$_{50}$ (95% CI) (μM)	E$_{max}$ (95% CI) (% OEA response)
Oleoyl ethanolamide (OEA)		7.65 (7.56 - 7.74)	100.0 (99.7 - 100.3)
Oleoyl dopamine (OLDA)		54.79 (37.64 - 79.75)[a]	43.15 (37.43 - 48.87)[a]
Oleamide		42.86 (37.06 - 49.58)[a]	33.48 (31.56 - 35.39)[a]
Oleoyl alanine		N.D.	N.D.
Oleoyl glycine		N.D.	N.D.
Oleoyl GABA		N.D.	N.D.

[a] Significantly different (P < 0.05) from OEA. N.D., not determined.

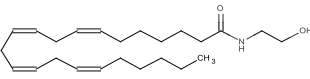**Table 4. The effects of different head groups on the ability of oleoyl amides to increase cAMP levels in HEK293 cells stably expressing G protein-coupled receptor 119.**

Drug	Structure	EC$_{50}$ (95% CI) (μM)	E$_{max}$ (95% CI) (% OEA response)
Oleoyl ethanolamide (OEA)		7.65 (7.56 - 7.74)	100.0 (99.7 - 100.3)
Oleoyl dopamine (OLDA)		54.79 (37.64 - 79.75)[a]	43.15 (37.43 - 48.87)[a]
Oleamide		42.86 (37.06 - 49.58)[a]	33.48 (31.56 - 35.39)[a]
Oleoyl alanine		N.D.	N.D.
Oleoyl glycine		N.D.	N.D.
Oleoyl GABA		N.D.	N.D.

[a] Significantly different (P < 0.05) from OEA. N.D., not determined.

Discussion

Agonist binding to G protein-coupled receptor 119 leads to Gs coupling and activation of adenylate cyclase (Dhayal & Morgan, 2010; R. M. Jones et al., 2009; Overton et al., 2008; Shah & Kowalski, 2010). As a result, there is an increase in intracellular cAMP levels which was measured as a decrease in HTRF signal. The dissertation reporting these results has shown that the cell-based HTRF cAMP assay is a suitable technology for assaying ligands that may act on G protein-coupled receptor 119.

The Z´factor is a standard statistical parameter used to evaluate the robustness of a high throughput assay (Zhang et al., 1999). The Z´factor value can range between 0 and 1, with values approaching 1 indicates excellent assay robustness. In the dissertation reporting these results, the calculated Z´factor for the assay was 0.71. Since Z´ factor greater than 0.5 indicates a suitable difference between signal and background values with low variability, the dissertation reporting these results demonstrate that the cell-based, HTRF cAMP assay is robust and suitable for testing ligands that activate G protein-coupled receptor 119.

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 assayed before DMSO reaches a concentration that is too high to be tolerated by the assay (Williams, 2004). Therefore, the effect of DMSO on the cell-based HTRF cAMP assay was determined. DMSO was tested 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 testing ligands that may act on G protein-coupled receptor 119 at a DMSO concentration of less than 1 %.

To validate that the cell-based HTRF cAMP assay is suitable for assaying ligands that may activate G protein-coupled receptor 119 concentration-response studies for three previously reported G protein-coupled receptor 119 agonists AR231453, OEA, and PSN632408 were performed. Both the rank order of potency and the rank order of efficacy of these three known G protein-coupled receptor 119 agonists in enhancing cAMP levels in G protein-coupled receptor 119-expressing HEK293 cells are consistent with previous reports (Overton et al., 2006; Semple et al., 2008). These results also confirmed the suitability of this cell-based HTRF cAMP assay for testing ligands for G protein-coupled receptor 119.

Recently, the fatty acid ethanolamide OEA has been reported to be a putative endogenous ligand for G protein-coupled receptor 119 (Overton et al., 2006). However, not all groups have observed OEA agonism on G protein-coupled receptor 119 (Brown, 2007). Overton and coworkers have also tested the endogenous cannabinoid agonist AEA and the saturated fatty-acid ethanolamide PEA for G protein-coupled receptor 119 activity in a yeast-based assay. Their results showed that OEA was the most efficacious, followed by PEA and then AEA. Based on the data with OEA, PEA, and AEA, it has been proposed that the degree of saturation in fatty acid aryl chain might be important for these fatty-acid ethanolamides to activate G protein-coupled receptor 119 (Chu et al., 2010; Overton et al., 2006). The results on OEA, PEA, and AEA with the cAMP assay demonstrated rank orders of both potency and efficacy of OEA > PEA > AEA. Thus, these data on these three fatty acid amides with the human G protein-coupled receptor 119 stably expressed in HEK293 cells are consistent with those reported by Overton et al. with the yeast-based assay (Overton et al., 2006).

The dissertation reporting these results was the first report of the detailed potency and efficacy analyses of a novel 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, the new data in the present dissertation provides direct evidence to support the hypothesis that the degree of saturation in the acyl chain of fatty acid ethanolamides affects the ability of these compounds to activate G protein-coupled receptor 119.

OEA, LEA, DLEA, DTEA, EPEA and DHEA contain one, two, three, four, five, and six double bonds in their fatty acid acyl chain, respectively. The results indicate that increasing the number of double bonds reduces the ability of these ligands to activate G protein-coupled receptor 119; with compounds containing 1-2 double bonds have significantly higher efficacy and potency than those compounds containing 3-6 double bonds.

Chu and coworkers reported that a diverse set of lipid amides, including N-oleoyldopamine (OLDA) and oleamide, activate G protein-coupled receptor 119 (Chu et al., 2010). Thus, they suggested that there might be a broad permissiveness in the amine-derived moieties (the head groups) of lipid amides for being an agonist for G protein-coupled receptor 119 (Chu et al., 2010). In the present dissertation reporting these results, it was demonstrated that both OLDA and oleamide activate G protein-coupled receptor 119, with a rank order of potency of OEA > OLDA = oleamide, and a rank order of efficacy of OEA > OLDA > oleamide. These new data on the potency and efficacy of these fatty acid amides confirm the notion that there is a considerable level of

permissiveness in the head group of oleoyl amides. However, the data also demonstrate that to achieve the maximum efficacy in activating G protein-coupled receptor 119, the ethanolamide head group is necessary.

