Universidade de Lisboa
Faculdade de Farmácia

Identification and Pharmacological Evaluation of Surrogate Ligands of Cannabinoid Receptors

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Mestrado Integrado em Ciências Farmacêuticas

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Abbreviations

Δ^9-THC	Δ^9-tetrahydrocannabinol
2-AG	2-arachidonyl glycerol
2-AGPI	2-arachidonoyl-sn-glycero-3-phosphoinositol
5-HT	Serotonin
$5HT_{1A}R$	Serotonin receptor 1A
$5HT_{2A}R$	Serotonin receptor 2A
$5HT_6R$	Serotonin receptor 6
$5HT_7R$	Serotonin receptor 7
AC	Adenylyl cyclase
abn-CBD	Abnormal-cannabidinol
AD	Alzheimer's disease
AEA	N-arachidonyl ethanolamine
ALS	Amyotrophic lateral sclerosis
b-gal	β-galactosidase
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CaSR	Ca^{2+}-sensing receptor
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CCR5	C-C chemokine receptor type 5
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene related peptide
CHO	Chinese hamster ovary
CNR1	CB1 receptor gene
CNR2	CB2 receptor gene
CNS	Central nervous system
CREB	cAMP response element binding protein
CXCR4	C-X-C chemokine receptor type 4
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DRD2	Dopamine Receptor D2

EA	Enzyme acceptor
EC	Extracellular
ECL	Extracellular loop
ECS	endocannabinoid system
ED	Enzyme donor
EFC	Enzyme fragment complementation
ERK	Extracellular-signal-regulated kinase
F12	Nutrient mixture F-12
FCS	Fetal calf serum
FDA	Food and Drug Administration
FFAR1	Free fatty acid receptor 1
$GABA_B$	Gamma-aminobutyric acid
GDP	Guanosine diphosphate
GLP1	Glucagon-like peptide-1
GLP1R	Glucagon-like peptide-1 receptor
GnRH	Gonadotropin-Releasing Hormone
G-protein	GTP binding protein
GPCR	G protein-coupled receptor
GPR18	G-protein coupled receptor 18
GPR23	G-protein coupled receptor 23
GPR35	G-protein coupled receptor 35
GPR55	G-protein coupled receptor 55
GPR84	G-protein coupled receptor 84
GPR92	G-protein coupled receptor 92
GPR119	G-protein coupled receptor 119
GRK	G protein-coupled receptor kinase
GSK	GlaxoSmithKline Company
GTP	Guanosine-5'-triphosphate
H2R	Histamine Receptor 2
HBSS	Hank’s buffered saline solution
HD	Huntington’s Disease
HIV-1	Human Immunodeficiency Virus 1
IC	Intracellular
ICL	Intracellular loop

IP3	Inositol triphosphate
LPA	Lysophosphatidic acid
LPI	L-α-lysophosphatidylinositol
mAbs	Monoclonal antibodies
mGluR	Metabotropic glutamate receptor
MS	Multiple Sclerosis
NAGly	Acylamino acid N-arachidonoylglycine
NC IUPHAR	International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification
NIH	United States National Institutes of Health
NK1R	Neurokinin 1 receptor
OEA	Oleoylethanolamida
P2Y5	Lysophosphatidic acid receptor 6
PBS	Phosphate buffer saline
PD	Parkinson´s disease
PEA	Palmitoylethanilamida
PLA1	Ca^{2+}-dependent phospholipase A1
PLA2	Ca^{2+}-independent phospholipase A2
PK	ProLink™
PKA	cAMP - protein kinase A
PKC	IP3 – protein kinase C
PLCγ	Phosphoinositide phospholipase Cγ
PTH1R	Parathyroid hormone 1 receptor
RGSs	Regulators of G protein signalling
RL	Radioligand
RvD2	Resolvin D2
S1P1	Sphingosine 1-phosphate receptor 1
siRNA	Short interference RNA
SMO	Smoothened
T2D	Type II diabetes
TAS1R	Amino acid taste receptor
TM	Transmembrane
TRPV1	Transient receptor potential vanilloid type-1 channel

Abstract

G protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors. Essential for the control of several physiologic and pathophysiologic processes, such cancer, hypertension, abnormal bone development, and others, GPCRs are one of the most studied families of receptors. The number of drugs targeting these receptors has been increasing, counting with more than 475 FDA approved drugs.

In this work we focused in four GPCRs: Cannabinoid Receptor 1 (CB1) and 2 (CB2), two cannabinoid receptors, G protein coupled receptor 18 (GPR18) and 55 (GPR55), two orphan receptors. CB1 and CB2 belong to the GPCR family class A. CB1 is highly expressed in the central nervous system (CNS), while CB2 is abundant in peripheral organs with immune function. GPR55 belongs to the GPCR family class A, and it is suggested as a therapeutic target in obesity, diabetes, osteoporosis and cancer. GPR18 also is a class A GPCR, and may be a therapeutic target in inflammatory diseases, cancer, and intraocular high pressure.

Therefore, the aim of this study was to identify and evaluate pharmacologically surrogate ligands of these cannabinoid receptors.

Previous studies from our lab also identified some compounds, from the in-house library, that were able to modulate these receptors' activity. Based on these molecular structures, we screened the agonist and antagonist effect of these new compounds, from our in-house library. For that initial screening was performed, followed by selectivity and potency studies, using the β-arrestin recruitment assay, radioligand binding assay and cAMP accumulation assay. For GPR18, two series of compounds were tested (compound library 1 and 2), while for CB2 only one series of compounds (compound library 3) were tested. Selectivity studies were performed using with GPR55 and CB1, respectively.

Our results identified one promising compound for GPR18 (EC_{50}(compound 56)=0.0302±0.004 μM) and another one for CB2 (Ki(compound 76)=0.339±0.061 μM).

In the future, more studies need to be done to understand how these compounds modulate these receptors' activity and to optimize their activity, with the goal of developing new molecules that could be used as a therapeutic approach to several diseases related with dysfunctions in CB1, CB2, GPR18 and GPR55.

Key-Words: G-protein coupled receptors; Cannabinoid Receptors; GPR55; GPR18; Surrogate ligands.

Resumo

Os recetores acoplados à proteína G (GPCRs) constituem a maior família de recetores membranares, sendo essenciais no controlo de processos fisiológicos e patofisiológicos, como cancro, hipertensão, entre outros. O número de fármacos direcionados para estes recetores tem vindo a aumentar, contando com mais de 475 aprovados pela FDA.

Neste estudo focámo-nos em quatro recetores: Recetor Canabinoide 1 (CB1) e 2 (CB2) e recetor acoplado à proteína G 18 (GPR18) e 55 (GPR55), dois recetores órfãos. CB1 e CB2 pertencem à classe A da família de GPCRs. A expressão de CB1 é elevada no sistema nervoso central, enquanto que CB2 é abundante em órgãos periféricos com função imunitária. GPR55 pertence à classe A da família de GPCRs, sendo sugerido como alvo terapêutico na diabetes, obesidade, osteoporose e cancro. GPR18 pertence também à classe A e poderá ser alvo em terapias para cancro e doenças inflamatórias.

O objetivo deste estudo consistiu na identificação e avaliação farmacológica de ligandos destes recetores.

Estudos anteriores do nosso laboratório identificaram alguns compostos, de uma biblioteca de compostos *in-house*, capazes de modular a atividade destes recetores. Tendo por base a estrutura molecular destes, fizemos o *screening* do efeito antagonista e agonista destes novos compostos, pertencentes também à nossa biblioteca. Seguidamente ao *screening* inicial, foram realizados estudos de seletividade e potência, recorrendo a ensaios de recrutamento de β-arrestina, ligação de radioligandos e acumulação de cAMP. Para o GPR18 foram testadas duas séries de compostos (biblioteca de compostos 1 e 2), enquanto que para o CB2 foi testada apenas uma série de compostos (biblioteca de compostos 3). Os estudos de seletividade foram realizados com GPR55 e CB1, respetivamente.

Os nossos resultados identificaram um composto promissor para GPR18 (EC_{50}(composto56)=0.0302±0.004 μM) e um para CB2 (Ki(composto76)=0.339±0.061 μM). No entanto, mais testes devem ser executados, de forma a clarificar se estes compostos conseguem efetivamente modular a atividade destes recetores.

Futuramente, será necessária a realização de novos estudos, permitindo compreender como estes compostos modulam a atividade destes recetores e otimizar a mesma, com objetivo de desenvolver novas moléculas a serem usadas como terapêuticas em várias disfunções relacionadas com CB1, CB2, GPR18 e GPR55.

Palavras-Chave: Recetores acoplados à proteína G; Recetores Canabinoides; GPR55; GPR18; Ligandos.

Introduction

1. G Protein-Coupled Receptors (GPCRs)

G protein-coupled receptors (GPCRs) comprise about 1000 of distinct receptors, being the most common and the largest family of membrane receptors. These receptors are encoded by more than 800 genes in the human genome, comprising about 1% to 5% of the entire vertebrate's genome. GPCRs recognize several ligands including neurotransmitters, small molecules, hormones, peptides, biogenic amines, amino acids, nucleosides, elemental ions, lipids and photons of light (1,2).

More than 140 GPCRs are considered orphan receptors, since they have a similar structure to others identified receptors but they have unknown endogenous selective and exclusive ligand(s) (1,3).

When GPCRs are overactive or inactive, they can be associate to several diseases and syndromes, such as abnormal bone development, night blindness, stroke, cancer, thyroid dysfunction, congenital bowel obstruction, hypertension, congestive heart failure and neonatal hyperparathyroidism (2).

The superfamily of GPCRs can be divided into six principal families. Class A or 1, the rhodopsin receptor family, includes around 85% of GPCRs and this class responds to exogenous and endogenous ligands, and can be divided in further three subclasses (I, II and III) according to the type of ligands. Subclass I include ligands like neurotransmitters and light photons, subclass II includes receptors for peptides and subclass III for glycoprotein hormones. Class B or 2, the secretin receptor family, respond to large proteins and peptide hormones, such as gastrointestinal hormones, calcitonin, parathyroid hormone, growth hormone-releasing factors, and others. Class C or 3, includes metabotropic glutamate receptors (mGluRs), gamma-aminobutyric acid ($GABA_B$) receptors, pheromone receptors, Ca^{2+}-sensing receptors (CaSRs) of the brain, parathyroid and kidney, sweet and amino acid taste receptors (TAS1R) and odorant receptors in fish. Class D or 4, corresponds to the fungal mating pheromone receptors, and Class E or 5, cyclic adenosine monophosphate (cAMP) receptors, are involved in the developmental control. Finally, F or 6 correspond to frizzled/smoothened, receptors that are involved in embryonic development and other cellular and physiologic processes. The classes D and E are not found in vertebrates (1,2).

GPCRs are composed by an extracellular (EC) domain, being the most variable region between receptors, contrasting with the transmembrane region and the ligand-binding pocket, and, finally, by the intracellular (IC) domain (since its binding partners have a limited diversity, this region is moderately conserved) (Fig. 1) (2).

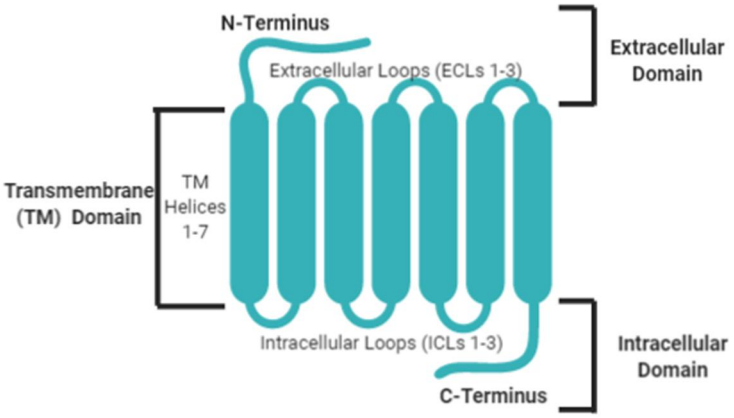


Figure 1: Structural characteristics of GPCRs. GPCRs have common structural characteristics, being composed by an N-terminus, followed by seven α-helices (TM helices 1-7), interconnected by loops of different lengths at both extracellular (ECL 1-3) and intracellular (ICL 1-3) sides, and a C-terminus. Most of the differences observed between classes of GPCRs are localized at the intra and extracellular domains of the protein *(Adapted from: Latorraca et al. 2017)*.

The transduction of the signal across the cell membrane is made by the transmembrane domain. This domain has a common structure between the different GPCR's classes, and it is composed by a bundle of seven α-helices, inserted in the cell membrane (termed TM1 through TM7), connected by three intracellular and three extracellular loops (termed ICL1 through ICL3 and ECL1 through ECL3, respectively). It is also important for the mediation of interactions of the receptor and the intracellular components, including GTP binding proteins (G-proteins) and arrestins The rearrangement of the transmembrane helices plays a crucial role in the transmission of the signal to the interior of the cell (2,3,4).

The GPCR's ability to change between different conformations, allows these receptors, to detect the presence of several molecules, to transmit a signal through the cell membrane and to initiate a variety of intracellular signalling cascades in response to ligand biding. Thus, the study of the structural dynamics of GPCRs is an important instrument to the design of targeted drugs (4).

Different ligands binding induces multiple conformational states, with different abilities to recruit intracellular binding partners, which can lead to the activation of distinct signalling pathways. Generally, binding of a ligand to a GPCR alter the fraction of time that the receptor remains in a certain conformational state (4).

GPCRs have a complex response process. The majority of these receptors tend to have baseline activity, even in the absence of any external stimulus. The response of the receptor can be different according to the type of ligand (Fig.2A). A full agonist induces maximal receptor activity by stabilizing an active conformation, while a partial agonist just induces a partial activity of the receptor and has affinity to the active and inactive conformations. An inverse agonist differs from a antagonist (or neutral agonist) because, while an antagonist prevent others ligands from binding and activating the receptor, an inverse agonist decrease the constitutive activity by stabilizing the inactive conformation of the receptor. Moreover, ligands can bind to a GPCR at the same site as the native ligand or to other sites, being called as

orthosteric ligands or allosteric ligands/modulators, respectively. Allosteric ligands can alter several aspects of the dynamics, structure and function of a GPCR, such as: increase or decrease the affinity or influence the efficacy of orthosteric ligands; induce the activation of the receptor, even when there is not a orthosteric ligand (Fig.2B). When different receptors present identical orthosteric sites allosteric modulators may achieve selectivity between receptor subtypes. The GPCR's activity is influenced by oligomerization and by the localization on certain membrane region (2,3).

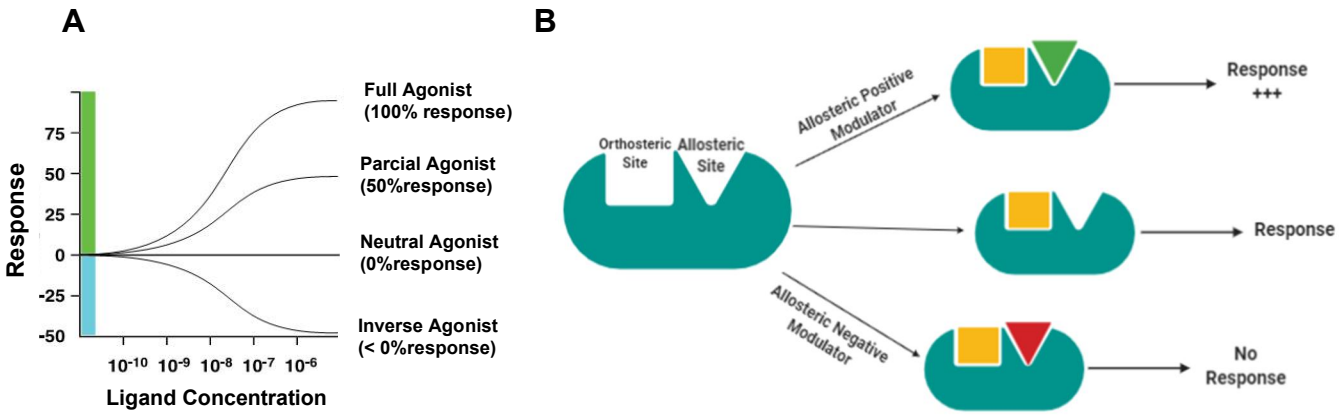


***Figure 2: The type of ligands influences the response of the GPCR**. (A) Dose-response curve of a cellular response according to the type of ligand*. (*B) Orthosteric and Allosteric ligands*. (*Adapted from*: *Saengsawang & Rasenick 2015; Latorraca et al. 2017;Vavers et al. 2019)*.

GPRCs transmit signals into cells via activation of the G-proteins. These proteins activate effector proteins, initiating a cascade of events and culminating with an activation or inhibition of certain proteins, such as enzymes, proteins associated with vesicular transport and ion channels. The messenger molecule, the associated G-protein, and the GPCR type influence the activated pathway. Indeed, the activation of a receptor by an agonist induces the modulation of the activity of a large number of enzymes and ion channels such as potassium channels, calcium channels, phospholipases, adenylyl cyclase, Ser/Thr protein kinases, and G protein-coupled receptor kinases (GRKs) to activate further downstream signalling cascades. This event can also cause various intracellular alterations in the levels of second messenger molecules such as cAMP, intracellular Ca^{2+}, inositol triphosphate (IP3), cAMP response element binding protein (CREB) and diacylglycerol (DAG) (1,2).

G-proteins are composed by three subunits, α, β and γ and Gαs can be classified into distinct families: Gαs activates adenylyl cyclase, inducing the cAMP - protein kinase A (PKA) signaling cascade and can be over-activated by the cholera

toxin; $G\alpha i_{/o}$ inhibits adenylyl cyclase (AC), activates c-Src tyrosine kinases and can be inactivated by the pertussis toxin; $G\alpha q_{/11}$ that activates phosphoinositide phospholipase Cγ (PLCγ), inducing the IP3 – protein kinase C (PKC) signaling cascade, $G\alpha_{12/13}$ allows Rho (a family of small signalling G proteins) activation, and $G\alpha_{transducin}$ which activates cyclic guanosine monophosphate (cGMP) phosphodiesterase in the retina (Tab. 1) (2).

Table 1: Signalling pathways activated according to the Gα Family

Gα Family	Signaling Pathway Activated
Gαs	Activation of adenylyl cyclase → Induction of cAMP-PKA signaling cascade
$G\alpha i_{/o}$	Inhibition of adenylyl cyclase → Activation of c-Src tyrosine kinases
$G\alpha q_{/11}$	Activation of PLCγ → Induction of IP3-PKC signaling cascade
$G\alpha_{12/13}$	Rho activation
$G\alpha_{transducin}$	Activation of cGMP phosphodiesterase in the retina

When in a resting state, the G-protein can bind to an active or inactive GPCR or drift in the membrane. The three subunits of G-proteins are bound tightly to each other and to the membrane. Gα is bound to the guanosine diphosphate (GDP) nucleotide and Gβ is tightly bound to Gγ via nonpolar interactions. The Gα and the Gγ subunits are covalently attached to the membrane (2).