Furthermore, the dissertation reporting these results was the first report that demonstrated that oleoyl alanine, oleoyl glycine, and oleoyl GABA were unable to activate G protein-coupled receptor 119. These data suggest that although there are certain levels of permissiveness, in order to activate G protein-coupled receptor 119, 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 G protein-coupled receptor 119 have a carboxyl group. This suggests that a plausible explanation that these ligands failed to activate G protein-coupled receptor 119 might be due to either the steric hindrance or the acidic nature of the carboxyl group.

Desensitization is the attenuation of receptor responsiveness to agonist after prior agonist exposure and represents an important feedback mechanism for preventing receptor overstimulation (Kohout & Lefkowitz, 2003). Although it is a well-known phenomenon for GPCRs, receptor desensitization has not been reported for G protein-coupled receptor 119. To our knowledge, this is the first characterization of agonist-induced desensitization of the G protein-coupled receptor 119 receptor which appears to be due to a reduction both in potency and in efficacy of OEA to elevate cAMP.

In the dissertation reporting these results, the data demonstrate that the degree of receptor desensitization produced by a certain agonist correlates well with the potency and efficacy of the agonist. For example, the most potent and efficacious G protein-

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coupled receptor 119 agonist AR231453 induced the greatest degree of receptor desensitization. Furthermore, among a subset of fatty acid amides, the degree of receptor desensitization follows the order of OEA > LEA > DLEA > DTEA > EPEA=DHEA, which correlates closely with their ability to activate G protein-coupled receptor 119. These results indicate that increasing the number of double bonds reduces the ability of these fatty acid amides to activate, as well as to desensitize G protein-coupled receptor 119.

The dissertation reporting these results have shown that pretreatment with OEA, PEA, LEA, DLEA, DTEA, EPEA, or DHEA is able to inhibit the OEA-induced response to different extents that correlate with their ability to activate G protein-coupled receptor 119. These results suggest that these fatty acid amides share the same binding sites. This suppression of OEA-induced response could be due to (1) desensitization of the receptor, (2) competition between pre- and post-treated ligands, (3) both receptor desensitization and competition between the pre- and post-treated ligands. With the experimental protocol that was employed in the present dissertation , possibility number 3 is most likely to be the mechanism. To further differentiate and/or exclude these possible mechanisms, and to further confirm that the suppression of OEA response was from ligand binding to the orthosteric rather than allosteric site, one of the critical experiments needed is the radioligand binding experiment with pre-treated and post-treated cells. However, currently there is no commercially available radioligand for G protein-coupled receptor 119 for us to conduct ligand binding experiments. Even though the present dissertation was unable to differentiate/exclude the possible mechanisms at the present time, the main conclusion regarding the degree of saturation in the acyl chain

and the head group of the fatty acid amides are still strongly supported by the structure-activity relationship data.

CHAPTER 5

A CATEGORICAL STRUCTURE-ACTIVITY RELATIONSHIP ANALYSIS OF
GPR119 LIGANDS

INTRODUCTION

G protein-coupled receptors (GPCRs) are a large family of seven-transmembrane

domain receptors that respond to diverse external signals and transmit information to

signaling pathways inside the cell. GPCR activation via ligand binding often results in the

generation of second messengers that regulate a broad range of physiological functions.

G protein-coupled receptor 119 (GPR119) is a member of the class A (rhodopsin-type)

GPCR family, which is highly expressed in pancreatic β-cells and in enteroendocrine

cells of the gastrointestinal tract (Dhayal & Morgan, 2010; R. M. Jones et al., 2009;

Overton et al., 2008; Shah & Kowalski, 2010). GPR119 agonists have been shown to

increase insulin secretion and inhibit appetite (Dhayal & Morgan, 2010; R. M. Jones et

al., 2009; Overton et al., 2008; Shah & Kowalski, 2010). As a result, GPR119 has

recently emerged as a novel and promising therapeutic target for both type 2 diabetes

(T2D) and obesity (Dhayal & Morgan, 2010; R. M. Jones et al., 2009; Overton et al.,

2008; Shah & Kowalski, 2010).

Agonist binding to GPR119, which is coupled to Gαs, a heterotrimeric G protein,

results in an increase in intracellular cyclic adenosine monophosphate (cAMP) by

activating adenylate cyclase (Z. L. Chu et al., 2007). There are at least two mechanisms

by which GPR119 agonists stimulate insulin release: 1) Increased cAMP signaling results directly in an enhancement of glucose-dependent insulin release (Flock et al., 2011; L. Lauffer et al., 2008) or 2) Increased glucagon-like peptide 1 (GLP-1) levels which further stimulates glucose-dependent insulin secretion (Flock et al., 2011; L. Lauffer et al., 2008). Furthermore, GLP-1 inhibits glucagon secretion, appetite, and gastric emptying (Baggio & Drucker, 2007; L. Lauffer et al., 2008).

The broad interest in discovering novel, orally effective GPR119 agonists as potential therapies for T2D and obesity has resulted in the development of many synthetic GPR119 agonists over the past several years (Dhayal & Morgan, 2010; R. M. Jones et al., 2009; Overton et al., 2008; Shah & Kowalski, 2010). Recently, GPR119 agonists have already reached the stage of being investigated for clinical use (Katz et al., 2011; Katz et al., 2012; Nunez et al., 2014; Polli et al., 2013).

Structure-activity relationship (SAR) modeling is a method designed to ascertain relationships between chemical structure and qualitative biological activity of ligands. Quantitative SAR and qualitative SARs are relationships that are derived from continuous data (*e.g. biological potency*) and non-continuous data (*e.g. active or inactive*), respectively.

The lack of an x-ray crystal structure for GPR119 hinders the ability to understand how ligands bind and interact with this receptor. As such, the aim of the current dissertation was to vigorously investigate the relationship between chemical structure and biological activity by employing a fragment based qualitative SAR expert system, cat-SAR, to study GPR119 ligand characteristics.

The cat-SAR expert system is flexible and user-friendly in the development of the learning set and model parameterization (Cunningham, Cunningham, Consoer, Moss, & Karol, 2005). Cat-SAR analysis permits the user to designate adjustable modeling parameters including the selection of the size of the 2-dimensional fragments, inclusion or exclusion of hydrogen atoms in the analysis, and rules for selecting important fragments for the final model (Cunningham et al., 2005). Hence, the selection of compounds included in the learning set and control over various model parameters provides the user with the ability to more thoroughly investigate the relationship between chemical structure and biological activity (Cunningham et al., 2005).