When a GPCR is activated, the G-protein switches to an active state, emerging a conformational change in the Gα, leading to: 1) the conversion of GDP into guanosine-5'-triphosphate (GTP) by Gα (this subunit has GTPase activity); 2) the departure of Gβγ complex, which, in its turn, can bind to effectors equivalent or different from those of Gα; 3) the binding of Gα to an effector protein; 4) and activation of the effector protein (Fig. 3 – 1,2,3) (2).

After a short time, Gα suffers a conformational change, caused by the hydrolysis of GTP to GDP. Since the GTPase activity of Gα it is not too fast enough for the cellular response requirements, in order to accelerate this process, G-proteins interact with proteins called ‘regulators of G protein signalling’ (RGSs). This allows the reattachment of Gα with Gβγ complex, restoring the resting state and preparing the system to another cycle (Fig.3 - 4) (2).

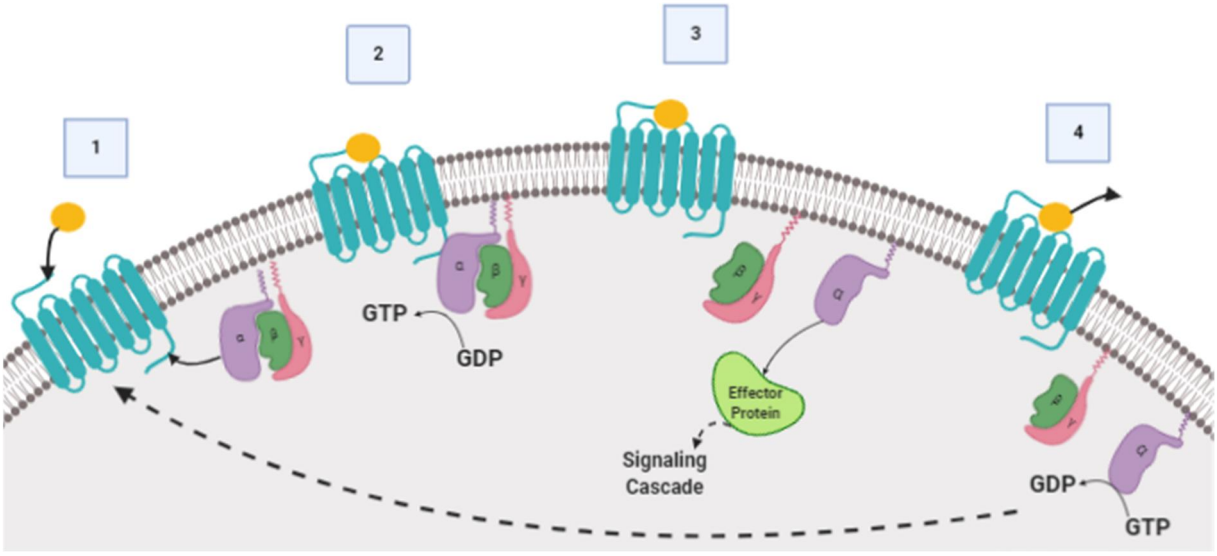


Figure 3: GPCR signaling cycle. (1) When an agonist binds to extracellular domain of the inactive GPCR, the G-protein binds to the intracellular domain of the receptor. (2) *Conversion of* GDP into GTP in G*α*'s nucleotide binding, site, (3) leading to the separation of G*α* from G*βγ* and, successively, to the binding of the G*α* subunit to an effector protein, initiating the signalling cascade.(4) After a short time, the bound between GTP and G*α* is hydrolysed and this subunit separates from its effector protein, re-binding to G*βγ* complex. This last step restores the resting state and the system is prepared for another cycle (*Adapted from: Saengsawang & Rasenick 2015)*.

The formation of heterodimers (dimers or oligomers) is also possible. This suggests that intracellular signalling can change under different conditions and it may have an important role in GPCR signalling transduction, crosstalk between signalling pathways and regulation of GPCR activity (2,4).

In order to avoid the over-stimulation, after activation of the downstream signalling pathway, the receptor is desensitized by two distinct processes: homologous desensitization or heterologous downregulation. Homologous desensitization is mediated by the phosphorylation of the C-terminal of the receptor, which allows the interaction between the GPCR and arrestin proteins, ending the signalling, by preventing the interaction of the receptor with its related G-protein, and inducing the internalization by clathrin-coated vesicles, that can by either recycled to the cell membrane or lead to the degradation of the receptor. Heterologous downregulation acts on other membrane receptors (and not on the active GPCR) and its mediated by second messenger-activated kinases, like PKA or PKC. When the receptors stimulation is prolonged, the downregulation mechanisms are more complex and include modulation of gene transcription and translation (2,3,5).

1.1. GPCRs as therapeutic targets

These receptors have a large membrane distribution and can be involved in numerous physiologic and pathophysiologic processes, the GPCRs are targets of about 60% of the clinically prescribed drugs (2,6).

Table 2: Examples of GPCR-targeting drugs *(Adapted from: Saengsawang & Rasenick 2015)*

Disease/Disorder	Drug	Target
Schizophrenia	Aripiprazole	Dopamine Receptor D2 (DRD2)
Hypertension	Valsartan	Angiotensin Receptor
Asma or Chronic Obstructive Pulmonary Disease (COPD)	Salmeterol	β_2-adrenergic Receptor
Diabetes	Albiglutide	GLP1 Receptor (GLP1R)
Gastric Ulcers	Ranitidine	Histamine Receptor 2 (H2R)
Prostate Cancer	Leuprolide	GnRH Receptor

The drugs can bind directly to the GPCR or can change the GPRC's activity by affecting other proteins, being called directly or indirectly acting drugs, respectively. Directly- acting drugs, in its turn, can be classified into orthosteric drugs, when the molecule binds to the orthosteric site of the receptor, acting as agonist, antagonist or inverse agonist, or into allosteric drugs, binding to the allosteric site and modulating the activity of GPRC or changing its affinity for the natural ligand (2).

The interest of the pharmaceutical industry in the development of allosteric drugs is enormous. This type of drugs allows more precise control of the activity of the GPCR and, since the allosteric sites are less conserved than orthosteric sites, allosteric drugs tend to be more subtype selective. However, the design of these drugs is complex since it necessitates the knowledge of active and inactive conformations, requiring the crystallization of the receptor with several ligands. Cinacalcet, for the treatment of hyperparathyroidism, and Maraviroc, for the prevention of cellular entry of Human Immunodeficiency Virus 1 (HIV-1), are examples of a positive allosteric modulator of the CaSR and of a negative allosteric modulator of the chemokine receptor C-C chemokine receptor type 5 (CCR5), respectively (2,8).

GPCR drug discovery is moving towards finding more effective, selective

molecules that minimize adverse effects. Thus, in order to achieve these goals, the focus is to design biased-ligands – that activate a specific signalling pathway, increasing the specificity and decreasing side effects -, dual acting drugs – bind to different receptors or other proteins producing a combined effect, potentializing him-, monoclonal antibodies (mAbs) – bind to the desired GPCR and modulate their activity -, and drugs that affect the trafficking or desensitization of the receptor (2).

By the end of the year 2017, were identified 475 approved drugs that act at 108 different GPCRs, accounting for approximately 34% of all Food and Drug Administration (FDA) approved drugs. 321 molecules (68% of the total number of approved drugs) were in clinical trials, of which about 20% targeted 66 potentially novel GPCR targets without an approved drug. Between 2013 and 2017, 69 new GPCR-targeting drugs were approved by FDA (8).

Table 3: Examples of new molecules acting via GPCR approved by the FDA between 2014 and 2017 *(Adapted from: Hauser et al. 2017)*

Substance	Indication(s)	Target
Abaloparatide	Osteoporosis	PTHR1
Brexpripazole	Depression	$5HT_{2A}R$, $5HT_{1A}R$, DRD2 and $5HT_{7}R$
Dulaglutide	Type 2 Diabetes	GLP1R
Netupitant	Nausea and/or vomiting	NK1R

Orphan GPCRs serve as possibly novel targets for several pathologies and may provide more selective therapeutic targets, resulting in the potential reduction inside effects. The study of currently unknown signalling transduction pathways may allow the development of new drug design strategies. Examples of orphan receptor that are promising therapeutic targets include G-protein coupled receptor 55 (GPR55) as antispasmodic target, G-protein coupled receptor 35 (GPR35) as a target in allergic inflammatory condition or G-protein coupled receptor 84 (GPR84) in ulcerative colitis. The number of orphan GPCR-target drugs entering into clinical trials is increasing (3,8).

The therapeutic indication of GPCR-targeting drugs covers several and diverse diseases. The number of indications seems to be increasing, expanding from areas such as hypertension, depression, analgesics and schizophrenia, to new areas such as diabetes, obesity and several central nervous system (CNS) disorders (8).

More than 50% of non-olfactory GPCRs are expressed in the cerebral cortex and disfunctions in the neurotransmission mediated by these receptors can lead to

several disorders. GPCRs can be a target for diseases like Alzheimer's Disease (AD), Multiple Sclerosis (MS), Huntington's Disease (HD), and others. Therapies for CNS diseases account with approximately 124 (26%) of all FDA approved GPCR-targeting drugs, and 79 molecules are in clinical trials (8).

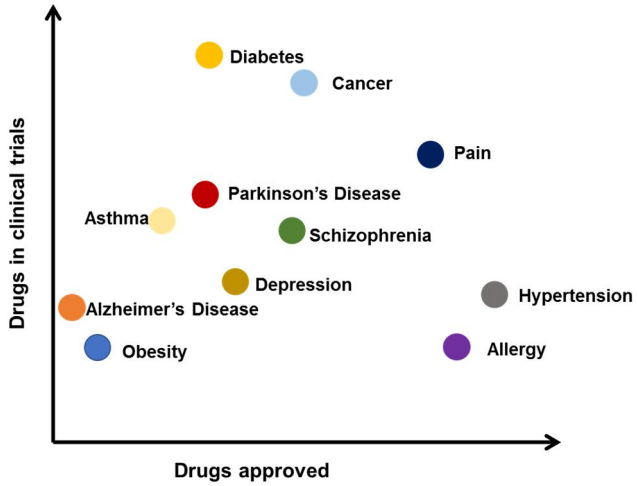


Figure 4: Number of agents approved versus number of drugs in clinical trial according to the pathology. Allergy and hypertension are the pathologies with more drugs approved, while diabetes and cancer are the diseases with more agents in clinical trials (*Adapted from:* Hauser et al. 2017).

For instance, fingolimod, a sphingosine 1-phosphate receptor 1 (S1P1) modulator, is a therapeutic approach in MS, reducing relapse and slowing down the progression, and it is a GPCR-targeting drug (8). In AD, Serotonin (5-HT) receptor modulators raise huge interest for scientists, comprising serotonin receptor 6 ($5HT_6$) receptor antagonists. These last molecules increase the release of acetylcholine, improving the disease symptoms (8).

Diabetes is another pathology whose therapies include a high percentage of GPCR-targeted agents: 9% of the total number of drugs in clinical trials in 2017 belongs to GPCR-targeting drugs for diabetes and obesity. The glucagon-like peptide 1 (GLP1) receptor agonist exenatide was the first GPCR-targeted agent approved for type II diabetes (T2D), followed by liraglutide, dulaglutide, albiglutide and lixisenatide. In recent years, several other receptors are being studied as GPCRs targets for diabetes, such as G-protein coupled receptor 119 (GPR119) and free fatty acid receptor 1 (FFAR1) (8,9).

Opportunitie for GPCR-targeted agents for cancer's therapy are also emerging, playing an important role in cancer initiation, progression and metastasization. Degarelix, a gonadotrophin realising hormone (GnRH) receptor antagonist used for advanced prostate cancer, sonidegib and vismodegib, both a smoothened (SMO) receptor inhibitors and approved for basal cell carcinoma, are examples of these type of drugs (8,10).

2. Cannabinoid receptors

The endocannabinoid system (ECS) has an important role in several physiological and pathological processes, as pain modulation, fertility, neuroprotection, immune function, cancer, cardiovascular diseases and appetite. It is composed by the endocannabinoids, the cannabinoids GPCRs and by the enzymes responsible for its biosynthesis and degradation (11).

Endocannabinoids are endogenous lipid-signalling molecules that act like agonists of cannabinoid receptors. In 1992 the first endocannabinoid were identified, the N-arachidonyl ethanolamine (anadamide or AEA), followed by the identification of 2-arachidonyl glycerol (2-AG) in 1995(11). Also in the early 1990s, two cannabinoid receptors were identified, by molecular cloning, that can bind with high-affinity to anandamide and 2-AG: cannabinoid receptor type 1 and type 2 (CB1 and CB2, respectively)(11). These cannabinoid receptors belong to class A of GPCR subfamily. Later on, three orphan GPCRs, G-protein coupled receptor 18 (GPR18), GPR55 and GPR119, have been linked to the ECS, since they exhibit homology with the established CB1 and CB2 (11,12).

3. Cannabinoids compounds and endocannabinoids

In 1965, Raphael Mechoulam identified the Δ^9-tetrahydrocannabinol (Δ^9-THC), a lipophilic phytocannabinoid extracted from cannabis plant. This compound and other lipids also derived from cannabis plant showed the capacity of activate GPCRs, which generated the hypothesis that an endogenous ligand, most probably a lipid, must be produced (13).

Mechoulam's group identified, in 1992, an endogenous analogue of Δ^9-THC from porcine brain, the AEA. This endocannabinoid has a molecular structure similar to endogenous N-acyl amide signalling molecules, a family of novel endogenous signalling molecules with a wide-range of cellular signalling potential. Both Δ^9-THC and AEA showed ability to activate the CB1 and induce physiological responses as hypothermia, analgesia, hypoactivity and catalepsy. AEA also binds to the CB2 and activates the transient receptor potential vanilloid type-1 channel (TRPV1)(13). 2-AG was the second endogenous cannabinoid lipid identified, in rat brain and canine gut. It also binds to both CB1 and CB2(13). The identification of both AEA and 2-AG opened a path to the identification of other agonists, which are primarily N-acyl amides in structure (13).

Five structurally distinct classes of cannabinoid compounds have been identified: classical cannabinoids, like Δ^9-THC, Δ^8-THCdimethylheptyl (HU210); bicyclic cannabinoids, as CP55.940; indole-derived cannabinoids, as WIN 55,212; eicosanoids, including the endogenous ligands AEA and 2-AG; and antagonist/inverse agonists, like SR141716A for CB1 and SR145528 for CB2 (14).

4. CB1 and CB2: two cannabinoid receptors

CB1 and CB2 are two cannabinoid receptors that were identified about 20 years ago. They belong to the GPCR family class A and are coupled to Gi/o heteromeric G protein. CB1 receptor could also couple to G_s protein (12,15,16).

These receptors mediate the effects of Δ^9-THC and of the endocannabinoids AEA and 2-AG, however, they also have differences in their pharmacology (14).

The human CB1 receptor gene (*CNR1*) is located on human chromosome 6q14-15, while the human CB2 receptor gene (*CNR2*) is located at chromosome 1p36. Several polymorphisms and alternative splice variants for both receptors have been identified, and these variations have been associated with the development of some diseases or to different pharmacologic responses. For example, several studies linked have polymorphisms of the human *CB2* gene to osteoporosis (14).

Interestingly, the CB1 receptors are highly conserved among vertebrate species and have also been found in some invertebrates. For instance, the human CB1 receptor presents an homology of 93% at the nucleic acid level and 97% at the amino acid level to the rat receptor (14). The CB2 receptor, initially isolated from a type of human leukaemia cell line called HL60 cells, shows a homology of 44% at the amino acid level with the CB1 receptor, representing a receptor subtype, with similar binding profile. Nevertheless, this receptor is less conserved between species than CB1 (14).

The CB1 receptors are highly expressed in the central nervous system, being one of the most abundant GPCRs in the brain. They mediate most of the psychoactive effects and play a role in the control of motor function, cognition, memory and analgesia, what can be correlated with their distribution: they are primarily localized in the terminals of central and peripheral neurons, where they mediate inhibition of neurotransmitter release (12,14,15).

CB1 is expressed in lower levels in several peripheral tissues, as the heart, lung, liver, bone marrow, thymus, adrenal gland, prostate, uterus, ovary, testis, vas deferens and tonsils. It was detected in many circulating immune cells, being that the expression of CB1 receptors appears to increase or decrease during immune cell activation (12,14,15).

CB2 receptors are abundantly expressed in peripheral organs with immune function, like spleen, thymus, tonsils, macrophages, leukocytes, lung and testes, mediating immunosuppressive effects. In situations of inflammation and immune cell activation, CB2 receptors are upregulated. Besides the fact that these receptors are expressed in lower levels than CB1, they also play a clear role in CNS activity and in immune responses (12,14,15,17).

4.1. Ligands and therapeutic applications

When a compound can target CB1 or CB2 receptor, it is classified as a cannabinoid. According to their functionality, they can be divided as agonist (after binding to the receptor, is verified an increase in the basal level of signalling), inverse agonists (downregulate the signalling when binding to the receptor) or antagonists

(act on the agonist or inverse agonist activity, by decreasing their effect). According to their origin, they can be classified as plant derived or phytocannabinoids, endogenous or endocannabinoids, and chemically synthesized or synthetic cannabinoids. Chemical structures of some cannabinoids are shown in Figure 5 (4,8).

Endogenous ligands of CB1 and CB2 receptors, such as AEA, 2-AG and noladin ether, are produced in several peripheral tissues, including liver, bone, gastrointestinal tract, skin, reproductive and immune system (18).

Several CB2 ligands are only relatively selective, because most of the commonly CB2 ligands have evolved from molecules that have appreciable affinity for CB1 receptors. For instance, both receptors can be activated by (–) 11-hydroxy-Δ8-THC-dimethylheptyl (HU-210), CP55.940, and R-(+)-WIN55212 (11). The majority of the agonists show little selectivity between the CB1 and CB2 receptors, while highly selective antagonists have been found, allowing the discrimination of CB1 and CB2- mediated effects *in vivo* and *in vitro* (12,13).

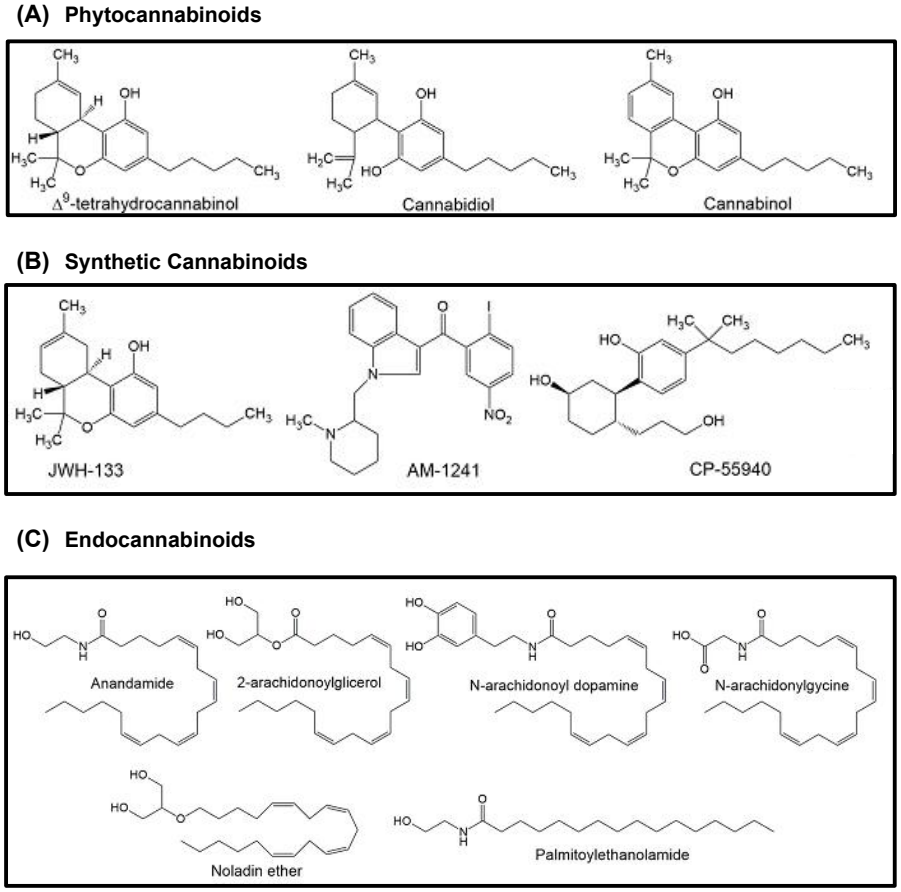


Figure 5: Chemical structures of some cannabinoids. (A) Examples of Phytocannabinoids; (B) Examples of Synthetic Cannabinoids; (C) Examples of Endocannabinoids. *(Adapted from: González-Hernández et al. 2019)*

Δ^9-THC structure was the starting point for synthesizing and studying chemical analogues. After the discovery of Δ^9-THC and anandamide, in 1994, the first CB1 receptor selective antagonist, the SR141716A (Rimonabant), with a 1000-fold higher affinity for CB1 than for CB2 was reported. In 1997, SR144528 was discovered as a

antagonist/inverse agonist for the CB2 receptor, sharing the pyrazole core structure as SR141716A (14,17).

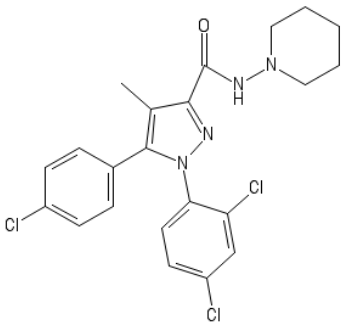


Figure 6: Chemical Structures of SR141716A*. (Adapted from:* Han et al. 2014)

CB1-selective antagonists include AM251 and AM281, while agonists are, for example, R-(+)-methanandamide, arachidonyl-2′-chloroethylamide, arachidonyl-cyclopropylamide and O-1812. Nevertheless, CB1 ligands have a limited use since they can provoke serious psychotropic side effects. For example, since CB1 receptor has an important role in the control of food intake and energy balance, several antagonist and inverse agonists were developed in order to treat obesity and diabetes, which was the case of Rimonabant. This compound was used as an anti-obesity drug, although the profile between benefit and risk was not favourable, due to psychiatric side effects, and Rimonabant was withdrawn from the market in 2008 (5,13,19).

In contrast, activation of CB2 receptors does not appear to produce these psychotropic effects, so selective modulation of CB2 receptor has being considered an interesting therapeutic approach, being devoid of central side effects (13,19).

Selective agonists of CB2 have been considered a promising therapeutic for the treatment or management of a range of painful conditions, for treat neuroinflammatory or neurodegenerative diseases, such as Parkinson´s Disease (PD), AD, MS, amyotrophic lateral sclerosis (ALS), HD and stroke, for peripheral disorders that involve inflammation, including atherosclerosis, inflammatory bowel diseases, ischemia/reperfusion injury, renal fibrosis and liver cirrhosis. CB2 agonists have shown efficacy in preclinical cancer models and there are epidemiologic and preclinical data that suggest that these compounds show a protective role in osteoporosis and arthritis (13,19).

There are many identified CB2 receptor agonists, including classic cannabinoids (Δ9-THC, L759633, L759656, JWH133, KM233), nonclassic cannabinoids (CP55940, HU308), aminoalkylindoles (WIN55212-2, AM1241, STS135, JWH015, GW405833, UR144, MAM2201, AM2232, AM2233, AM1248), thiazoles (A836339), thiazoles (A836339), tricyclic pyrazole (GP1a), cannabilactone (AM1710), carboxamides (SER601, 4Q3C), pyrimidine analog (GW833972A), eicosanoids (2AG, methanandamide), plant products (4-Methylhonokiol, (E)b-caryophyllene (BCP)). The CB2 receptor antagonists include AM630, JTE907, SR144528. The CB2-selective agonists are JWH133, HU308 and AM1241 and selective antagonists are SR144528 and AM630 (5,15).

In 2007, the GlaxoSmithKline (GSK) research group reported a selective agonist for the CB2 receptor, the compound GW842166X. The phase II studies of this compound have been completed for evaluating its analgesic efficacy for dental surgery and for the treatment of osteoarthritis pain of the knee, according to the United States National Institutes of Health (NIH)(15). The CB2 receptor selective agonist S-777469 and inverse agonist JTE-907 were found to be effective in suppressing spontaneous itch-associated responses in the mice model of atopic dermatitis, respectively. Recently, S-777469 has also been reported on completing its phase II study in subjects with allergic contact dermatitis (20).

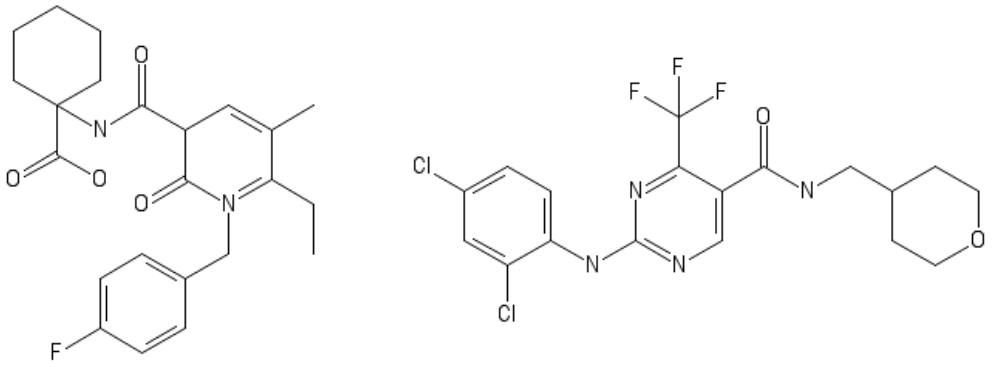


Figure 7**: **Chemical Structures of the CB2 receptor selective agonists S-777469 (left) and GW842166X (right)
(Adapted from: Han et al. 2014)

Despite of all the studies and developments, CB2 agonists still have a limited success in clinical trials, due to diverse factors including the immune system side effects, the lack of translation from preclinical models and the differences between species (21).

5. GPR55 and GPR18: two distinct orphan receptors

Despite the fact that GPR55 and GPR18 directly or indirectly interact with cannabinoids, the Subcommittee on Cannabinoid Receptors of the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC IUPHAR) decided that GPR55 and GPR18 do not meet all the requirements to be consider as novel cannabinoid receptors. Thus, they have been classified as orphan receptors (5). Nevertheless, many studies challenge this classification.

5.1. GPR55

The orphan GPR55 was identified in 1999 by Sawzdargo et al., that isolated and cloned the human GPR55 (hGPR55). This receptor belongs to the rhodopsin-like (Class A) family of GPCRs. It is composed by a 319 a.a. sequence, encoded by the *GPR55* gene was mapped to human chromosome 2q37. Has been reported that GPR55 couples to $G\alpha_{12/13}$ or Gαq proteins (1,11,13,14,17).

GPR55 exhibit some similarity in terms of the amino acid sequence with GPR35 (27%), lysophosphatidic acid receptor 6 (P2Y5) purinergic receptor (29%), G-protein coupled receptor 23 (GPR23) (30%), and C-X-C chemokine receptor type 4 (CXCR4) (26%). Nevertheless, although it shares many cannabinoid ligands with CB1 and CB2, GPR55 has low a.a. identity with these two cannabinoid receptors, having a similarity of 13.5% with CB1 and 14.4% with CB2 (13,14).

hGPR55 is highly expressed in the CNS, and is also expressed in peripheral tissues such as endothelial cells, adrenal glands, kidney, bladder, gastrointestinal tract, liver, lung, uterus, bone and other tissues/organ systems. More recently, high levels of GPR55 were also found on lymphocytes and spleen, as well as on many cancer cells, in which the expression levels correlate with the rate of cancer cell proliferation (11,13,14).

5.1.1. Ligands

L-α-lysophosphatidylinositol (LPI) was the first endogenous ligand identified for this receptor, by Oka et al. in 2007. LPI is produced from membrane phosphatidylinositol through the catalytic activity of Ca^{2+}-dependent phospholipase A1 (PLA1) or Ca^{2+}-independent phospholipase A2 (PLA2). In 2009, the same authors demonstrated that the addition of an arachidonic fatty acid chain (2-arachidonoyl-sn-glycero-3-phosphoinositol; 2-AGPI) to LPI produced the most biologically active LPI species. Interestingly, the structurally related endogenous lipid, the lysophosphatidic acid (LPA), does not activate GPR55. Even though LPI and 2-AGPI are ligands for GPR55, they do not activate CB1 or CB2 receptors, being non-cannabinoid ligands (5,11,13,22).

Actually, GPR55 binds to several cannabinoid compounds, so after being initially deorphanized as a cannabinoid receptor, it still is presented as the main candidate to be considered as the “third” cannabinoid receptor (13,14).

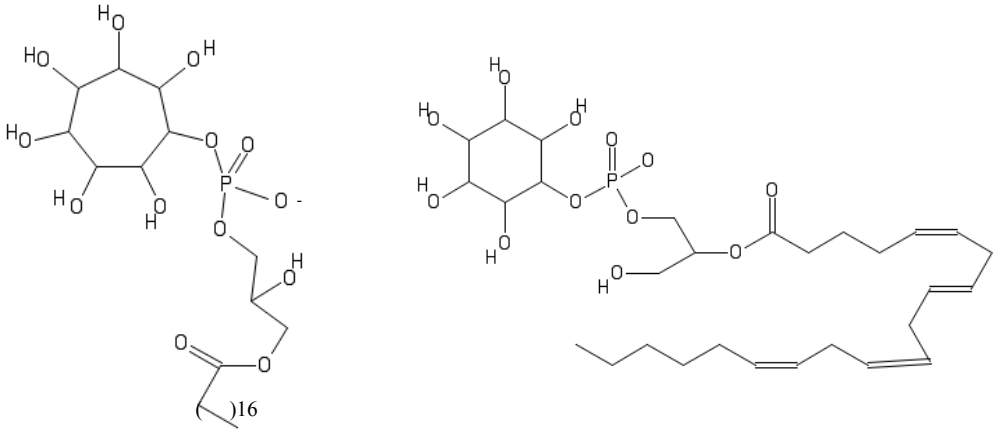


Figure 8: Chemical structures of the GPR55 ligands LPI (left) and 2-AGPI (right). *(Adapted from: Reggio & Shore 2015)*

GPR55 signaling is modulated by the endocannabinoids 2-AG, virodhamine, noladin-ether, oleoylethanolamida (OEA) and palmitoylethanilamida (PEA) and by the atypical cannabinoids cannabidiol (CBD) and abnormal-cannabidinol (abn-CBD; 4-[(1R,6R)-3-methyl-6-prop-1-en-2-yl-1-cyclohex-2-enyl]-5-pentylbenzene-1,3-diol) (3,20).

Several studies described HU210 (a synthetic analogue of Δ^9-THC), JWHO15 (a CB2 receptor agonist), AEA (in a much higher concentration than the one required to CB1 activation) and R-methanandamide, CBD and its synthetic analogue O-1602 (5-methyl-4-[(1R,6R)-3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]benzene-1,3-diol) as GPR55 agonists. Although, other studies indicated that CBD may act as a GPR55 antagonist (3,20).

Other cannabinoid ligand, the CB1 antagonist AM251 also showed high affinity for GPR55, acting as an agonist on this receptor (3,20).

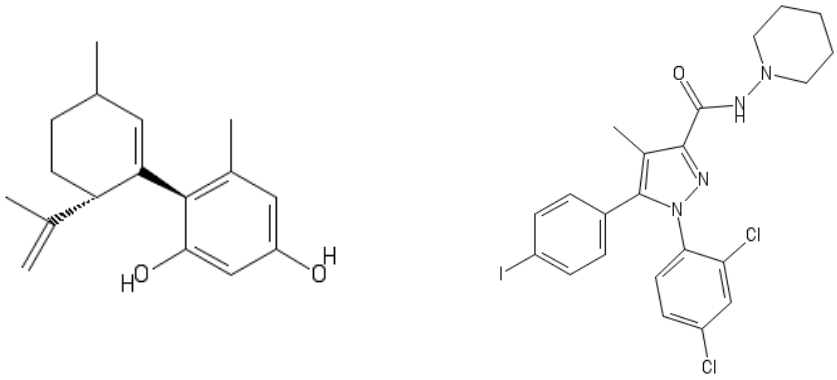


Figure 9: Chemical structures of O-1602 (left) and AM251 (right). *(Adapted from: Reggio & Shore 2015)*

Rimonabant is another CB1 antagonist that demonstrated agonist activity on GPR55. However, similarly to what was described for CBD, there are studies that report a blockage of GPR55 signaling responses to agonist compounds, like AEA or JWH015, by Rimonabant. CP55940, a cannabinoid agonist, showed partial agonist/antagonist activity in GPR55 (3,20).

GPR55 shows affinity and responds to non-cannabinoid ligands. For example, GSK494581A (1-{2-fluoro-4-[1-(methyloxy)ethyl]phenyl}-4{4-fluoro-4-(methylsulfonyl)-2-biphenylyl]carbonyl} piperazine) activates selectively the GPR55, at low nanomolar potency. Compounds such as ML191 (CID23612552), ML192 (CID1434953) and ML193 (CID1261822) act as non-cannabinoid GPR55 antagonists. CID16020046 is another non-cannabinoid GPR55 antagonist, although this compound inhibit selectively this GPRC, not having shown any activity on CB1 or CB2 (3,20).

5.1.2. Therapeutic applications

LPI had been associated to essential biological processes such as induction of insulin release, mediation of hepatic vitamin D3-modulating Ca^{2+} metabolism and mitogenic modulation in neuronal and endothelial cells. Conjugating LPI and the processes mentioned above, with GPR55 expression and distribution, one can

envision that GPR55 can be a therapeutic target in the several diseases such as obesity and diabetes, osteoporosis, in cancer therapy and others (3,20).

The expression of GPR55 in β-cells of the islets of Langerhans indicates that these receptors play an important role in glucose homeostasis by the regulating insulin secretion. Therefore, using GPR55 agonists might be a therapeutic approach in T2D, increasing insulin secretion and sensitivity. In agreement, some studies showed that abn-CBD has glucose-lowering and insulinotropic potential (20,23).

This receptor can also be a potential therapeutic target in PD due to a possible alteration on its expression in the basal nuclei, where it is related to procedural memories. Pharmacological studies showed that CBD might prevent the pro-inflammatory glial cell activation induced by the beta-amyloid administration and might attenuate the induced dopaminergic system impairment in rats. However, clinical trials with CBD showed lack of pro- or anti-parkinsonian effects. Furthermore, it is possible that some antiepileptic actions observed with phytocannabinoids involve the blocking of GPR55 (5,23).

Recent studies revealed that LPI and GPR55 are important factors in cell proliferation and migration, and a correlation between GPR55 expression and/or LPI levels and cancer progression/aggressiveness has already been detected. For example, LPI can be considered a marker of poor prognosis for patients with ovarian cancer. There are also evidences of GPR55 overexpression in several tumour cells, including breast cancer, melanoma, ovarian cancer, glioblastoma, and others Several studies described that the β2-adrenergic agonist AMF ((R,R´)-40-methoxy-1-naphthylfenoterol), a GPR55 inhibitor, blocked the GPR55 agonist effect of O-1602 and AM251 in cancer cell lines, reducing chemoresistance of cancer cells (20,22).

GPR55 is expressed in osteoblast and osteoclasts, and LPI has been shown to stimulates bone reabsorption. Therefore, inhibiting GPR55 signaling might be beneficial in osteoporosis (22).

5.2. GPR18

GPR18 belongs to the class A or rhodopsine like GPCRs. It consists in a sequence of 331 amino acids, encoded by the *GPR18* gene which is localized in the chromosomal region 13q32.3. This receptor was described for the first time in 1997 and interacts with Gαi/o, Gαq/11, and Gα12/13, but it has been suggested that GPR18 modulate the transduction pathways of these G proteins in a ligand dependent way (11,12,13,19,21).

The expression of hGPR18 varies according to the tissue or organ: GPR18 mRNA has moderate to high expression in testes, thyroid, peripheral blood leukocytes, lungs and specific brain regions, such as hypothalamus, cerebellum, brain stem and striatum; while in the spleen, thymus, ovaries, uterus, stomach and intestines it shows a low to moderate expression. Finally, low or undetectable hGPR18 mRNA levels were detected in the heart, lungs, liver, kidney, pancreas, colon, skeletal muscle, skin, placenta, prostate, adrenal medulla, adrenal cortex and

in some brain areas like amygdala, frontal cortex, hippocampus, cerebellum, thalamus and brain stem (5,11,12,21)

Based upon the expression pattern of this receptor, it is suggested that GPR18 has a potential role in the control of immune system activity, such as leukocyte trafficking during acute inflammation, and accordingly inflammation (12).

5.2.1. Ligands

Kohno and collaborabors identified the acylamino acid N-arachidonoylglycine (NAGly) as an endogenous agonist for GPR18, by screening a bioactive lipid library against various cells recombinantly expressing human GPR18 (5,12,15).

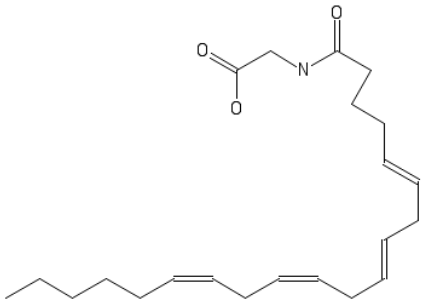


Figure 10:Chemical Structure of N-arachidonoylglycine (NAGly) *(Adapted from: Schoeder et al. 2018)*

NAGly is a metabolite of the endocannabinoid anandamide and is a member of a subfamily of lipoamino acids. It can be detected in high levels in mammalian nervous tissues, especially in spinal cord and brain. Some studies showed that cells are able to convert anandamide to NAGly. The chemical similarity between NAGLy and anandamide created the hypothesis that GPR18 could be the third cannabinoid receptor. Besides that, GPR18-mediated cellular signalling, in response to NAGly and anandamide, was describe through an inhibition of cAMP, phosphorylation of extracellular-signal-regulated kinase (ERK), and other effects such as regulation of cellular migration and proliferation/apoptosis. The characterization of GPR18 as a cannabinoid receptor is also supported by studies that found that the phytocannabinoid Δ^9-THC and the synthetic cannabinoids abn-CBD and O-1602 act like GPR18 agonists (5,11,12,15,17,19,21).