Cat-SAR models are built through a comparison of structural features found amongst categorized compounds (active and inactive) in the model's learning set (Cunningham et al., 2005). Fundamentally, the cat-SAR approach is transparent in the development of the learning set, the identification of fragments, and the determination of important fragments (Cunningham et al., 2005). Moreover, the approach permits a high degree of user involvement and model optimization during the modeling process. This method includes the ability to examine the entire fragment base, investigate and optimize the fragments that have hypothetical biological relevance. In previous analyses, the cat-SAR program was able to achieve an overall concordance between observed and predicted values of 92% for a set of chemicals assessed for their ability to induce respiratory hypersensitivity (Cunningham et al., 2005) and 78%–84% for a set of rat mammary carcinogens (Cunningham et al., 2008).

Moreover, since cat-SAR is based on the analysis of categorical data and 2-dimensional fragments versus intact chemicals, the program can examine data sets that

are divided into categories of activity rather than degrees of potency as in the case of QSAR (Cunningham et al., 2005). Thus, in contrast to Hansch and conformational molecular field analysis (CoMFA) approaches which require continuous-type data, cat-SAR functions by identifying molecular attributes associated with biological activity by comparing characteristics of active (*e.g.*, compounds known to act as agonists for GPR119 to inactive (*e.g.*, compounds known to not activate GPR119) compounds. The models and subsequent predictions based on this can be used to examine structural features associated with activity and predict the probability of activity of unknown compounds, respectively (Cunningham et al., 2005).

Recently, a hierarchical virtual screening study (Saxena & Roy, 2012) was carried out to identify novel agonists for the β_3-adrenergic receptor, a GPCR. The approach consisted of pharmacophore modeling, docking and virtual screening which resulted in the identification of possible leads as novel β_3-adrenergic receptor agonists (Saxena & Roy, 2012).

In the dissertation reporting these results, the development of several novel GPR119 SAR models using the cat-SAR expert system to analyze the structural attributes of compounds that activate GPR119 and report predictive and mechanistically insightful SAR models for GPR119 activation were described. Overall, the cat-SAR models discussed herein for GPR119 activation demonstrate a high degree of predictive ability and mechanistically interpretability and may be useful for screening new drug candidates for this GPCR. These models can potentially be used to virtually screen large compound libraries to identify novel GPR119 ligands.

MATERIALS AND METHODS

Materials

The cat-SAR models are generated through an evaluation of structural features found amongst two designated categories of compounds in the model's learning set: Active or Inactive. The cat-SAR learning set consists of the chemical name, its structure as a MOL2 file, and its categorical designation (*e.g.*, one or zero for active and inactive, respectively). Typically, organic salts are included as the freebase and simple mixtures and technical grade preparations may be included as the active component. Metals, metalo-organic compounds, polymers, hydrogen atoms, and mixtures of unknown composition are excluded.

The active data set consisted of 222 compounds that were collected from literature sources. The inactive data set consisted of compounds determined not to activate GPR119 (less than 10 % of AR231453 activity) at a concentration of 10 μM. Previous reports have validated a high throughput cAMP assay for screening GPR119 ligands (Kumar, Kumar, & Song, 2014). Using this assay we experimentally tested the compounds in three commercially available libraries (FDA-approved drug library, NIH clinical collection, and Tocriscreen) as potential GPR119 agonists. The FDA-approved drug library was purchased from Enzo Life Sciences (Farmingdale, NY). The NIH clinical collection was purchased from Evotec, Inc. (San Francisco, CA). The Tocriscreen library was purchased from R and D Systems, Inc. (Minneapolis, MN).

The experimental screen did not result in the identification of any agonists, but resulted in the determination of 1000 inactive compounds. Four sets of 222 randomly

selected inactive compounds were produced to generate four replicate models (standard 222 active and random 222 inactive). Therefore, the dissertation reporting these results was able to assess the stability of the derived models. This approach prevented the chance of selecting 222 inactive compounds that produced a "good" model. Therefore, four models consisting of 222 active and random sets of 222 inactive compounds were built.

The cat-SAR program provides for a number of user-specified options, so there is no *a priori* determination of the parameters in the final model. As such, the present dissertation reports the development of four different cat-SAR GPR119 models. With the ability to vary modeling parameters some can extend past the structural range of the learning sets and must be taken into consideration For example, the fragment length parameter for the models described herein was set from three to seven heavy atoms (described below). Thus, chemicals of only three heavy atoms contributed their entire chemical structure as one fragment. Likewise, compounds consisting of less than three heavy atoms contributed no fragments to the model.

In silico chemical fragmentation and fragment clustering

Previous cat-SAR models used the Tripos Sybyl HQSAR module to generate chemical fragments. The dissertation reporting these results describes the development of a novel algorithm for the *in silico* fragmentation of compounds. For each compound the respective MOL2 file was used to generate a computational unordered graph, represented by $G(V,E)$ where V is the set of vertices (atoms) and E is the set of edges (bonds) that connect a given pair of vertices. Next, each vertex was iterated over and all unique, connected subgraphs within six edges – the maximum fragment length-

containing that vertex were identified, after which the given root vertex was removed from the graph for the remaining iterations. These subgraphs serve as mathematical representations of the chemical fragments. To convert the subgraphs to usable canonical SMILES, a Depth First Search of each subgraph was performed and the resulting SMILES was assigned using methodology derived from the CANGEN process of Daylight Chemical Information Systems.

As in previous cat-SAR models (Cunningham, Carrasquer, & Mattison, 2009; Cunningham et al., 2008; Qamar, Carrasquer, Cunningham, & Cunningham, 2011), chemical fragments that serve as valuable descriptors of activity/inactivity were identified and retained. However, there remained a high degree of redundancy between many of these fragments (based on similar chemical structures and derivation from mostly the same compounds). To ease in model interpretation and increase model accuracy and efficiency, this redundant fragment information was condensed by clustering the fragments. The clustering methodology utilizes the Tanimoto Similarity Coefficient and compound derivation similarity to determine relatedness between any two fragments. If two fragments share a Tanimoto Coefficient ≥70% and are present in ≥70% of the same compounds those two fragments are then determined to be related. Once every possible combination of two fragments in the model was tested for relatedness, a second graph was generated with the vertices representing fragments and the edges representing relationships (either related or non-related). A clustering algorithm was then used to generate all fragment clusters. The clusters contained anywhere from a single fragment to over a hundred fragments, with each clusters activity being representative of the activity of each of their members.