One of the most significant data impeding the deorphanisation of GPR18, is the non-totally overload distribution of GPR18 by the different tissues and NAGly: the highest levels of NAGly, in rat, were found in the spinal cord, small intestine, kidneys and skin, while lowest levels were found in testes and spleen (organs that present a moderate expression of GPR18). Nevertheless, it can be hypothesized that NAGly is synthesised when a system actively induces its synthesis, as it happens with AEA and 2-arachidonyl glycerol (18).

The activation of GPR18 by NAGly leads to apoptosis of inflammatory leukocytes, which in turn reduces local inflammation. This is an example of a finding that suggests a physiological function of NAGly via GPR18 in an inflammatory process. Nevertheless, the responsivity of GRP18 to activation by NAGly has been brought into question Several reports described GPR18 to be completely unresponsive towards NAGly and other studies suggest that signaling properties of GPR18 may be cell type specific (5,12,15,19,21).

Despite the similarity among NAGly and anandamide, NAGly did not showed affinity for either CB1 and CB2, what can be explained by the structural modification in NAGly (oxidation state of the beta carbon to the amide nitrogen). NAGly is not a specific agonist for GPR18, it can also activate other receptors, such as G-protein coupled receptor 92 (GPR92). GPR18 is also activated by the endocannabinoid AEA and THC, demonstrating to be full agonists at GPR18. While CBD and AM251 act as weak partial agonists, a number of other agonists of CB1 and CB2 cannabinoid receptors were inactive (WIN55212-2, CP55940, JWH015, and JWH133) (5,11,15,19,21).

In recent studies, resolvin D2 (RvD2) was proposed as an endogenous agonist of GPR18. RvD2 is a natural lipid that showed capacity to activate recombinant human GPR18 receptors in a receptor- and ligand-dependent manner and it was found that this compound exhibit ant-inflammatory effects on macrophages, promoting the resolution of bacterial infections and organ protection (13,14).

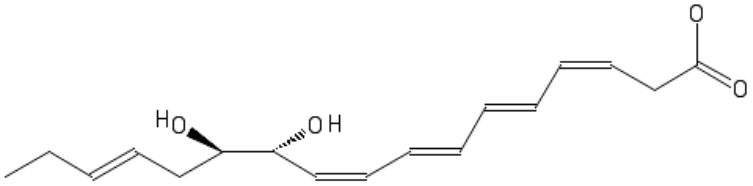


Figure 11: Chemical Structure of Resolvin D2 (RvD2) *(Adapted from: Schoeder et al. 2018)*

The only GPR18 receptor antagonist described until the date is amauromine, a natural product (24).

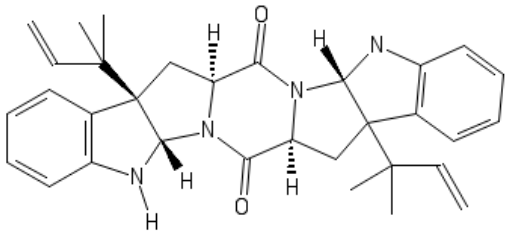


Figure 12:Chemical Structure of Amauromine *(Adapted from: Schoeder et al. 2018)*

Repel and coworkers and Schoeder and collaborators developed several synthetic GPR18 antagonists. The first potent GPR18 antagonist developed by this group was the (Z)-2-(3-(4-chlorobenzyloxy)benzylidene)-6,7-dihydro-2H-imidazo[2,1-b][1,3]thiazin-3(5H)-one (PSB-CB-5). PSB-CB-5 behaved as a partial antagonist, and

it showed a certain degree of selectivity. After the developedment of the PSB-CB-5, several analogs were synthesized through modifications of the heterocyclic core and variations of substituents at the benzylidene in order to design a molecule more potent and selective. These analogs include PBS-CB-27, the compound with good potency and a higher selectivity when comparing to the PBS-CB-5 (24).

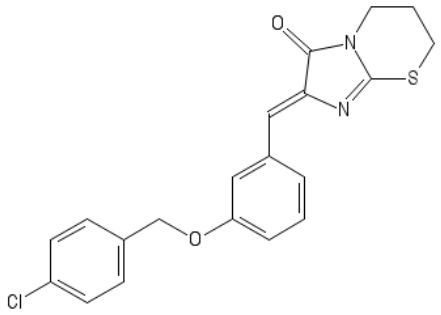


Figure 13: Chemical Structure of PSB-CB-5 *(Adapted from: Schoeder et al. 2018)*

5.2.2. Therapeutic applications

Although potential therapeutic approaches targeting GPR18 need further investigation, the distribution and expression of GPR18 suggests that this receptor is implicated in the pathophysiology of several disorders and its regulation plays an important role in these ones.

GRP18 expression was found significantly increased in proinflammatory M1 mouse peritoneal macrophages and its activation also regulates leukocyte trafficking during acute inflammation. NAGly was also found to reduced cell viability of the same type of cells, not having any effect on M2 anti-inflammatory macrophages. These studies indicate that GPR18 is involved in the attenuation of the proinflammatory M1 stage of macrophages and that NAGly is a potential anti-inflammatory factor. So, GPR18 is important in the regulation of cell death and in the immunological function and it might be a relevant therapeutic target for several inflammatory diseases (5,13,25).

In the case of osteoarthritis, GPR18 is expressed in the degenerated chondrocytes within the deep zone of cartilage, suggesting that this receptor might be a target for the treatment of this pathology (5).

The role of this receptor in pain has also been a research focus. Some reports showed that a nerve injury enhances GPR18 mRNA expression in the spinal cord, mediating a few analgesic effects and modulating neuropathic pain. NAGly upregulated GPR18 mRNA in spinal cord after sciatic nerve injury as well (1,5,16).

Other disorders where GPR18 might be used as a target are obesity and diabetes. Obesity is considered a chronic inflammation and, endogenous GPR18 ligands, have been shown to be modulated by dietary factors. GPR18 ligands are also linked to insulin resistance: for example, studies performed in rat models showed that NAGly increases insulin secretion. Recently, it was additionally

emphasized that GPR18 activation in diabetes reverse myocardial oxidative stress and dysfunction, protecting the cardiovascular system (25).

Supporting the theory that GPR18 mediates cell proliferation and antiapoptotic signalling, a group of scientists described the increase of apoptosis rate of melanoma cells when treated with short interference RNAs (siRNAs) against GPR18 mRNA. This finding supports the hypothesis of targeting GPR18 for the treatment of several type of cancers (25).

More therapeutic approaches are being studied and the hypothesis are being explored, however the need of understanding the complex GPR18 signaling mechanism is huge a barrier.

Materials and Methods

1. Cell Culture

The experiments were performed using Chinese Hamster Ovary (CHO) cells stably expressing the receptors of interest.

In order to ensure cell viability, the cells were maintained in T75 culture flasks at 37ºC under a 5% CO_2 atmosphere, and sub-cultured at different densities, depending on the experimental goal, when the degree of confluence reached 80-95%.

The growth mediums used varied according to the type of cells: for GPR18 and GPR55 expressing cells the medium was F-12 supplemented with 10% of Fetal calf serum (FCS), 100 µg/mL or 100 U/mL of Penicillin/Streptomycin, 0.3 mg/mL of hygromycin and 0.8 mg/mL of Geneticin® (G418) Selective Antibiotic (Invitrogen™, Thermo Fisher); while for CB1 and CB2 expressing cells the medium was Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM:F12) supplemented with 10% of FCS, 100 µg/mL or 100 U/mL of Penicillin/Streptomycin and 0.8 mg/mL of Geneticin® (G418).

To split the cells, after the removal of the medium and after washing with 10 mL of Phosphate Buffer Saline (PBS), 3 mL of a 0.05% trypsin solution was added to the cells. This solution is responsible for the enzymatic dissociation of cell-cell adhesion bonds and the disconnection of adherent cells from the growth surface. After confirming the cells detachment from the flask surface, 7 mL of new culture medium was added to the culture flask. The cell suspension was homogenized, to assure the complete dissociation of cells. The desired volume was taken to a new flask and were added 25 mL of medium. Cells were kept at 37ºC under a 5% CO_2 atmosphere.

2. β-Arrestin Assay

β-Arrestin PathHunter™ assay (DiscoveRx, Fremont, CA, USA) is a functional assay that uses PathHunter β-Arrestin cell lines (stable clonal cell lines), allowing the screening and the study of agonists and inhibitors of GPCRs. The fundament of this assay relies on the β-Arrestin recruitment to activated GPCRs.

In order to study the effect of the compounds of interest in the GPCRs, measuring the activation status of the target receptor, an Enzyme Fragment Complementation (EFC) technology was used. This assay uses two fragments of β-galactosidase (b-gal) enzyme, the Enzyme Donor (ED) and Enzyme Acceptor (EA). The target GPCR is tagged with a small fragment of b-gal called ProLink™, that works like the ED, while the β-Arrestin is tagged with the EA. Even though these two fragments separately have no enzymatic activity, when they are brought together, they form an active b-gal enzyme, which is capable of hydrolyze the substrate present in the PathHunter detection reagents, to generating light.

To seed the cells, after the removal of the medium and washing with 10 mL of PBS, 10 mL of a dissociation buffer (2 mM EDTA-NaX2H_2O, 10 mM glucose) were added to a 175cm^2-flask of cells. After confirming the cells detachment from the flask surface, the cell suspension was homogenized, to assure the complete dissociation

of cells. The total volume was transferred to a 50mL tube and an aliquot of the cell suspension was used to count cell number using a Neubauer chamber. After a 5 minutes centrifugation, the supernatant was discarded, and the pellet was resuspended in 10 mL of growth medium (F12 growth medium with 2% of FCS, 100 µg/mL or 100 U/mL of Penicillin/Streptomycin, 0.3 mg/mL of hygromycin and 0.8 mg/mL of G418).

Cells were seeded into 96-well plates at a density of 40 000 cells/well for the cells expressing GPR18 and of 30 000 cells/well for the cells expressing GPR55, 24h before performing the assay, with 90 µL of growth medium (F12 growth medium with 2% of FCS, 100 µg/mL or 100 U/mL of Penicillin/Streptomycin, 0.3 mg/mL of hygromycin and 0.8 mg/mL of G418). The cells were kept at 37ºC under a 5% CO_2 atmosphere.

After 24 h, medium of GPR18 expressing cells were changed for 90 µL of the same medium without FCS, followed by an incubation of one hour to one hour and a half at 37ºC. For GPR55 expressing cells the assay was started immediately.

For the preparation of the reagents, the compounds were firstly diluted in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, and then diluted in PBS with 10% of Bovine serum albumin (BSA), being the final DMSO concentration in the assay equal to 1%.

For the agonist test, cells were incubated, for one hour and a half, with 10 µL of each compound. For the antagonist test, cells were incubated, for one hour, with 5 µL of the compound of interest, followed by an one hour and a half incubation with 5 µL of a known agonist of the receptor in study.

After this period of time, 50 µL of detection buffer were added to each well and incubated for one hour. This buffer is composed by lysis buffer (for GPR18 cells: 5mM K_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, 10 mM MgAcetate, 2% CHAPS, pH 7.5; for GPR55 cells: 10 mM Tris, 1 mM EDTA-Na x $2H_2O$, 100 mM NaCl, 5 mM MgCl, 1% Triton – X, pH 8), Emerald II ® (Invitrogen™, Thermo Fisher) and Galacton Star ® (Invitrogen™, Thermo Fisher).

The luminescence was measured in a TopCount NXT (Packard, Perkin-Elmer), for 1 second per well.

Data were obtained from three independent experiments, performed in duplicates.

3. Membrane Preparation

This complex process allows the preparation of a membrane suspension, being that the membrane fraction contains the receptors of interest.

One 175cm^2-flask of cells was used to seed 20 dishes, and 1 mL of the cells of each 175cm^2-flask was seeded in dishes with 20 mL of growth medium (DMEM-F12 growth medium supplemented with 10% of FCS, 100 µg/mL of

Penicillin/Streptomycin and 0.8 mg/mL of G418). After the desired confluence has been reached, the medium was discarded, and the cells were washed with 5 mL of PBS. At this step, cells were frozen at -80ºC and the procedure was restarted the next day. To harvest the cells, 1.5 mL of scrapping buffer (2 mM Na_2EDTA, 5 mM Tris) was added to each dish, and the resulting suspension was collected to a tube.

Afterwards, the cells were homogenized using Ultra-Turrax® homogenizer (IKA Labortechnik). The homogenate was then centrifuged at high speed (50 000 *g*) for 5-10 minutes. The supernatant was discarded, the pellet was resuspended in the same buffer, and centrifuged again. After this last centrifugation, the supernatant was discarded, the pellet was resuspended in 50 mM Tris pH 7.4 and the membrane suspension was frozen at -80ºC.

4. Lowry Method

Lowry method is a colorimetric method used in the determination of the protein levels in several preparations. The first step consisted in the preparation of several solutions with crescent concentrations of BSA, in order to make a calibration curve. Then, the samples, in duplicate, were also diluted, in n Tris–HCl buffer, 50 mM, pH 7.4

Afterwards, 1000 µL of the reagent C (fifty parts of 2% Na_2CO_3 with 0.1 n NaOH, and one part of 0.5% $Cu_2SO_4.5H_2O$ with 1% Na-Tartrate in 50 mL of water) were added, followed by an incubation of 20 minutes. After this period, the reagent D/Folin & Ciocalteau's Phenol Reagent Working Solution (18 mL of Folin – Reagent (F-9252) and 72 mL of H_2O) were added and incubated by 30 minutes. Absorvances were measured in a spectrophotometer, at 500 nm (protein > 25 µg/mL) or at 750 nm (protein < 25 µg/mL).

5. Radioligand Binding Assay

Radioligand (RL) binding assay is an assay that uses radioactively labeled drugs, being that these labelled molecules can bind to the receptor, transporter, enzyme or to the protein of interest. This assay allows the measuring of the rate and extension of binding, providing information about the number of binding sites, and their affinity and accessibility for various compounds.

[^{3}H] CP55,940 was used as radioligand (final concentration 0.1 nM; specific activity = 141.2 Ci/mmol) and to calculate the volume of radioligand the following formula was used:

$$V_{RL}\ (\mu L) = \frac{(total\ well + 9) * Specific\ Activity\ \left(\frac{Ci}{mmol}\right) * Final\ concentration\ of\ RL\ in\ the\ assay\ (nM) * Total\ final\ volume\ (mL)}{1000 * Concentration\ of\ RL\ (\frac{Ci}{mmol})}$$

The final concentration of the protein for each well was 16 µg/well for CB2 expressing cells, and 30 µg/well for CB1 expressing cells. To prepare the dilutions of the membrane preparation buffer (50 mM Tris, pH 7.4) was used.

All compounds were diluted in DMSO, and the final concentration of vehicle was 2.5%.

The assay layout included 2 wells with 100% DMSO (representing the Total Binding of RL), 2 wells with 10 µM unlabelled CP 55.940 (final concentration; in DMSO; representing Non-Specific Binding of RL) and duplicates of the test substances in the established concentration. 465 µL of assay buffer (50 mM Tris, 3 mM $MgCl_2$, 0.1% BSA, pH 7.4) were pipetted into each well of a 96 well assay plate. Then, 15 µL of the test compounds, DMSO and unlabelled CP 55.940 were added at the corresponding wells, followed by the addition of 60 µL of RL. Finally, 60 µL of diluted membrane were added into each well to begin the reaction, following an incubation of 2 hours. About 30 minutes before harvest the RL-protein complex, the GF/C glass fiber filter was placed into 0.3% aq. polyethyleneimine solution, in order to reduce the binding of RL into the filter.

A Brandel 96-channel cell harvester (Brandel, Gaithersburg, MD), was used to harvest the cells with cold (4ºC) washing buffer composed by 50 mM Tris, 0.1% BSA, pH 7.4. Each well was washed 3 times with 1 mL of cold washing buffer.The filter was dried at 50ºC for 1 hour. Afterwards, 50 µL of scintillation cocktail (Multiscint 25, PerkinElmer) were added to each well and the determination of the radioactivity bound to the receptor was performed in a liquid scintillation counter (Topcount NXT, Packard/Perkin-Elmer) using a 10 hours preincubation program.

Data were obtained from three independent experiments, performed in duplicates.

6. cAMP Accumulation Assay

Inhibition of adenylate cyclase activity was determined in CHO cells stably expressing the CB1 and CB2 receptor subtype, using a competition binding assay for cAMP, adapted from the procedure described by Nordstedt *et al* (26).

Cells were seeded, according to the method described above, into a 24-well plate at a density of 200 000 cells/well 24 hours before performing the assay with the growth medium (F12 growth medium with 2% of FCS, 100 µg/mL or 100 U/mL of Penicillin/Streptomycin and 0.8 mg/mL of G418). After the incubation, the cells were washed with Hank's buffered saline solution (HBSS) consisting of NaCl (13 mM), HEPES (20 mM), glucose (5.5 mM), KCl (5.4 mM), $NaHCO_3$ (4.2 mM), $CaCl_2$ x $2H_2O$ (1.25 mM), $MgSO_4$ (0.8 mM), $MgCl_2$ (1 mM), KH_2PO_4 (0.44 mM) and Na_2 PO_4 (0.34 mM) dissolved in deionized, autoclaved water.

After the addition of 200 µL or 185 µL of HBSS per well for agonist or antagonist test, respectively, cells were incubated for 2 hours at 37ºC. After this period of time, 20 µL of the phosphodiesterase inhibitor Ro-20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imodazolidinone), final concentration 40 µM, dissolved in HBSS, was added and the suspension incubated for 10 minutes. 15 µL of the test compound was diluted in HBSS containing 10% DMSO, to obtain the desired concentration, and added to the suspension. After 5 minutes of incubation, 15 µL of forskolin (final concentration: 10 µM), prepared with HBSS containing 10% of DMSO, were added to each well. Antagonists were added at the desired concentration 20 minutes before

adding the agonist. The final DMSO concentration was 1.9%. The suspension was incubated for 10 minutes after the addition of Ro-20-1724, again for 5 minutes after the addition of the test compound, and for another 15 minutes after adding forskolin.

cAMP accumulation was stopped by removing the supernatant from the cell suspension and subsequently lyzing the cells with 500 µL of hot lysis buffer (120ºC; 4 mM EDTA, 0.01% Triton X-100). Aliquots of 50 µL of cell suspension were transferred to 2.5 mL tubes, 30 µL of [^{3}H]cAMP (3 nM) and 40 µL of cAMP-binding protein (50 µg per well) were added, followed by 1 hour incubation on ice. Bound and free radioligand were separated by rapid filtration through GF/B glass fiber filters using a Brandel 48-channel cell harvester (Brandel, Gaithersburg, MD). Radioactivity on the filters were determined in a liquid scintillation counter (TRICARB 2900TR, Packard/Perkin-Elmer) after 6 hours of preincubation with 3 mL of scintillation cocktail (LumaSafePlus, Perkin-Elmer).