Identifying 'important' fragment and fragment clusters of activity and inactivity

As mentioned, four fragment models were developed leading to the ultimate development of one cluster model (the final model). These four fragment models were used for preliminary analysis and the best model was chosen for cluster analysis and final model (cluster model) development. The general mechanism for identifying and selecting fragments or fragment clusters are similar and are described together.

To determine any association between each fragment or fragment cluster and biological activity (or inactivity), a set of rules was implemented to select 'important' active and inactive clusters. The first selection rule- or the number rule- is the number compounds in the learning set that contain fragment(s) derived from a given cluster, which- in this exercise- was set at between three and five compounds. Looking at clusters that come from between three and five compounds in the learning set, models derived in the three to five range would be more inclusive (*i.e.*, higher coverage), while those in the four to five range would be more accurate (*i.e.*, higher concordance).

The second rule concerns the proportion of active or inactive compounds that contribute to each cluster and in the dissertation reporting these results ranged from between 85% to 95%. Even if a particular cluster is associated with activity, there may be other factors (*i.e.*, clusters) that contribute to it being inactive, and would not be expected to be found in 100% of the active compounds. For inactive fragments, a comparable argument can be made. Thus, by taking into account clusters toward the lower high end of the proportion scale (*e.g.*, derived from 60% active and 40% inactive) model would be expected to again be more inclusive (*i.e.*, higher coverage) while those derived from the

higher end of the proportion scale (*e.g.*, 90% active and 10% inactive) would be more accurate (*i.e.*, higher concordance).

Rule optimization

As in previous cat-SAR models (Cunningham et al., 2009; Cunningham et al., 2008; Qamar et al., 2011), setting of parameters for selecting important fragments (fragment compound counts and fragment activity proportion values) was used, with this experiment applying the same rules to fragment clusters. For these analyses, a rule optimization routine was employed wherein the Number Rule varied between 1 and 9 fragments or fragment clusters and the Proportion Rule varied between 0.50 and 0.95. Leave-one-out (LOO) validations were then performed for each model. The final models were chosen that were both highly accurate (*i.e.*, had a high concordance between experimental and predicted values) and highly inclusive (*i.e.*, made predictions on >90% of the chemicals in the learning set).

Model validation

A self-fit (*i.e.*, leave-none-out (LNO)) and two cross-validations (*i.e.*, LOO and multiple leave-many-out (LMO)) were performed for each model. The purpose of the self-fit analysis was to determine if the model that was built could be used to predict the activity of the chemicals in its learning set to confirm that the model could at least fit its own data (Qamar et al., 2011) as well as mechanistic studies since all available data is used to generate a final model.

For the LOO cross-validation, each chemical, one at a time, was removed from the total fragment or cluster set and the n−1 model was derived. For the LMO cross-validation, randomly selected sets of 10% of the chemicals were removed from the total

cluster set and the n–10 % model was derived. Using the same criteria described above, the activity of the removed chemical was then predicted using the n–1 model or n-10 % model. Predicted vs. experimental values for each chemical or for the chemicals in the left out sets were then compared and the model's concordance, sensitivity, and specificity were calculated (Qamar et al., 2011), where Concordance = Correct predictions / Total predictions, Sensitivity = Correct positive predictions / Total positive predictions, Specificity = Correct negative predictions / Total negative predictions.

The cat-SAR predictions are based on the active and inactive fragment clusters. The predicted activity of a chemical is calculated based on the average probability of all the active and inactive compounds contributing to its fragment clusters. One method to classify compounds back to an active or inactive category is to determine an optimal cutoff point that best separates the probabilistic prediction of active and inactive compounds derived from the LOO validations (Cunningham et al., 2009). Depending on the purpose of the model, the cutoff point can be adjusted wherein a model with the best overall concordance can be selected (*i.e.*, a most predictive model), one with equal sensitivity and specificity (*i.e.*, a balanced model that does not overly predictive active compounds at the cost of wrongly predicting inactive ones and vice versa), or one with high sensitivity.

Predicting activity

The resulting list of fragment clusters can then be used for mechanistic analysis, or to predict the activity of an unknown compound from the final model (Cunningham et al., 2009; Qamar et al., 2011). In order to predict the activity of an unknown compound, the cat-SAR program determines which, if any, clusters from the model's collection of

important fragment clusters are present in the unknown or test compound (Cunningham et al., 2009; Qamar et al., 2011). If none are present, no prediction of activity can be made for the compound (*i.e.* there are no default predictions of inactivity or activity). If one or more clusters are present, the number of active and inactive compounds containing each cluster is determined and the probability of activity or inactivity is then calculated based on the total number of active and inactive compounds that went into deriving each of the fragment clusters (Cunningham et al., 2009; Qamar et al., 2011).

The probability of activity was calculated with the cat-SAR FragSum routine (Cunningham et al., 2009; Qamar et al., 2011). This method calculates the average probability of the active and inactive clusters contained in each compound and is weighted to the number of active and inactive compounds that contribute to each cluster. For example, if a compound contains two clusters, one being found in 9/10 active compounds in the learning set (*i.e.*, 90% active) and the other being found in 3/3 inactive compounds (*i.e.*, 0% active), the unknown compound will be predicted to have a probability of activity of 69% (*i.e.*, 9/10 actives + 0/3 actives=9/13 actives or 69% chance of activity).

Results

Overview of predictive performance of the cat-SAR GPR119 models

The self-fit analysis of all models yielded concordance between experimental and predicted results averaging 99%. Considering the LOO validations with the FragSum method to calculate the probabilities of activity, the best GPR119 model had a concordance of 99%, a sensitivity of 99%, and a specificity of 100%. This model made predictions on 438 of the 440 chemicals in the learning set (no default prediction, see section 2.6 for description). The GPR119 models were also cross-validated with LMO. The GPR119 Model 1 had a concordance of 97%, a sensitivity of 95%, and a specificity of 99%.

Comparison of models

Using the difference between two proportions test, analysis of each set of four models derived from the random selection of inactive compounds indicated that the models had approximately the same concordance. For example, there was no significant difference between the four models. Model 1 correctly predicted 438 correct compounds out of 439 predictions (99%), and Model 2 correctly predicted 434 compounds out of 435 predictions (99%) ($p = 0.02$). Likewise, Model 3 correctly predicted 437 compounds out of 439 predictions (99%), and Model 4 correctly predicted 434 compounds out of 435 predictions (99%) ($p = 0.01$). This indicates that the accurate predictions made by the models were not spurious events based on a fortuitous random selection of "good" compounds (i.e., random selections of 222 inactive compounds from the 1000 compound inactive set) and thus provides assurance that the models are based on a sound foundation and are not providing arbitrary predictions or mechanistic assertions. Since there was no

significant difference between the four fragment models, Model 1 was used for cluster analysis and final model (cluster model) development.

Analysis of compounds in the training set

The two significant pharmacophores consist of: (1) an aryl or heteroaryl moiety substituted with a hydrogen bond accepting group on one part of the molecule and (2) a piperidine moiety N-capped with a carbamate or an isosteric heterocycle on the opposite side of the molecule. These two motifs are connected via an appropriate central spacer containing a heterocyclic ring or an acyclic chain (R. M. Jones, Leonarda, James N, 2009).

The clustering analysis of AR231453 indicates that the nitro-pyrimidine core and the presence of a sulfone moiety are responsible for AR231453 agonist activity. Furthermore, analysis of AR231453 resulted in several clusters that indicated the presence of critical hydrogen bond acceptors that is consistent with a previous report (Semple et al., 2008). In addition, several clusters contained a sulfone group that has previously been described as a key functional group for AR231453 as shown in Figure 5.1 Specifically, clustering analysis of AR231453 resulted in the generation of five clusters (Cluster 53, Cluster 113, Cluster 129, Cluster 177, and Cluster 503), of which a representative fragment from each group is shown (Figure 1), which are associated with the activity of this compound.

The SAR analysis of PSN632408 has also been described (R. M. Jones, Leonarda, James N, 2009). Through extensive SAR analysis, it was determined that the N-capped piperdine motif is required for GPR119 agonist activity. The clustering analysis of PSN632408 resulted in the generation of five clusters (Cluster 54, Cluster 135, Cluster

188, Cluster 234, and Cluster 444), of which a representative fragment from each group is shown, that are associated with the activity of this compound (Figure 5.2). This analysis is consistent with a previous report (R. M. Jones, Leonarda, James N, 2009), wherein the N-capped piperidine core with a carbamate was determined to be responsible for PSN632408 agonist activity.

Analysis of compounds not in the training set

Several of the known GPR119 agonists have evolved from the prototypical compounds 2-fluoro-4-methanesulfonyl-phenyl)-{6-(4-(3-isopropyl-(1,2,4)oxadiazol-5-yl)-piperidin-1-yl}-5-nitro-pyrimidin-4-yl}-amine (AR231453) (Semple et al., 2008) and tert-butyl 4-{(3-pyridin-4-yl-1,2,4-oxadiazol-5-yl)methoxy}piperidine-1-carboxylate (PSN632408) (Overton et al., 2006).

In addition to the internal validations performed (LOO, LMO), an external validation was employed to predict the activities of 45 compounds (external test set) which were not present in the training set. The purpose of this was to ensure the robustness of the model by testing the hypothesis that it could accurately predict the activity of compounds not in the training set.

Of these 45 compounds, 14 were known GPR119 agonists while 31 were not agonists for GPR119 confirmed by cAMP assay. The 14 known agonists consisted of compounds that can be structurally classified in two groups: 1) bicyclic amine scaffolds and 2) pyrazolopyrimidine scaffolds. These 14 known GPR119 agonists were selected from patents and literature sources. The 31 inactive compounds consisted of compounds which do act as agonists for GPR119 at concentrations up to 10 μM, which were experimentally confirmed using the established HTRF assay (Kumar et al., 2014). These inactive chemicals consisted of compounds originating from three distinct chemical

libraries (FDA, NIH clinical collection, Tocriscreen) ensuring structural diversity. The GPR119 model 1 had a 78.6 % success rate in correctly predicting 11 out of 14 as GPR119 agonists. Furthermore, the model had a 90.3 % success rate in correctly predicting 28 / 31 as inactive compounds (not GPR119 agonists). Overall, the model achieved an 86.7 % success rate in correctly predicting the activity of compounds in the test set.

Recently, Wu et al. 2010 synthesized a series of piperazinylpyridine derivatives as GPR119 agonists (Wu et al., 2010). Through SAR analysis, compounds with alkylsulfonamide and isopropylcarbamate end groups displayed potent GPR119 receptor activity (Wu et al., 2010). The clustering analysis of propan-2-yl 4-([6-[4-(propane-1-sulfonyl)piperzin-1-yl]-oxy)methylpiperidine-1-carboxylate (Wu Compound 19A) resulted in the generation of three clusters (Cluster 36, Cluster 64, and Cluster 435) of which a representative fragment from each group is shown which are responsible for the activity of this compound (Figure 5.3). The fragment analysis indicates that the sulfonamide and carbamate groups are important for Wu Compound 19A agonist activity, which is consistent with previously reported SAR of this compound (Wu et al., 2010).

Previously, in a separate study, Sakairi et al. 2012 disclosed a novel series of GPR119 agonists based on a bicyclic amine scaffold (Sakairi et al., 2012). Through SAR analysis of Wu Compound 19A, it was determined that the basic nitrogen atom of the bicyclic amine played an important role in the production of GPR119 agonist activity (Sakairi et al., 2012). Furthermore, Sakairi and coworkers showed that the carbonyl group on the bicyclic core represented a better pharmacophore than a sulfonyl group (Sakairi et al., 2012) which is consistent with the results.

The first ligand-based pharmacophore model of GPR119 was developed by Zhu and coworkers to obtain a hypothetical picture of the chemical features responsible for activity (Zhu et al., 2011). Pharmacophore models were generated with 24 known GPR119 agonists using Discovery Studio V2.1 (Zhu et al., 2011). The application of this model was able to predict the activity of 25 known GPR119 agonists with a correlation coefficient of 0.933.