Data were obtained from three independent experiments, performed in duplicates.

7. Data Analysis

All data were processed with Microsoft Excel (2016) and further analysed using IBM SPSS® Statistics version 24.0 (SPSS Inc., IBM, USA).

Results

1. Compound library 1 on GPR18

1.1. Compounds from compound library 1 did not show agonist activity on GPR18

In order to identify new compounds that act as agonists or antagonists of GPR18 we performed an initial screening using the β-arrestin recruitment assays. The compound library tested, is a proprietary in-house library of compounds that have structural similarities to previously identified ligands of GPR18, and to other in-house compounds that also showed capacity to activate or inhibit GPR18 in previous studies from our lab (24). To test if these compounds can act as agonists, CHO cells expressing GPR18 were incubated with each compound at a concentration of 10 μM, for 90 minutes (Fig. 14). For the antagonist test, cells were pre-treated with each compound at a concentration of 10 μM, for 60 minutes, and then they were further incubated with 0.1 μM peptide-like agonist, a GPR18 agonist, for another 90 minutes (Fig. 15). The activation or inhibition was assessed by chemiluminescent output of β-galactosidase activity. All data result from three independent experiments (N=3), performed in duplicate. Data represents mean ± SEM and is expressed as percentage of GPR18 activation, or inhibition of receptor activation, for the agonist test or antagonist test, respectively.

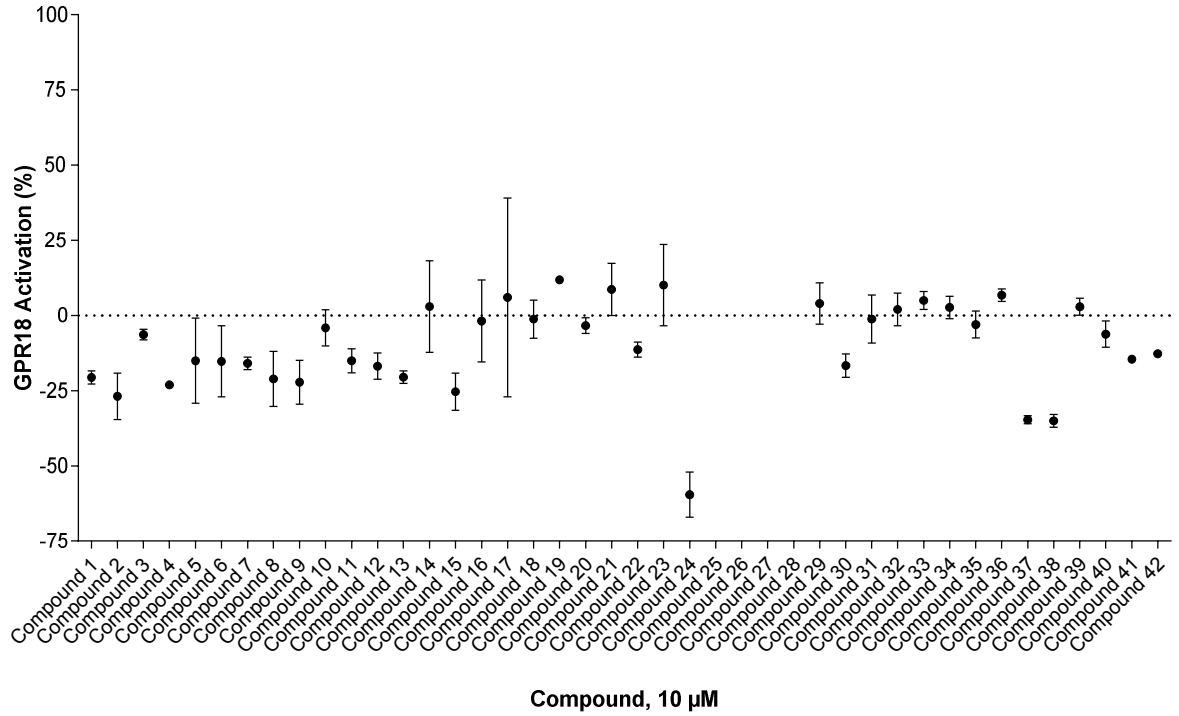


Figure 14: Screening of agonist activity of compound library 1 on GPR18. CHO cells expressing GPR18 were treated with each compound at 10 μM for 90 minutes. 0.1% BSA in PBS and 0.1 μM peptide-like agonist were used as blank and positive control, respectively. The activation was assessed by the measurement of the luminescence produced and is expressed as percentage of GPR18 activation. Data represents mean ± SEM from three independent experiments (N=3), performed in duplicate.

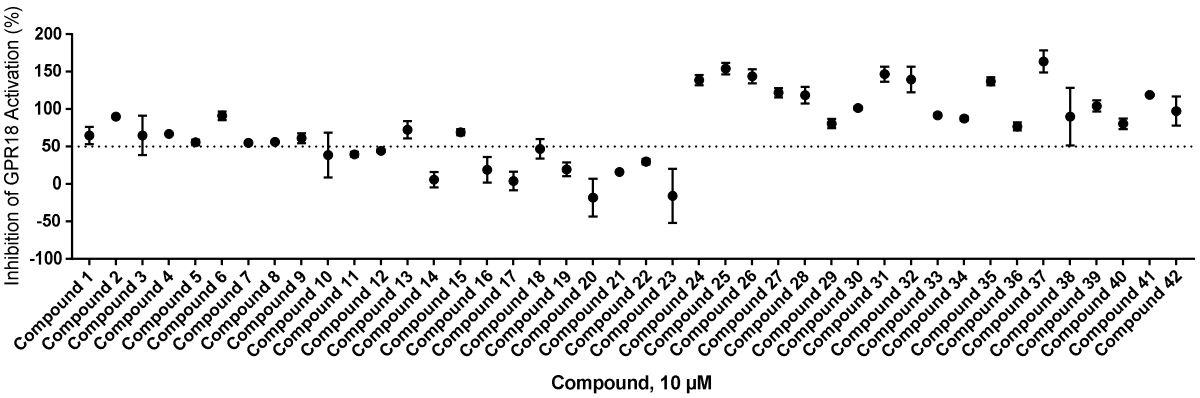


Figure 15: Screening of antagonist activity of compound library 1 on GPR18. CHO cells expressing GPR18 were pre-treated with each compound at a concentration of 10 µM, for 60 minutes, and then they were further incubated with 0.1 µM peptide-like agonist, a GPR18 agonist, for another 90 minutes. 0,1% BSA in PBS and 0.1 µM peptide-like agonist were used as blank and positive control, respectively. The inhibition was assessed by the measurement of the luminescence produced and is expressed as percentage of inhibition of GPR18 activation. Data represents mean ± SEM from three independent experiments (N=3), performed in duplicate.

Analysing the results of the study of the agonist activity, it is possible to observe that none of the compounds promoted a GPR18 activation higher than 50%.

On the contrary, in the study of the antagonist activity several of compounds showed an inhibition of GPR18 activation higher than 50%, including compound 1, compound 2, compound 3, compound 4, compound 5, compound 6, compound 7, compound 8, compound 9, compound 13, compound 15, compound 24, compound 25, compound 26, compound 27, compound 28, compound 29, compound 30, compound 31, compound 32, compound 33, compound 34, compound 35, compound 36, compound 37, compound 38, compound 39, compound 40, compound 41 and compound 42.

We decided to proceed with our studies with compounds that showed a percentage of GPR18 activation, or inhibition of receptor activation, equal or higher than 50%.

1.2. Compounds from compound library 1 showed to be selective for GPR18

In the second phase of our work, we evaluated the selectivity of compounds from compound library 1 for GPR18, by analysing the activity of the same compounds on GPR55, using the same methodological approach. Therefore, once again, we performed agonist and antagonist tests in CHO cells expressing GPR55 by the procedure described above. In this case we used 2 μM LPI as a positive control. All data result from three independent experiments (N=3), performed in duplicate. Data represents mean ± SEM and is expressed as percentage of GPR55 activation, or inhibition of receptor activation, for agonist test or antagonist test, respectively.

As can be seen from the results showed in Figure 16, none of the compounds activated the GPR55 more than 50%. According to Figure 17, only the compound 31 showed a capacity to inhibit GPR55 activation higher than 50%.

We decided to pursue the studies with all the compounds, selective or not for GPR18.

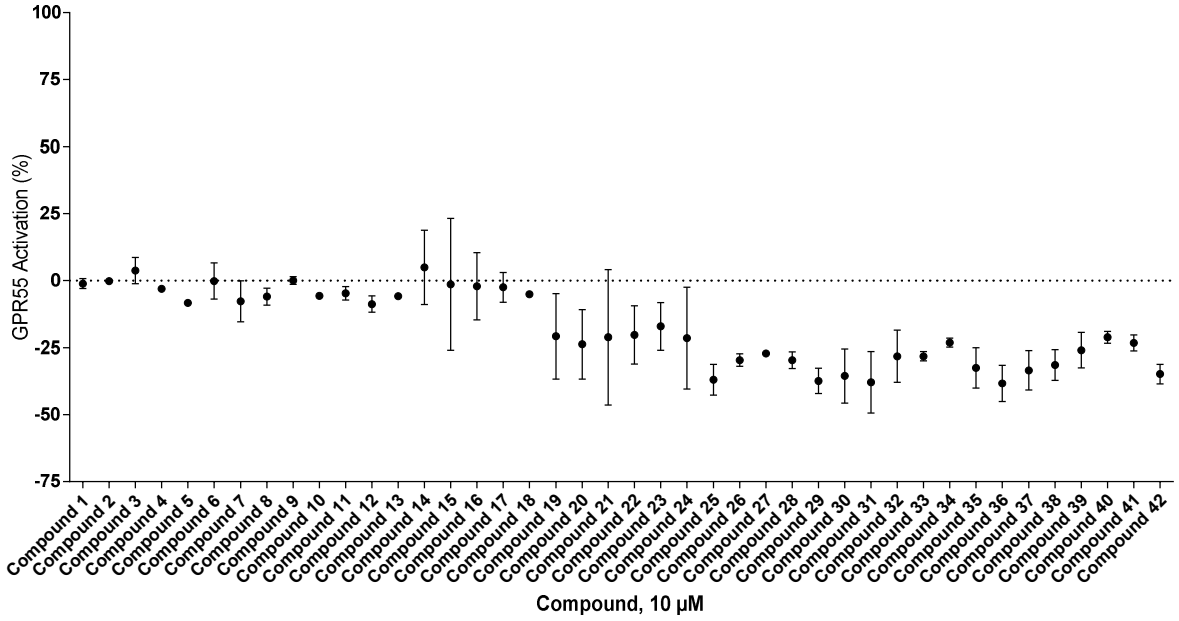


Figure 16: Screening of agonist activity of compound library 1 on GPR55 to study the selectivity for GPR18. CHO cells expressing GPR55 were treated with each compound at 10 μM for 90 minutes. 0,1% BSA in PBS and 2μM LPI were used as blank and positive control, respectively. The activation was assessed by the measurement of the luminescence produced and is expressed as percentage of GPR18 activation. Data represents mean ± SEM from three independent experiments (N=3), performed in duplicate.

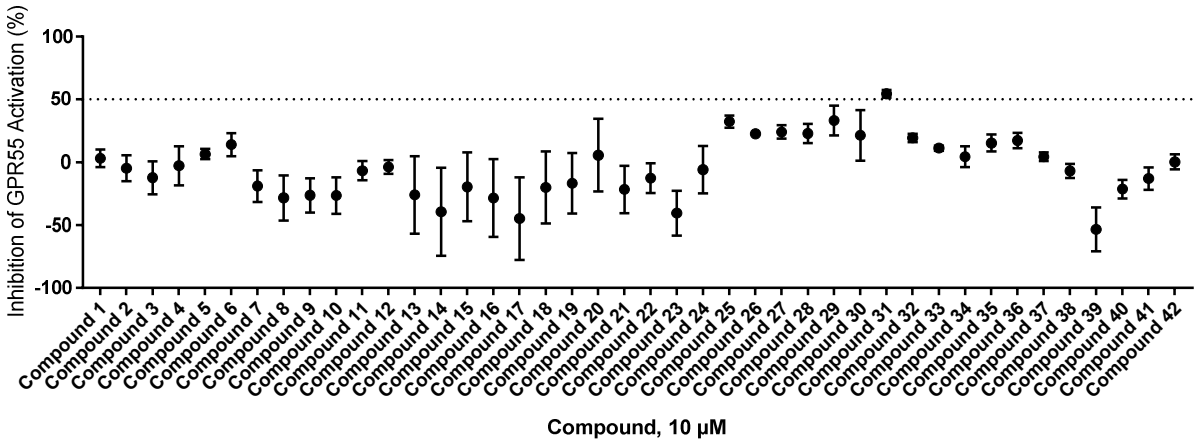


Figure 17: Screening of antagonist activity of compound library 1 on GPR55 to study the selectivity for GPR18. CHO cells expressing GPR55 were treated with each compound at 10 µM for 60 minutes, and then they were further incubated with 2 LPI, a GPR55 agonist, for another 90 minutes. 0,1% BSA in PBS and 2 µM LPI were used as blank and positive control, respectively. The inhibition was assessed by the measurement of the luminescence produced and is expressed as percentage of inhibition of GPR55 activation. Data represents mean ± SEM from three independent experiments (N=3), performed in duplicate.

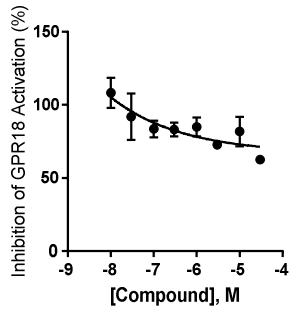
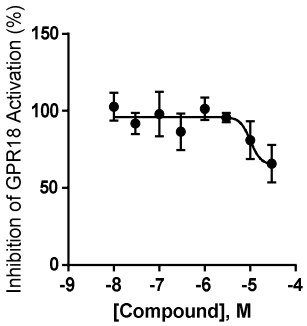
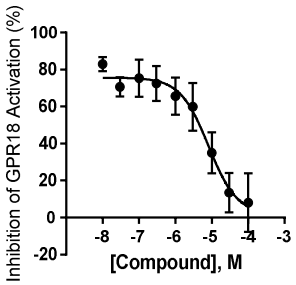
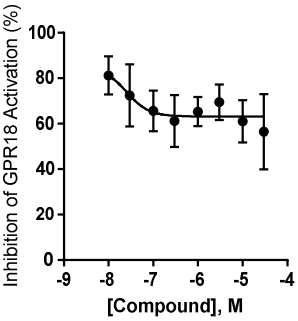
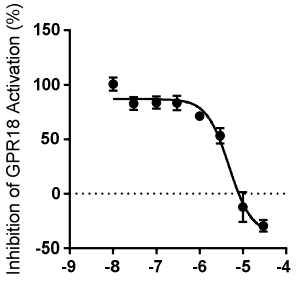
1.3. Some of the compounds from compound library 1 showed moderate antagonist potency

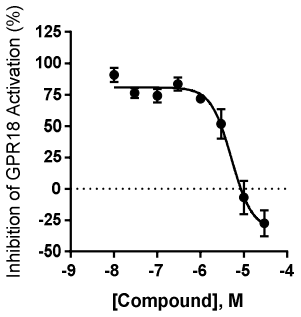
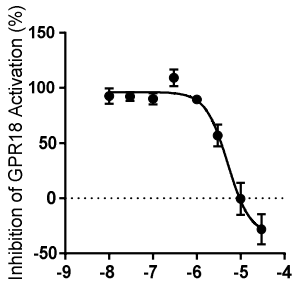
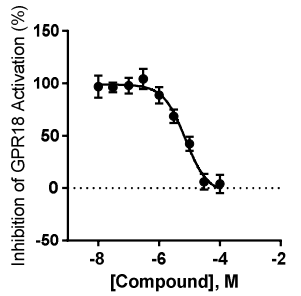
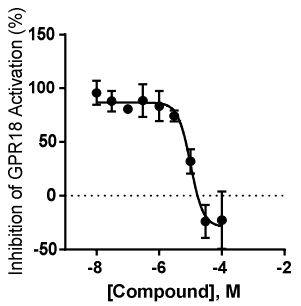
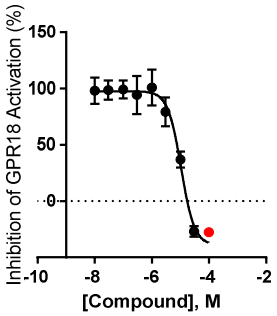
After the initial screening, the antagonist and agonist potencies of each selected compounds that showed antagonist or agonist activity, respectively, were evaluated. Once again, using the β-arrestin recruitment assays, the cells were treated with increasing concentrations of each compound, in the absence or presence of a known agonist, in order to obtain dose-response curves and to determine EC_{50} (μM) and IC_{50} (μM). All data result from three independent experiments (N=3), performed in duplicate. Data represents mean ± SEM and is expressed as EC_{50} (μM) or IC_{50} (μM) and as percentage of GPR18 activation, or inhibition of receptor activation, for the agonist test or antagonist test, respectively.

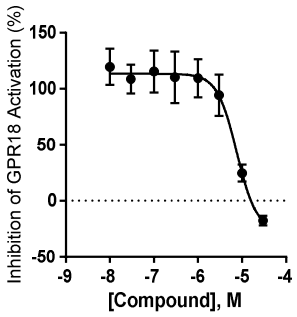
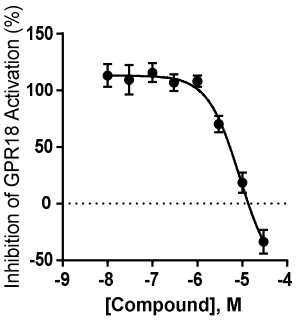
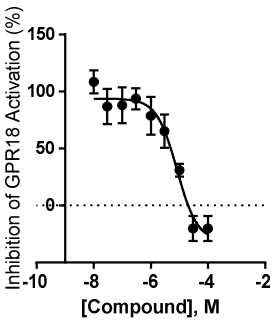
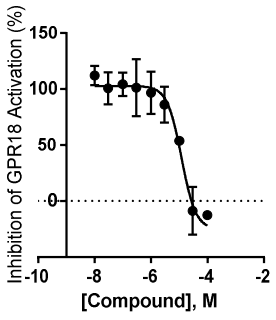
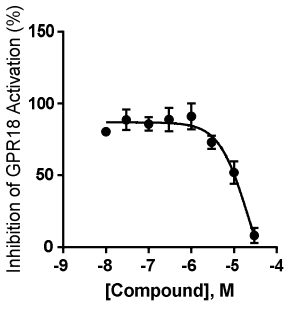
Since none of the compounds showed agonist activity higher than 50%, we only evaluated antagonist activity. Despite the fact that compound 5, compound 8, compound 9, compound 13 and compound 15 showed an antagonist activity higher than 50%, we decided to prioritize our study to compounds that showed a percentage of inhibition of GPR18 activation not close to 50%. Table 4 summarise the IC_{50} (μM) obtained for each compound and the respective dose-response curve. From these results it is possible to observe that the compound 3 showed the highest antagonist potency, presenting a IC_{50} of $4x10^{-10}$ $\pm 4x10^{-10}$ μM and a maximal inhibition of 62%, while the compound 34 showed the highest IC_{50} (34.5 ± 20.4 μM) and a maximal inhibition of 8%. Only compound 1, compound 3, compound 4 and compound 7 showed maximal inhibition higher than 50% (53%, 62%, 65%, and 56%, respectively).

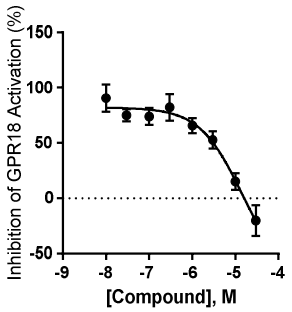
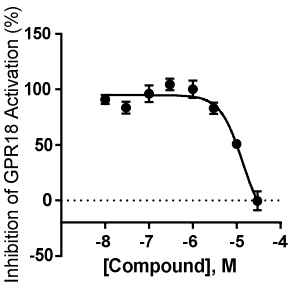
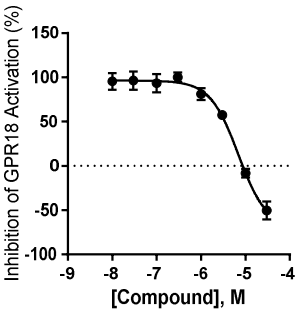
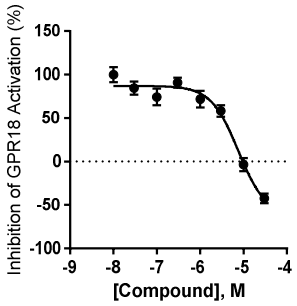
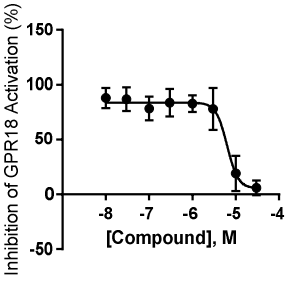
Table 4: Evaluation of the antagonist potencies of compound library 1 on GPR18.

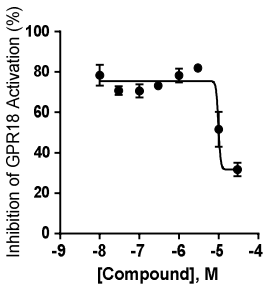
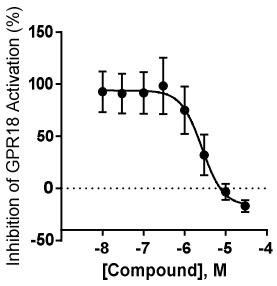
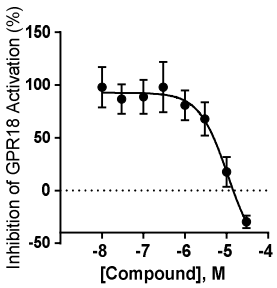
Compound	Human GPR18	
	IC_{50} ± SEM (μM)	
	(% of inhibition of peptide-like agonist activation)	**Curve**
Compound 1	**12.9** ± 1.6 (53 % of maximal inhibition)	
Compound 2	**8.83** ±2.32 (-4 % of maximal inhibition)	

Compound	Human GPR18	
	IC_{50} ± SEM (μM)	
	(% of inhibition of peptide-like agonist activation)	Curve
Compound 3	**>10** (62 % of maximal inhibition)	
Compound 4	**6.57** ±3.18 (65 % of maximal inhibition)	
Compound 6	**7.65** ±0.56 (8% of maximal inhibition)	
Compound 7	**0.0324** ±0.020 (56% of maximal inhibition)	
Compound 24	**6.27** ± 1.65 (-29% of maximal inhibition)	

Compound	Human GPR18	
	IC_{50} ± SEM (μM)	
	(% of inhibition of peptide-like agonist activation)	Curve
Compound 25	**5.63** ± 1.19 (-27% of maximal inhibition)	
Compound 26	**5.52** ± 0.896 (-28% of maximal inhibition)	
Compound 27	**9.96** ± 2.92 (4% of maximal inhibition)	
Compound 28	**10.5** ± 0.716 (-22% of maximal inhibition)	
Compound 29	**19.2** ±9.3 (-27 % of maximal inhibition)	

Compound	Human GPR18	
	IC_{50} ± SEM (μM)	
	(% of inhibition of peptide-like agonist activation)	Curve
Compound 30	**7.60** ±0.26 (-17 % of maximal inhibition)	
Compound 31	**9.94** ±1.86 (-33 % of maximal inhibition)	
Compound 32	**7.98** ±0.90 (-20 % of maximal inhibition)	
Compound 33	**32.5** ±16.0 (-12 % of maximal inhibition)	
Compound 34	**34.5** ±20.4 (8 % of maximal inhibition)	

Compound	Human GPR18	
	IC_{50} ± SEM (μM)	
	(% of inhibition of peptide-like agonist activation)	Curve
Compound 35	**28.3** ±13.1 (-20 % of maximal inhibition)	
Compound 36	**13.3** ±1.9 (-0.3 % of maximal inhibition)	
Compound 37	**7.36** ±0.87 (-50 % of maximal inhibition)	
Compound 38	**9.74** ±3.14 (-42 % of maximal inhibition)	
Compound 39	**6.46** ±1.47 (5 % of maximal inhibition)	

Compound	Human GPR18	
	IC_{50} ± SEM (μM)	
	(% of inhibition of peptide-like agonist activation)	Curve
Compound 40	**9.88** ±0.00 (31 % of maximal inhibition)	
Compound 41	**2.59** ±0.47 (-16 % of maximal inhibition)	
Compound 42	**11.2** ±2.7 (-29 % of maximal inhibition)	

2. Compound library 2 on GPR18

2.1. Only compound 57, compound 59 and compound 72 showed antagonist activity in the initial screening

Using the same methodological approach described for the compound library 1, we have screen a new proprietary library compound, the compound library 2. Once again, the first step was screening for compounds that may have an agonist or antagonist effect on GPR18, using β-arrestin recruitment assays.

Evaluating the results of the agonist activity screening (Fig. 18), we can observe that some compounds led to an GPR18 activation higher than 50%, including compound 50, compound 53, compound 56, compound 58, compound 60, compound 61, compound 62, compound 63, compound 64, compound 65, compound 66, compound 68 and compound 69.

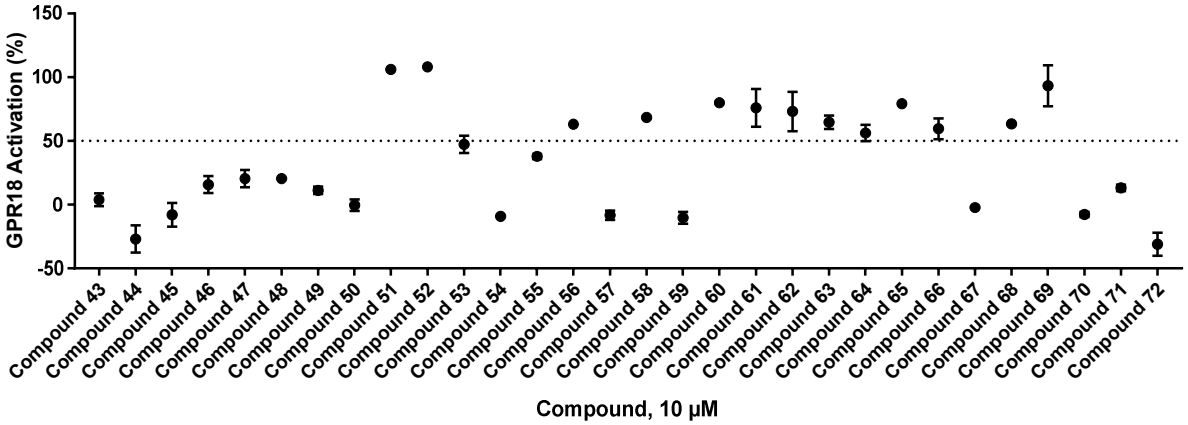


Figure 18: Screening of agonist activity of compound library 2 on GPR18. CHO cells expressing GPR18 were treated with each compound at 10 µM for 90 minutes. 0,1% BSA in PBS and peptide-like agonist (0.1 µM) were used as blank and positive control, respectively. The activation was assessed by the measurement of the luminescence produced and is expressed as percentage of GPR18 activation. Data represents mean ± SEM from three independent experiments (N=3), performed in duplicate.

On the other hand, only treatment with compound 57, compound 59 and compound 72 led to an inhibition of GPR18 activation higher than 50% (Fig. 19).

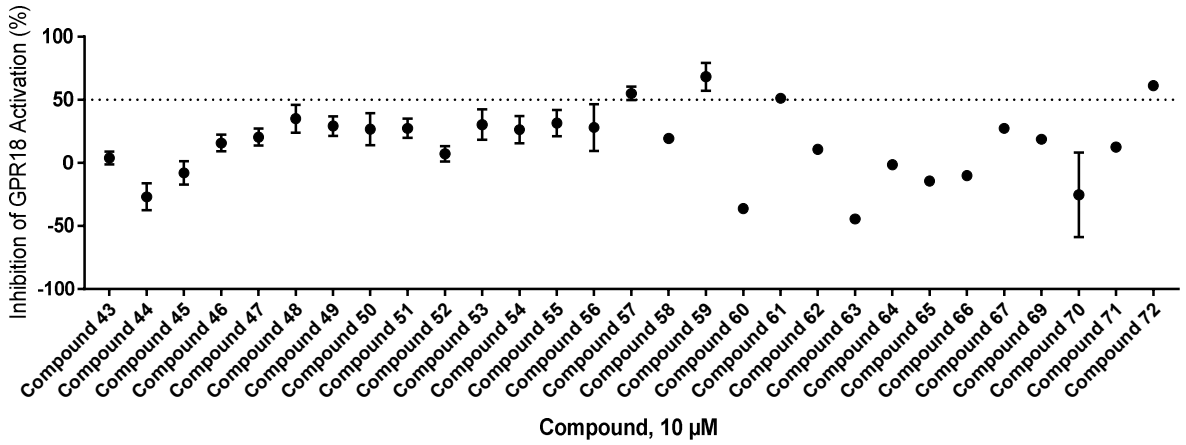


Figure 19: Screening of antagonist activity of compound library 2 on GPR18. CHO cells expressing GPR18 were treated with each compound at 10 µM for 60 minutes. 0,1% BSA in PBS and peptide-like agonist (0.1 µM) were used as blank and positive control, respectively. The inhibition was assessed by the measurement of the luminescence produced and is expressed as percentage of inhibition of GPR18 activation. Data represents mean ± SEM from three independent experiments (N=3), performed in duplicate.

We decided to proceed with our studies with compounds that showed a percentage of GPR18 activation, or inhibition of receptor activation, equal or higher than 50%.

2.2. None of the compounds from compound library 2 showed agonist activity on GPR55

After assessing the agonist or antagonist activity of the compounds from compound library 2 , the next step of our work was the evaluation of the selectivity of these compounds for GPR18, by analysing their activity on GPR55, through β-arrestin recruitment assays. Tests to determine the agonist and antagonist were performed in CHO cells expressing GPR55 as described above.

As can be seen from Figure 20, none of the compounds of the compound library 2 led to GPR55 activation higher than 50%.

However, compound 59, compound 60, compound 61, compound 67, compound 68, compound 71 and compound 72 showed a percentage of inhibition of GPR55 activation higher than 50% (Fig. 21).

We decided to pursue our studies with all the compounds that showed activity on GPR18, besides also activating or not GPR55.

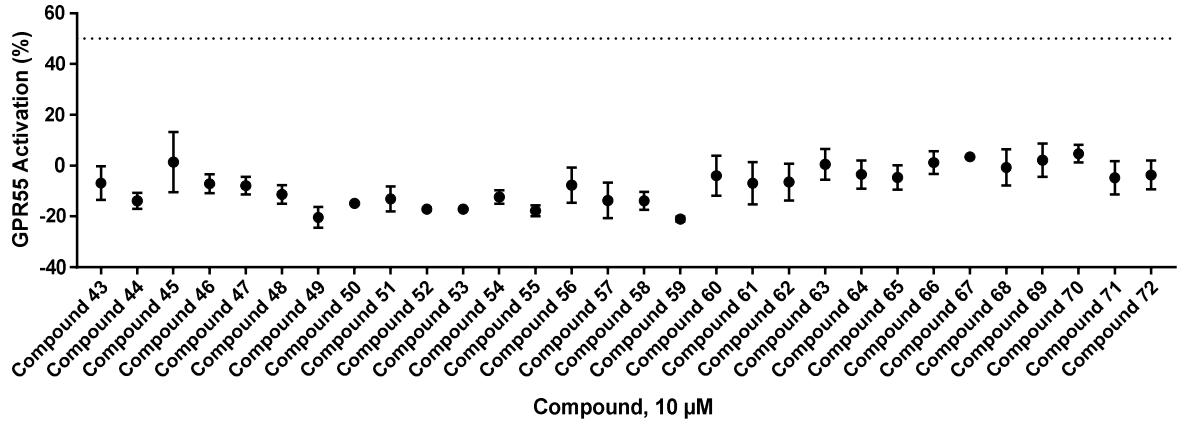


Figure 20: Screening of agonist activity of compound library 2 on GPR55 to study the selectivity for GPR18. CHO cells expressing GPR55 were treated with each compound at 10 µM for 90 minutes. Was used PBS+0,1% of BSA and LPI (2µM) as blank and positive control, respectively. The activation was assessed by the measurement of the luminescence produced and is expressed as percentage of GPR18 activation. Data represents mean ± SEM from three independent experiments (N=3), performed in duplicate.

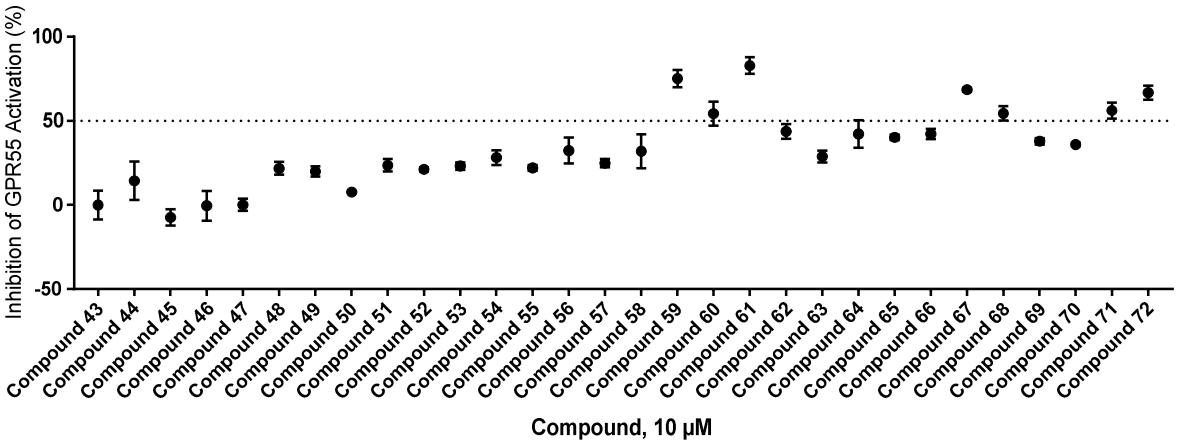


Figure 21: Screening of antagonist activity of compound library 2 on GPR55 to study the selectivity for GPR18. CHO cells expressing GPR55 were treated with each compound at 10 µM for 60 minutes. 0,1% BSA in PBS and LPI (2 µM) as blank and positive control, respectively. The inhibition was assessed by the measurement of the luminescence produced and is expressed as percentage of inhibition of GPR55 activation. Data represents mean ± SEM from three independent experiments (N=3), performed in duplicate.

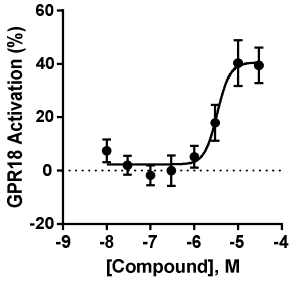
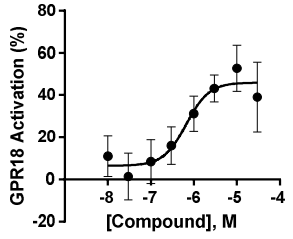
2.3. The compound 56 showed the highest agonist potency on GPR18

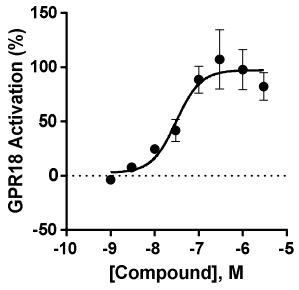
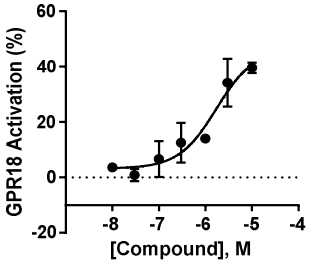
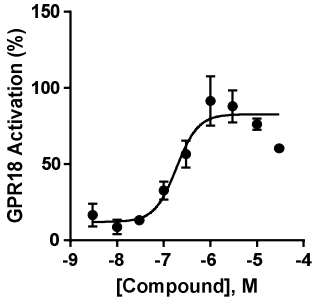
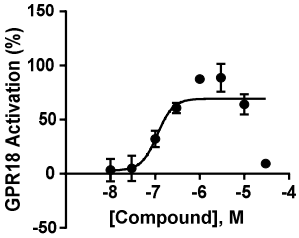
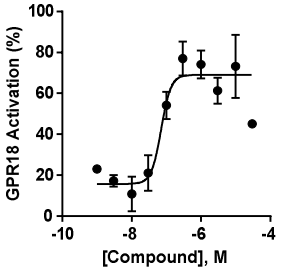
Similarly to what was performed for compound library 1, the antagonist and agonist potencies of the compounds from compound library 2 that showed antagonist or agonist activity, respectively, were evaluated. So, using β-arrestin recruitment assays once again, the cells were treated with increasing concentrations of each compound, in the absence or presence of a known agonist, in order to obtain a dose-response curves and to determine EC_{50} (μM) and IC_{50} (μM).