Wellenzohn and coworkers recently described the application of a virtual screening technique that was used to identify novel GPR119 agonists (Wellenzohn et al., 2012). The virtual screening process consisted of an activity anchor and the use of feature tree fragment space searches which was followed by a 3D post-processing step (Wellenzohn et al., 2012). The *in silico* results were then filtered and prioritized and combinatorial libraries of target molecules were synthesized. This method resulted in the discovery of two new structural classes of potent GPR119 agonists, one of which has progressed as a novel lead class [23].

A key difference between the modeling approach in the present dissertation and Zhu and coworkers (Zhu et al., 2011) is that cat-SAR is based on the analysis of categorical data and 2-dimensional fragments versus intact chemicals (Cunningham et al., 2009). This allows the program to examine data sets that are divided into categories of activity rather than degrees of potency (Cunningham et al., 2009).

Applicability domain of models

It should be noted that even though the models have high specificity, high sensitivity, and high concordance values, the predictive ability is limited by the model's applicability domain (AD). The applicability domain (AD) refers to a theoretical region

in the space defined by the descriptors of the model which provides insight into the development and applicability on which the training set can make reliable prediction for unknown compounds. As far as a potential virtual screening tool, the AD of the GPR119 models is somewhat constrained by the lack of diversity of the structures of the active set. To be useful as a virtual screening tool, it would be necessary to sort and rank the compounds that were predicted as potential ligands and compare the structures to the active compounds in the training set. Then, ad-hoc decisions on whether or not to test these potential ligands could be made.
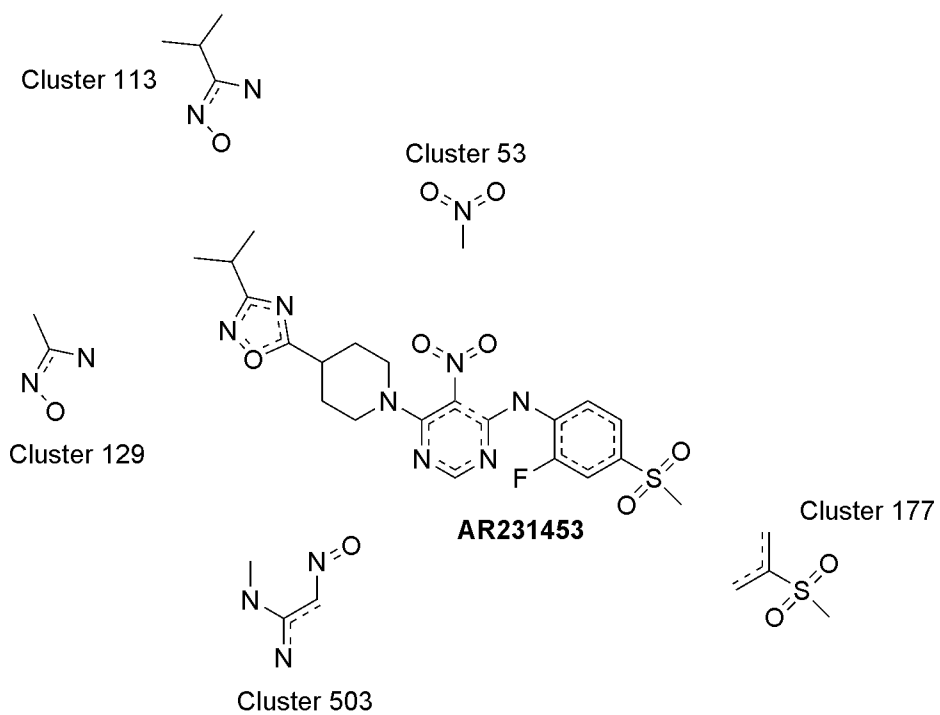
Fig. 5.1. Five representative clusters used to predict the activity of AR231453 by the GPR119 cat-SAR model.
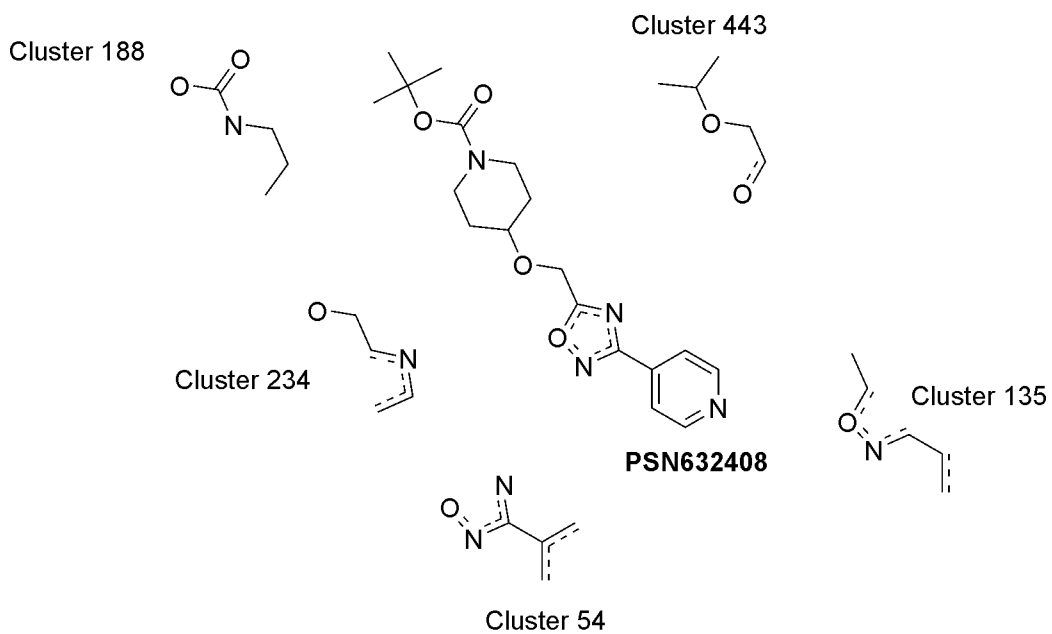
Fig. 5.2. Five representative clusters used to predict the activity of PSN632408 by the GPR119 cat-SAR model.
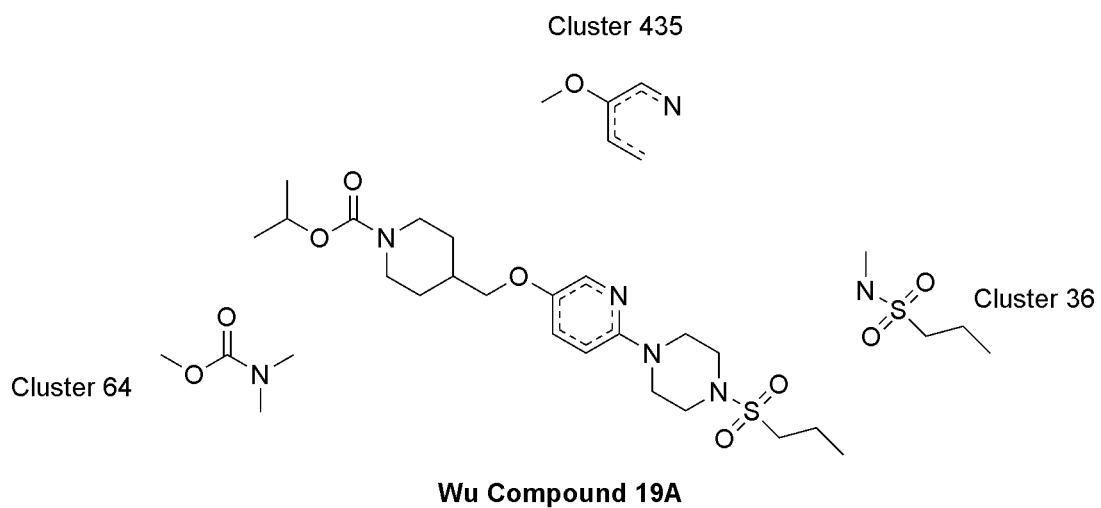
Fig. 5.3. Five representative clusters used to predict the activity of Wu Compound 19A by the GPR119 cat-SAR model.