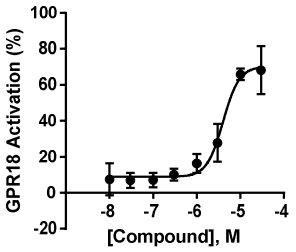
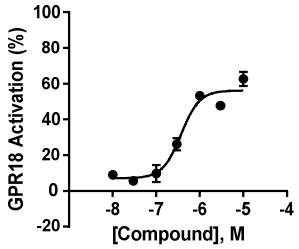
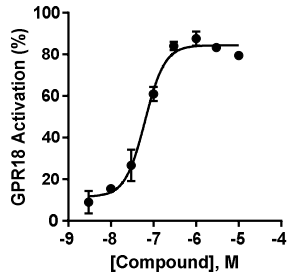
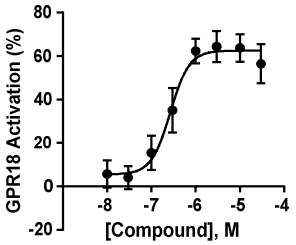
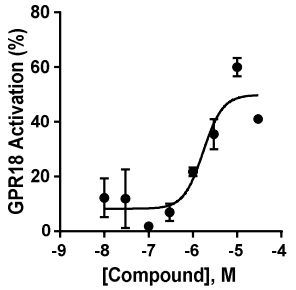
Even though the compounds 71 and 72 did not show activation of GPR18, a curve-dose response was performed to confirm their lack of activity. As expected, the agonist activity of these compounds was not significant.

Table 5 summarizes the EC_{50} (μM) obtained for each compound and the respective dose-curve response. Analysing these results and comparing the EC_{50} and the percentage of maximal activation, it is possible to observe that compound 56, compound 60, compound 61, compound 65 and compound 69 are the compounds that revealed the highest potency with EC_{50} of 0.0302 ± 0.004 μM, 0.219 ± 0.039 μM, 0.113 ± 0.003 μM, 0.0679 ± 0.018 μM and of 3.60 ± 0.19 μM, respectively. The compound that conjugated the lowest EC_{50} and the highest percentage of maximal activation was compound 56, showing the best agonist potency (EC_{50} = 0.0302 ± 0.004 μM and 107% of maximal activation). Only the compound 51 and the compound 58 showed a maximal activation lower than 50% (40% and 42% of maximal activation, respectively).

Table 5: Evaluation of the agonist potencies of compound library 2 on GPR18.

Compounds	Human GPR18	
	EC_{50} ± SEM (μM)	
	(% of peptide-like agonist activation)	Curve
Compound 51	**1.1** ± 1.92 (40 % of maximal activation)	
Compound 52	**1.65** ± 1.10 (52 % of maximal activation)	

Compounds	Human GPR18 EC_{50} ± SEM (μM) (% of peptide-like agonist activation)	Curve
Compound 56	**0.0302** ± 0.004 (107% of maximal activation)	
Compound 58	**1.68** ± 0.28 (43 % of maximal activation)	
Compound 60	**0.219** ± 0.039 (91 % of maximal activation)	
Compound 61	**0.113** ± 0.003 (87 % of maximal activation)	
Compound 62	**0.063** ± 0.001 (77 % of maximal activation)	

Compounds	Human GPR18 EC_{50} ± SEM (μM) (% of peptide-like agonist activation)	Curve
Compound 63	**4.02** ± 0.48 (68 % of maximal activation)	
Compound 64	**0.328** ± 0.027 (66 % of maximal activation)	
Compound 65	**0.0679** ± 0.018 (87 % of maximal activation)	
Compound 66	**0.285** ± 0.089 (64 % of maximal activation)	
Compound 68	**1.66** ± 0.13 (60 % of maximal activation)	

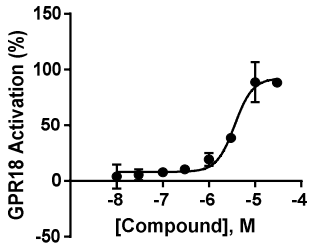
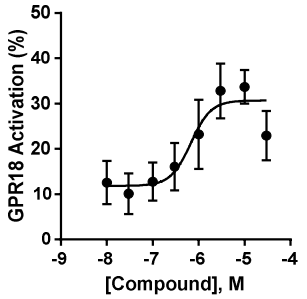
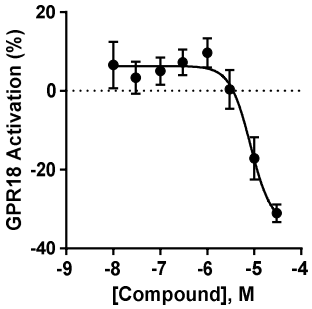
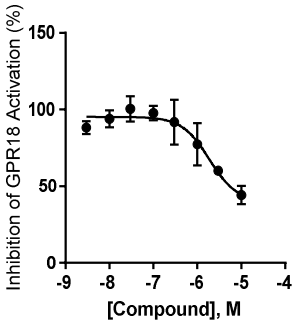
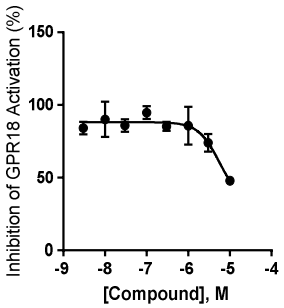
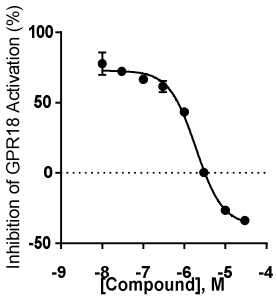
Compounds	Human GPR18	
	EC_{50} ± SEM (μM)	
	(% of peptide-like agonist activation)	Curve
Compound 69	**3.60** ± 0.19 (88 % of maximal activation)	
Compound 71	**0.801** ± 0.220 (33 % of maximal activation)	
Compound 72	**7.73** ± 1.41 (9 % of maximal activation)	

Table 6 summarizes the IC_{50} (μM) obtained for each compound and the respective dose-curve response. In the screening study, compound 57, compound 59 and compound 72 showed antagonist activity. Nevertheless, as can be noticed from results presented in Table 6, the assessment of their antagonist potencies did not show any significant inhibition, neither low IC_{50} values, with IC_{50} values of 1.70 ± 0.59 μM, 2.03 ± 1.19 μM and 1.91 ± 0.00 μM, and with percentages of maximal inhibition equal to 28%, 29% and -33% for compound 57, compound 59 and compound 72, respectively.

Table 6: Evaluation of the antagonist potencies of compound library 2 on GPR18.

Compound	Human GPR18	
	IC_{50} ± SEM (µM)	
	(% of inhibition of peptide-like agonist activation)	Curve
Compound 57	**1.70** ± 0.59 (28 % of maximal inhibition)	
Compound 59	**2.03** ± 1.19 (29 % of maximal inhibition)	
Compound 72	**1.91** ± 0.00 (-33 % of maximal inhibition)	

2.4. The antagonist potency on GPR55 of compound 61, compound 68, compound 71 and compound 72 was not significant

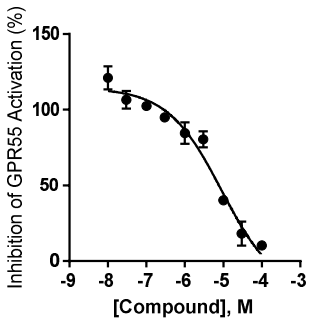
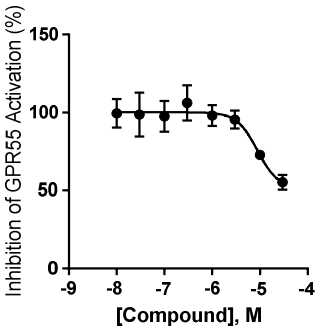
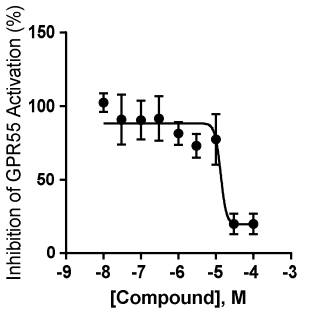
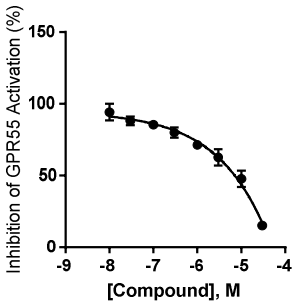
Since none of the compounds showed agonist activity on GPR55, we just evaluated the antagonist potency of the compounds on this receptor. For that, using β-arrestin recruitment assays, the cells were treated with increasing concentrations of each compound, in the presence of a known agonist, and the dose-response curve and the IC_{50} (μM) were obtained. All data result from three independent experiments (N=3), performed in duplicate. Data represents mean ± SEM and is expressed as IC_{50} (μM) and as percentage of GPR55 inhibition of receptor activation, respectively.

Although compound 59 and 67 had shown antagonist activity, it was not possible to calculate IC_{50} because there was not enough volume of these compounds to perform the assay.

In the case of compound 60, as the percentage of inhibition of GPR55 activation demonstrated was not striking, the potency evaluation of the other compounds was prioritized.

Table 7 summarizes the IC_{50} (μM) obtained for each compound and the respective dose-curve response. The results shown in this table indicate that the compounds did not have relevant antagonist potency on GPR55. The IC_{50} and the values of maximal inhibition were not significant, with IC_{50} values of 6.65 ± 0.13 μM, 9.44 ± 0.39 μM, 22.1± 9.6 μM and 9.92± 1.89 μM, and values of maximal inhibition of 10%, 55%, 19% and 20% for compound 61, compound 68, compound 71 and compound 72, respectively. The compound 68 was the only compound that showed a percentage of maximal inhibition higher than 50% (55%).

Table 7: Evaluation of the antagonist potencies of compound library 2 on GPR55.

Compound	Human GPR55	
	IC_{50} ± SEM (µM)	
	(% of inhibition of LPI activation)	Curve
Compound 61	**6.65** ± 0.13 (10 % of maximal inhibition)	
Compound 68	**9.44** ± 0.39 (55 % of maximal inhibition)	
Compound 71	**22.1**± 9.6 (19 % of maximal inhibition)	
Compound 72	**9.92**± 1.89 (20 % of maximal inhibition)	

3. Compound library 3 on CB2

3.1. None of the compounds studied showed antagonist activity on CB2

To identify new compounds that can bind to CB2 receptor, we performed an initial screening, using radioligand binding assays. The compound library tested is a proprietary in-house library of compounds that have structural similarities to previously identified ligands of CB2 and to other in-house compounds that also showed capacity to bind to CB2 in previous studies from our lab. Indeed, previously we identified several compounds from compound library 3 that had the capacity of binding to CB2. We decided to proceed the studies with the compounds that showed a percentage of inhibition of radioligand binding at 10 μM higher than 50%, including compound 73, compound 74, compound 75, compound 76, compound 77, compound 78, compound 79 and compound 80.

The following step was the assessment of the agonist or antagonist activity of the compounds from compound library 3 that had shown to be able to bind to the receptor in the previous assay, using cAMP accumulation assays. For that, CHO cells expressing CB2 were treated with 10 μM of each compound and incubated with it for 5 minutes. The controls used for both tests were 10% DMSO in HBSS as blank, 1 μM CP55,940 with 10 μM forskolin as positive control in the agonist test, and 10 μM forskolin as positive control in the antagonist test. The amount of cAMP produced was assessed by the determination of radioactivity present in each well. All data result from three independent experiments (N=3), performed in duplicate. Data represents mean ± SEM and is expressed as percentage of receptor activation or percentage of inhibition of receptor activation for agonist or antagonist test, respectively.

Examining the results shown in Figure 22B), it is possible to observe that none of the compounds showed antagonist activity on CB2, since inhibition of receptor activation was lower than 50% for all the compounds tested. Contrary to the antagonist activity study, in the agonist activity study (Fig. 22A)) most compounds showed an activation higher than 50%, including compound 73, compound 74, compound 75, compound 76, compound 77, compound 78 and compound 79.

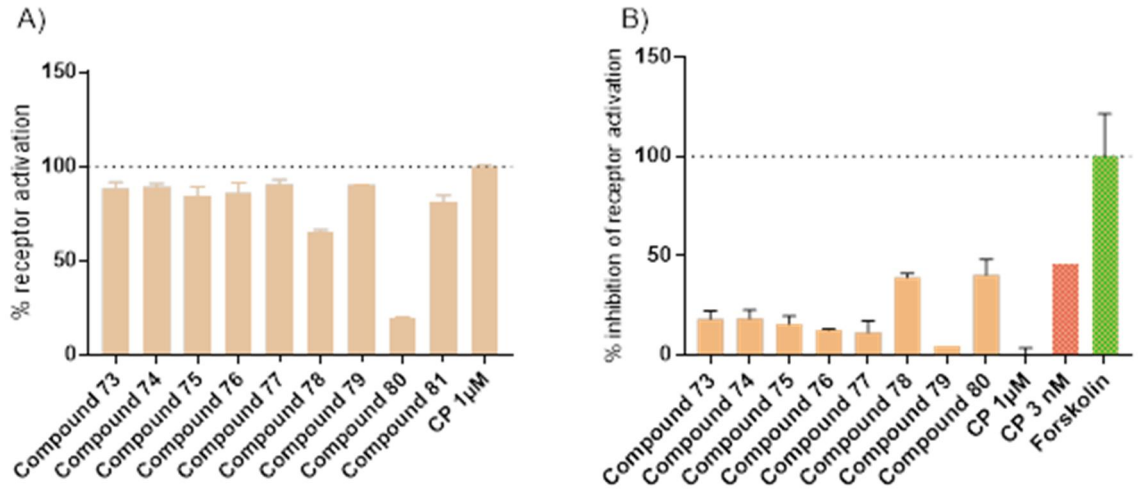


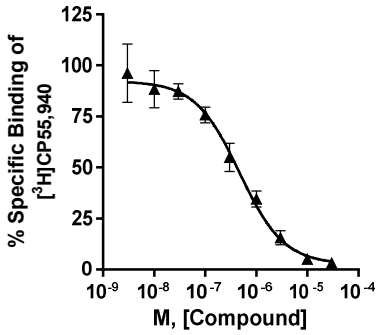
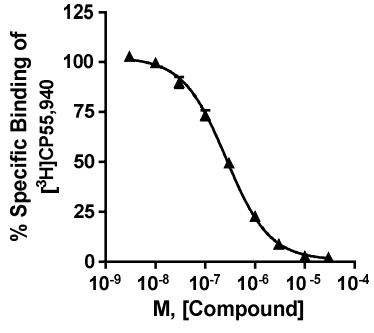
Figure 22: Screening of agonist (A) and antagonist (B) activity of compound library 3 on CB2. For the agonist test, the CHO cells expressing CB2 were incubated with 40 µM Ro-20-1724, 10 µM of each compound and 10 µM forskolin, added at different times in this order. For the antagonist test, the cells were incubated with 40 µM Ro-20-1724, with 3nM CP55.940, 10 µM of each compound, and 10 µM forskolin, added at different times in this order. The controls used for both tests were 10% DMSO in HBSS as blank, 1 µM CP55.940 with 10 µM forskolin as positive control at agonist test, and 10 µM forskolin as positive control at antagonist test. The radioactivity was determined in order to assess the amount of cAMP produced. All data result from three independent experiments (N=3), performed in duplicate. Data represents mean ± SEM and is expressed as percentage of receptor activation or percentage of inhibition of receptor activation for agonist or antagonist test, respectively.

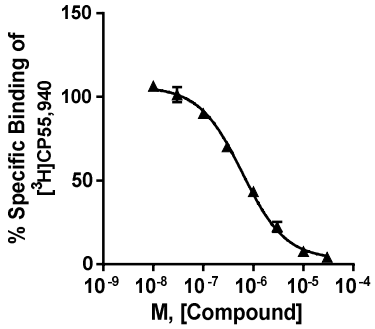
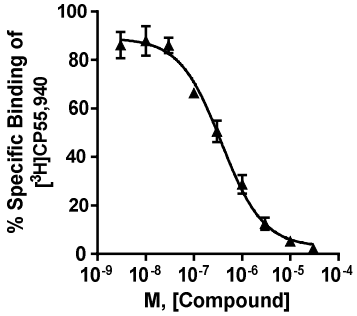
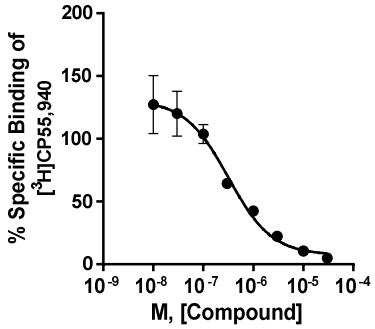
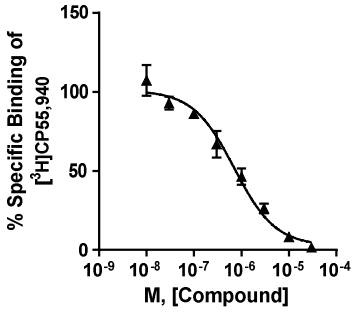
3.2. The compounds tested showed high potency on CB2

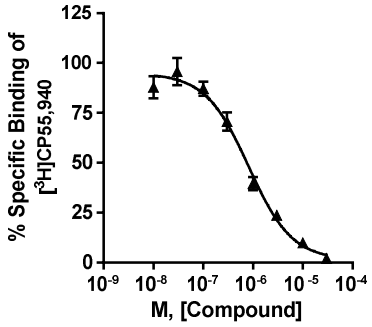
After the initial screening, and since none of the compounds showed antagonist activity on CB2, we just evaluated the agonist potency of the compounds on this receptor. Therefore, using cAMP accumulation assays, cells were treated with increasing concentrations of each compound, in order to obtain a dose-response curve and to determine the Ki (μM). All data result from three independent experiments (N=3), performed in duplicate. Data represents mean ± SEM and is expressed as Ki (μM) and as percentage of maximal inhibition of radioligand binding at 10 μM.

Table 8 summarizes the results of the agonist potencies of compound library 3 on CB2. As it is possible to observe from this table, all the compounds of compound library 3 tested were potent on CB2, having shown a maximal inhibition near to 100% and a Ki lower than 1 μM, with Ki values of 0.498 ± 0.176 μM, 0.237 ± 0.006 μM, 0.536 ± 0.058 μM, 0.339 ± 0.061 μM, 0.338 ± 0.146, 0.628 ± 0.07 μM and 0.716 ± 0.001 μM, and percentages of maximal inhibition of 98%, 98%, 96%, 99%, 99%, 97% and 99% for compound 73, compound 74, compound 75, compound 76, compound 77, compound 78 and compound 79, respectively. The compounds that conjugated the highest maximal inhibition with the lowest Ki were the compound 76 and 74.

Table 8: Evaluation of the agonist potencies of compound library 3 on CB2.