Discussion

The dissertation reporting these results was the first report of the application of a fragment-based modeling approach using the cat-SAR expert system to model GPR119. The good predictive ability of these models to understand 2-D molecular fragments indicates their potential usefulness in investigating the relationship between GPR119 ligand structure and activity as the model was able to correctly predict the activity of compounds outside of the training set.

It is expected that the generated information could be used to identify the chemical moieties specific to GPR119 activity. Thus the cat-SAR expert system produces models which are predictive and are based on mechanically sound attributes. Most importantly, the dissertation reporting these results was the first to demonstrate that the cat-SAR expert system can be used to model a GPCR. Overall, a model that can be potentially used to virtually screen large databases with high specificity, high sensitivity, and high concordance but the applicability domain must be taken into consideration.

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CURRICULUM VITAE

Pritesh Kumar

PhytoSciences Inc., Louisville, KY   USA   Present
Chief Executive Officer
- Lead team of 35 Global Research Scientists focused on R & D of novel medical cannabis-derived therapeutics
- Oversee all operations and ensure projects meet client deadlines
- Develop strategic alliances and partnerships to support corporate vision and goals
- Oversee product development of novel delivery systems for cannabinoid therapy

Vida Cannabis Corp., Ottawa, Ontario   Canada   April 2014 – Present
Chief Cannabinoid Research Scientist
- Ensure product quality and compliance with all Domestic and Global regulatory and corporate requirements
- Manage all QA/QC matters
- Oversee the QA/QC operations at the on-site laboratory
- Assure that operations meet requirements and cGMP/cGLP regulations in compliance with *Marijuana* for Medical Purposes Regulations (MMPR)
- Oversee the development and execution of the company's QA Management system
- Advise on new product development, review and authorize the release of finished products and raw materials
- Ensure that all sanitation, production, processing, packaging, testing, storage are in full compliance with MMPR and with the standards and practices.
- Determine cannabinoid profiles from various strains to develop illness-specific medications

Quantum 9 Inc., Chicago, IL USA   June 2013 – November 2014
Scientific consultant
- Prepare documentation pertaining to medical marijuana license acquisition (*e.g.* Sanitation protocols, GMPs) for U.S/Canada clients
- Advise U.S/Canada clients pertaining to laboratory equipment for operating a cannabis testing facility
- Advise U.S/Canada clients regarding the pharmacology of cannabinoid-based therapeutics

KY State Capitol, Frankfort, KY USA   November 2013 – Present
Scientific Adviser to Senator Perry Clark
- Advise on the development of effective medical cannabis policies
- Advise on the implementation of various medical cannabis legislative bills

- Prepare legislative bills as it pertains to medical cannabis

University of North Carolina, Greensboro, NC USA   September 2012 – Present
Cannabinoid Consultant and QA/QC Analyst
- Conduct pharmacological testing and analysis of novel CB2 antagonists/derivatives
- Prepare Certificates of Analysis (COA) as it applies to purity and testing of CB2 antagonists
- Responsible for issuing COA for each batch/lot

University of Louisville, Louisville, KY US   July 2009 – Present
Cannabinoid Pharmacologist / Laboratory and QA/QC Manager
- Conduct medical cannabinoid research as it pertains to the cannabinoid receptor 2 (CB2)
- Pharmacological testing of FDA approved drugs as potential ligands for CB2
- Investigating the pharmacology of cannabidiol (CBD) for CB2
- Responsible for all aspects of the laboratory: inventory management, ordering supplies and equipment maintenance
- Responsible for all aspects of QA as it pertains to the proper use, handling, and storage of cannabinoid ligands
- Responsible for all aspects of QC as it pertains to the pharmacological testing of synthetic cannabinoid ligands
- Developing, implementing, and reviewing SOPs pertaining to cannabinoid testing
- Responsible for maintaining a sterile working environment
- Oversee and implement a sanitation program to ensure laboratory grade cleanliness
- Responsible for performing weekly radiation, biosafety, and hazardous waste tests to ensure a safe working environment
- DQ/IQ/OQ/PQ validation as it pertains to cannabinoid testing systems (*e.g.* TECAN Genius Pro, Brandel Systems)
- Developed SOPs for recalls as it pertains to cannabinoid ligands
- Responsible for initiating recalls for compound drug libraries in accordance with 21 CFR 7.46
- Lead laboratory investigations for material, in-process, and finished pharmaceutical cannabinoid ligands OOS procedures
- Write and approve laboratory SOPs as it pertains to analytical and microbiological testing for contaminants
- Manage and oversee the entire QA/QC system for the laboratory including testing for contaminants
- Perform QA training for laboratory technicians / employees
- Conducts microbiological assays for bacterial staining and plating methods (streak)
- Prepares cell culture media and all buffers needed for microbiological assays
- Implemented a protocol to completely remove *mycoplasma* contamination from cell culture media
- Performs routine testing on cell culture media for the presence of *S. Aureus, Salmonella, Clostridium,* and other common contaminants present in incubators

University of Kentucky, Department of Nutritional Biochemistry, Lexington, KY USA   2008 – 2009

Research Assistant
- Investigated the role nutrition plays in preventing vascular toxicity induced by polychlorinated biphenyls (PCBs)
- Performed QC on cell culture media
- Responsible for laboratory maintenance

Heartland Sweeteners & Lexington Pharmaceuticals LLC, Lexington, KY USA   2007 – 2008

QA/QC Analyst
- Verified purity among commercially available sugar products via HPLC
- Assisted in the development of a novel methodology to enhance sweetness of company's sugar product
- Conducted research regarding new techniques to monitor sugar synthesis reactions
- Performed QC on batches/lots to ensure purity for bulk production
- Maintained batch production records (batch production and control records)
- Calibrated lab equipment for HPLC analysis
- Managed raw material testing program / product formulation processes
- Implemented a complaint management system in compliance with ISO 10002
- Managed complaints related to commercially available sugar products
- Audited the compliant-handling process
- Oversaw and implemented a sanitation program to ensure pharmaceutical grade cleanliness

University of Kentucky, Department of Microbiology, Lexington, KY USA 2006 – 2007

Laboratory Technician
- Performed microbiological assays (PCR, DNA extraction, agar plate streaking) to identify sources of contamination
- Conducted bacterial staining (Gram +/-) to identify specific types of bacteria present in culture
- Performed bacterial culture identification and analyzed bacterial growth curves

Education

University of Louisville   2015

Ph. D. Pharmacology & Toxicology
Louisville, KY

University of Louisville   2011

M.S. Pharmacology & Toxicology, Cannabinoid Concentration

Louisville, KY

University of Kentucky   2005
B.S. Biology
Lexington, KY

Professional Organizations

Member, Technology Sub-Committee
- *Initiated policies to advance enrollment for online education at the University of Louisville*

IACM (International Association for Cannabis as Medicine)
2013 – present
NCIA (National Cannabis Industry Association)
2013 – present
LCME (Liaison Committee on Medical Education)
2012
- *Committee head for the documentation for the re-accreditation of University of Louisville School of Medicine*

ICRS (International Cannabinoid Research Society)
2009 – present
ASCB (American Society of Cell Biology)   2
NGS (National Glaucoma Society)
2009 – present

Honors / Awards

Travel Award for ICRS Conference, Vancouver BC, Canada from NIDA (National Institute on Drug Abuse)   2013
Award for Best Oral Presentation at ICRS Conference, Vancouver, BC, Canada
2013
Fellowship from Drug Discovery and Target, Boston, MA USA
2013
NIEHS (National Institute of Environmental Health Sciences) fellowship
2012 –present
Travel Award for ICRS Conference, Freiburg, Germany, from NIDA (National Institute on Drug Abuse   2012
IPIBS (Integrated Programs in Biomedical Sciences) fellowship
2009-2011
Travel Award for ASCB Conference, Philadelphia, PA from UL School of Medicine
2010
Travel Award for ICRS Conference, St. Charles, IL from NIDA (National Institute on Drug Abuse)   2011

Abstracts and Presentations

Guest Speaker at ComfyTree Cannabis Academy, Owensboro. KY USA
2014
Guest Speaker at ComfyTree Cannabis Academy, Louisville, KY USA
2014
Guest Speaker at ComfyTree Cannabis Academy, Cincinnati, OH USA
2014
Speaker for house-senate caucus as it relates to developing practical medical cannabis legislation   2014
Oral Presentation ICRS, Vancouver, BC Canada
2013
Poster Presentation Discovery on Target Conference, Boston, MA USA
2013
Poster Presentation CCC (Carolina Cannabinoid Conference), Richmond, Virginia USA
2013
Poster Presentation ICRS, Freiburg, Germany
2012
Poster Presentation ICRS, St. Charles, IL USA
2011
Poster Presentation ASCB (American Society of Cell Biology), Philadelphia PA USA
2010

Peer-Reviewed Publications

1)   Kumar A, Qiao Z, **Kumar P**, Song ZH. (2012). Effects of Palmitoylethanolamide on Aqueous Humor Outflow.   Invest Ophthalmol Vis Sci. Accepted doi: 10.1167/iovs.11-9294

2)   Kumar A, Qiao Z, **Kumar P**, Song ZH. (2012). Involvement of a non-CB1/CB2 cannabinoid receptor in the   aqueous humor outflow-enhancing effects of abnormal-cannabidiol. Exp Eye Res. Accepted http://dx.doi.org/10.1016/j.bbr.2011.03.031

3)   **Kumar P**, Song ZH. (2013). Identification of raloxifene as a novel CB2 inverse agonist. Biochem Biophys Res Commun. doi: 10.1016/j.bbrc.2013.04.040. [Epub ahead of print]

4)   Kotsikorou E, Navas F 3rd, Roche MJ, Gilliam AF, Thomas BF, Seltzman HH, **Kumar P**, Song ZH, Hurst DP, Lynch DL, Reggio PH (2013).The importance of hydrogen bonding and aromatic stacking to the affinity and efficacy of cannabinoid receptor CB2 antagonist, 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide (SR144528).

5) **Kumar P,** Song ZH. (2014). Structure-activity relationships of fatty acid amide ligands in activating and desensitizing G protein-coupled receptor 119. Eur J Pharmacol. doi: 10.1016/j.ejphar.2013.10.044.

6) **Kumar P,** Song ZH (2014). CB2 cannabinoid receptor is a novel target for third-generation selective estrogen receptor modulators bazedoxifene and lasofoxifene. Biochem Biophys Res Commun. Accepted.

7) **Kumar P,** Carrasquer C, Carter A, Song ZH, Cunningham AR (2014). A categorical structure-activity relationship analysis of GPR119 ligands. SAR and QSAR in environmental research. doi: 10.1080/1062936X.2014.967292.