Compound	Cannabinoid Receptor Human CB2	
	Ki ± SEM (μM) (or inhibition of radioligand binding at 10 μM)	**Curve**
Compound 73	**0.498** ± 0.176 (98% maximal inhibition)	
Compound 74	**0.237** ± 0.006 (98% maximal inhibition)	

Compound	Cannabinoid Receptor	
	Human CB2	
	Ki ± SEM (μM) (or inhibition of radioligand binding at 10 μM)	Curve
Compound 75	**0.536** ± 0.058 (96% maximal inhibition)	
Compound 76	**0.339** ± 0.061 (99% maximal inhibition)	
Compound 77	**0.338** ± 0.146 (99% maximal inhibition)	
Compound 78	**0.628** ± 0.07 (97% maximal inhibition)	

Compound	Cannabinoid Receptor	
	Human CB2	
	Ki ± SEM (μM) (or inhibition of radioligand binding at 10 μM)	Curve
Compound 79	**0.716** ± 0.001 (99% maximal inhibition)	

3.3. The compounds 74, 75 and 78 did not show specificity for CB2

To evaluate the selectivity of these compounds for CB2, we studied the activity of the same compounds on CB1.

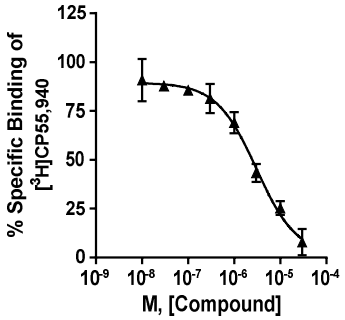
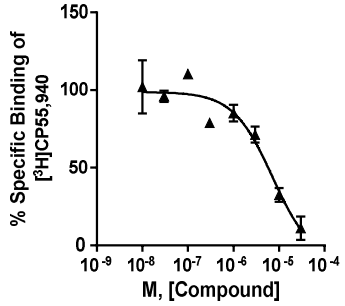
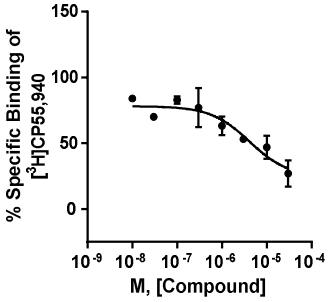
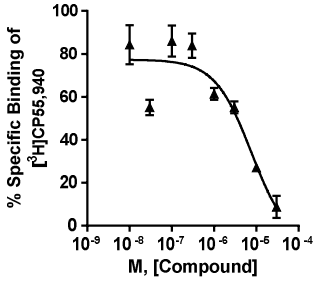
Therefore, the first step was performing a screening, using radioligand binding assays. In order to test the binding capacity of the compounds from compound library 3, a preparation containing the receptor of interest was incubated with 0.1 nM of radioligand and with 10 μM of each compound, for 120 minutes. DMSO was used to assess the Total Binding of radioligand and 10 μM unlabeled CP 55.940 was used to determine the Non-Specific Binding of radioligand. The inhibition of radioligand binding was determined by measuring the radioactivity present in each well and data was expressed as percentage of inhibition of radioligand binding at 10 μM. All data result from three independent experiments (N=3), performed in duplicate. Data represents mean ± SEM and is expressed as percentage of inhibition of radioligand binding at 10 μM.

Since none of the compounds showed antagonist activity on CB2, the selectivity study was performed with the compounds that showed agonist activity on this receptor.

So, in the next step, we performed the agonist test and the evaluation of the respective potencies, using cAMP accumulation assays. The procedures and the controls used were the same that the ones used for CB2 assays. All data result from three independent experiments (N=3), performed in duplicate. Data from the agonist screening is not shown. Data from the potency evaluation represents mean ± SEM and is expressed as Ki (μM) and as percentage of maximal inhibition of radioligand binding at 10 μM.

Table 9 summarizes the results of the screening and the evaluation of the agonist potencies of compound library 3 on CB1. The results showed that the compounds 73, 76 and 79 did not have significant binding capacity to the CB1 receptor, demonstrating a percentage of inhibition of radioligand binding lower than or close to 50% (35%, 47% and 53%, respectively). Compound 74, compound 75, compound 77 and compound 78 showed to act as agonists on CB1 (data not shown). As can also be seen from Table 9, the compounds 74, 75 and 78 were not potent agonists of CB1, besides showing a high percentage of maximum inhibition (92%, 89% and 93%, respectively), they showed a high Ki (4.07 ± 1.36 μM, 7.13 ± 1.68 μM, and 8.66 ± 3.82 μM, respectively). Compound 77 showed a low percentage of maximum inhibition (25%) and also a Ki higher than 1 μM (3.74 ± 0.07 μM).

Table 9: Screening and evaluation of the agonist potencies of compound library 3 on CB1.

Compound	Cannabinoid Receptor	
	Human CB1	
	Ki ± SEM (μM) (or inhibition of radioligand binding at 10 μM)	Curve
Compound 73	**>10** (35%)	-
Compound 74	**4.07** ± 1.36 (92% maximal inhibition)	
Compound 75	**7.13** ± 1.68 (89% maximal inhibition)	
Compound 76	**>10** (47%)	-
Compound 77	**3.74** ± 0.07 (75% maximal inhibition)	
Compound 78	**8.66** ± 3.82 (93% maximal inhibition)	
Compound 79	≈ **10 (53%)**	-

Discussion, Conclusions and Future Perspectives

GPCR family comprises more than 1000 receptors, involved in the control of several physiologic and pathophysiologic processes, including cancer, hypertension, abnormal bone development, thyroid dysfunction, and others. The complexity of its activation and signalling pathways, including heterodimerization, binding to several and distinct effector proteins and the possibility of allosteric modulation, raise several obstacles and challenges in drug discovery process. Though, the number of drugs targeting these receptors has been increasing, counting with more than 475 FDA approved drugs, acting at 108 different GPCRs, including drugs for the treatment of T2D, depression, hypertension, PD, and others (2,8,10).

Given the importance of this class of receptors, our work aimed to identify compounds that can be able to activate or inhibit four specific GPCRs, CB1, CB2, GPR55 or GPR18, and, consequently, could be used as therapeutic approaches in several pathologies, such as those mentioned above. In previous studies, our lab described several ligands for CB1, CB2, GPR55 and GPR18, and identified new compounds, from an in-house library, that were able to activate/inhibit these receptors. So, using the molecular structures of this recently identified hits as a starting point, we tested new compounds, also belonging to our in-house library, in order to search for novel molecules with therapeutic potential. Therefore, the first step was the screening of two different libraries, followed by selectivity studies and evaluation of the potency of the compounds.

CB1 and CB2 are two cannabinoid receptors, mediating the effects of Δ^9-THC, AEA and 2-AG. CB1 is one of the most abundant GPCRs in the brain, being involved in psychoactive effects, which limits its use as a therapeutic target. On the other hand, CB2 is highly distributed in peripheral organs with immune function. Since CB2 ligands do not appear to produce psychotropic effects, this receptor is considered a promising therapeutic for the treatment of diverse pathologies, including painful conditions, neuroinflammatory or neurodegenerative disorders, such as PD or MS, and other inflammatory disorders like atherosclerosis (2,12,16, 17,19).

GPR55 and GPR18 are two of the 140 GPCRs classified as orphan receptors. GPR55 is highly expressed in the CNS and in peripheral tissues such as endothelial cells, bone, lymphocytes, and in other systems, and LPI and 2-AGPI are ligands for this receptor. More recently, a correlation between GPR55 expression levels and the rate of cancer cells proliferation, was established. GPR55 may be a potential target to several disorders, including diabetes, obesity, cancer, osteoporosis and Parkinson's disease. In its turn, GPR18 may modulate the immune responses in acute inflammation situations. NAGly and RvD2 are considered endogenous agonists for GPR18, while amauromine is the only GPR18 antagonist described. There are evidences that places this receptor as a target for the treatment of several inflammatory diseases, osteoporosis, cancer and obesity (2,3,12,18,22,23,25).

In order to identify compounds that are be able to modulate the activity of these receptors, as previously mentioned, the first step was the screening of these compounds, followed by selectivity studies and evaluation of the potency of the compounds.

The agonist and antagonist activity of two distinct series of compounds (compound library 1 and 2), for GPR18 were screened, using the β-arrestin recruitment assays. For the compound library 1, 42 compounds were tested. Our results show that none of the compounds showed agonist activity, while 30 compounds exhibited capacity for inhibit GPR18 activity (Fig. 23). On the other hand, for compound library 2, 30 compounds were tested. The analysis of these results revealed that 13 of these compounds could activate GPR18, while only three of them showed antagonist activity (Fig. 24). We chose to continue the studies with the compounds that showed a percentage of activation of GPR18 or inhibition of receptor activation higher than 50%. Unfortunately, we were not able to amauromine, a GPR18 antagonist, as a negative control, in order to compare the activity between the different compounds, and this well-established antagonist.

The following step was the evaluation of the selectivity of these compounds for GPR18, performing a screening with the same compounds and analysing their activity on GPR55. Thus, the same methodological approach was used, and the agonist and antagonist tests were performed. Were studied the selectivity of the 42 compounds of compound library 1. The results did not show any compound with agonist activity on GPR55 and only one compound (compound 31) showed activation capacity of GPR55 slightly higher than 50%, not showing selectivity for GPR18 (Fig. 23). All the other compounds showed to be GPR18 selective. For compound library 2, 30 compounds were tested, and the results show that none of them activate GPR55, however, seven compounds were able to act as antagonist on GPR55 (Fig. 24). It is possible to conclude that compound 72 and compound 59 are not selective antagonists for GPR18, and compound 60 and compound 61, besides not being GPR18 selective, have opposite activities on the receptors, acting as agonists in GPR18 and as antagonists in GPR55. Further studies proceeded with all the compounds, selective or not for GPR18. Similarly to the screening for GPR18, a negative control should have been used.

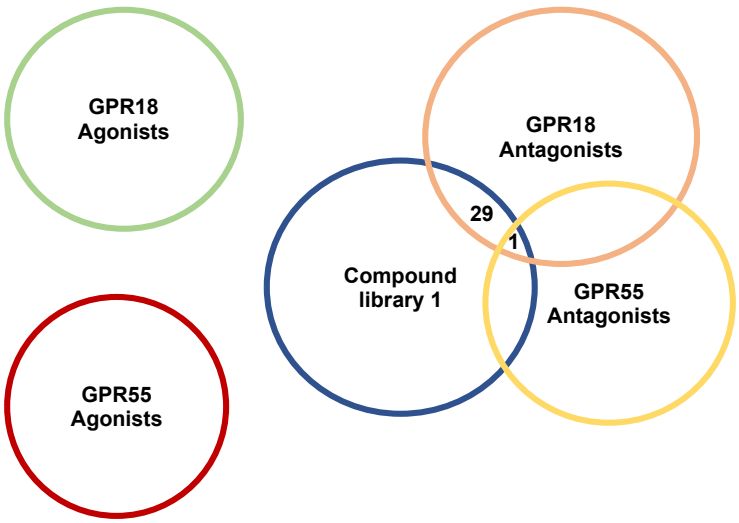


Figure 23:Representation of the number of compounds from compound library 1 vs type of modulation activity on GPR18 and GPR55. None of the compounds from this library showed to act as agonists on GPR18 or on GPR55. Three compounds revealed antagonist activity on GPR18, although one of these compounds also act as antagonist on GPR55.

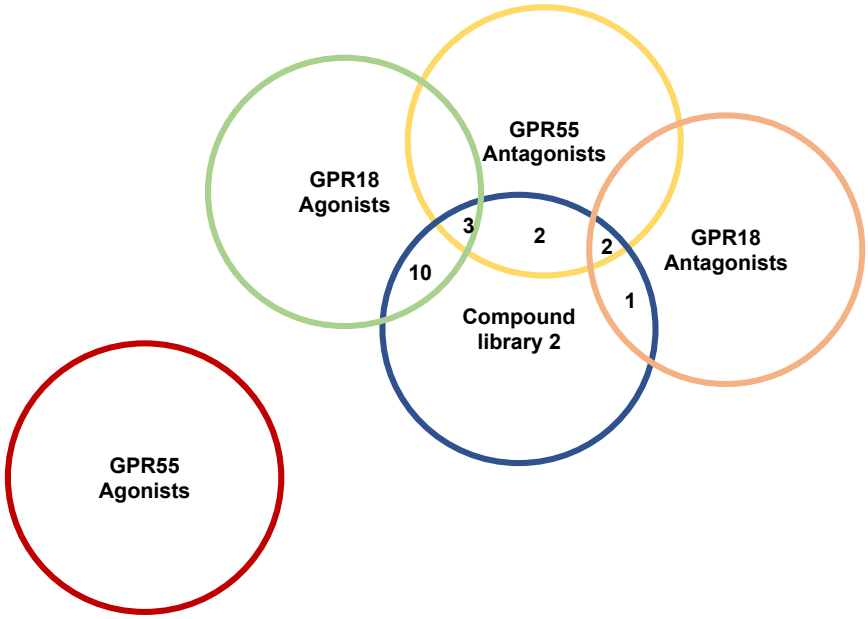


Figure 24: Representation of the number of compounds from compound library 2 vs type of modulation activity on GPR18 and GPR55. Thirteen of the compounds from this library activated GPR18, but only ten compounds were able to selectively activate GPR18, while the other three also act as GPR55 antagonists. Three of the compounds showed to be able to inhibit GPR18, however, only one of these compounds inhibit in a selective way this receptor.

Finally, the agonist and antagonist potencies of the compounds on GPR18 were evaluated, in order to obtain a dose-response curves and to determine EC_{50} (μM) and IC_{50} (μM) for each compound. Thereby, using once again β-arrestin recruitment assays, cells were treated with increasing concentrations of each compound, in the absence or presence of a known agonist. Analysing the results for compound library 1, it is possible to observe that compound 3 was the compound that showed the higher inhibitory potency, while compound 34 showed to be the less potent compound. Only compound 1, compound 3, compound 4 and compound 7 showed a percentage of maximal inhibition higher than 50%. However, it would be interesting to perform studies with more and lower concentrations of compound 1, compound 3, compound 4 and compound 7, in order to obtain a better defined dose-response curve and analysing the potency more accurately. The compounds from compound library 2 with agonist activity seem to be more promising, with more compounds showing high potencies. The results of agonist potency evaluation show that compound 56, compound 60, compound 61, compound 65 and compound 69 are the compounds that revealed the highest potency, being compound 56 the one that conjugate the lowest EC_{50} and the highest percentage of maximal activation. Though, the study of antagonist potencies showed that compound 57, 59 and 72 are not potent antagonists.

The antagonist potency on GPR55 was also evaluated for compound library 2 that showed antagonist activity on this receptor, however, none of the compounds showed to be potent antagonists.

Examining transversely the results obtained with compound library 1 and 2, of all the compounds tested for GPR18 the one that showed to be the most promising

molecule was compound 56, presenting selectivity for GPR18 and a high potency. So, compound 56 should be a focus of more and deeper studies and its' molecular structure should be a starting point for the development of other compounds.

Similarly of what was performed for GPR18, to study new compounds targeting CB2 that may have therapeutic potential, the first step was the screening of a series of compounds called compound library 3. Using radioligand binding assays, previous studies of our lab identified 8 compounds with capacity to bind to CB2. So, the next step was the screening of the agonist or antagonist activity of these compounds, using cAMP accumulation assays. The screening results revealed that none of the compounds from compound library 3 tested had antagonist activity on CB2, while seven of them presented agonist activity (Fig. 25). We decided to continue the studies with the compounds that led to a receptor activation higher than 50%.

The second step was the evaluation of the agonist potency of these seven compounds, using cAMP accumulation assays once again. Analysing the results, it was possible to observe that all the compounds from compound library 3 tested were potent on CB2, having shown a maximal inhibition near to 100% and a Ki lower than 1 μM, and the compounds that showed, simultaneously, the highest maximal inhibition and the lowest Ki values were compound 76 and compound 74.

Lastly, the selectivity of compound library 3 for CB2 was assessed by studying their binding capacity to CB1, followed by the screening of agonist activity and the potency study of these compounds, using radioligand binding assays and cAMP accumulation assays, respectively. The results showed that compound 73, compound 76 and compound 79 did not bind to CB1, being selective for CB2, while four compounds showed to act as non-potent agonists on CB1, not showing selectivity for CB2.

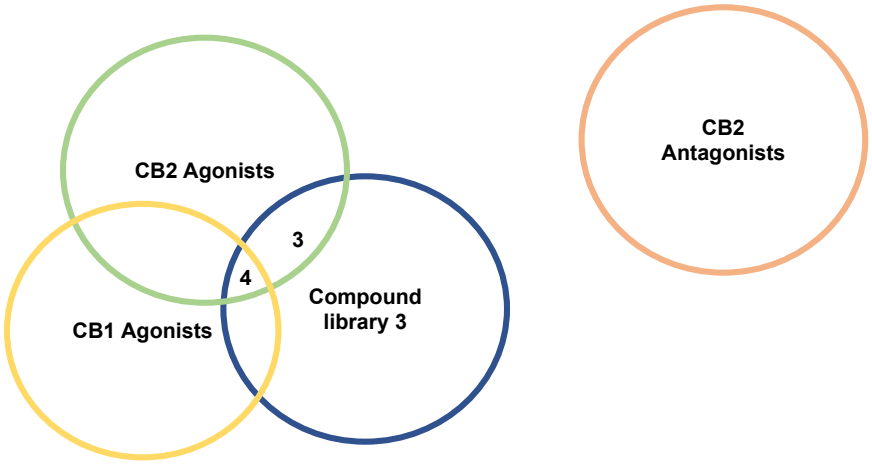


Figure 25: Representation of the number of compounds from compound library 3 vs type of modulation activity on CB2 and CB1. None of the compounds showed to have antagonist activity on CB2, while only three compounds revealed to selectively activate CB2.

Analysing the results obtain with compound library 3, compound 76 showed to be the compound with the best potential to agonizing CB2, being selective and a potent agonist for this receptor.

For all screenings with compound library 3, it would be interesting to use a negative control, in order to compare the activity between the different compounds. The antagonist test on CB1 should also be performed, but because of time constrain that was not possible to do.

The fact that the chemical structures of these compounds is under confidential agreement, did not make it possible to establish a relation between the molecular modifications in each structure and the effect on the receptors' activity, in order to identify important functional groups in this modulation. Possibly, receptor selectivity or agonist/antagonist activity are achieved by specific functional groups. It would be interesting to see if compounds 76 and 74 from compound library 3, have similar molecular structures since they are both potent agonists on CB2. It would also be interesting to compare compound 56 molecular structure with NAGly and RvD2 to study eventual molecular similarities.

At this point, a lot of questions need to be answered and more studies need to be performed. As mentioned before, the identification of functional groups involved in the receptors' activity is essential. It would also be interesting to analyse changes in receptors' gene expression in the presence of these compounds, which signalling pathway are being activated, as well as the conformational state of the receptor ideal to the binding of the compound. More selectivity studies, using other cannabinoid receptor-like GPCR, such as GPR119, and also cannabinoid receptors, should be performed, in order to achieve more selective therapies.

In the past few years, we have been observing a remarkable progress in GPRC drug discovery, and the direction that the future research is taking in this area looks promising. The advances in crystallography, molecular modelling, protein engineering and biophysical techniques, allowed the discovery of compounds targeting GPCRs and their structure-based optimization. The future of the development of novel therapeutic approaches targeting GPCRs, including CB1, CB2, GPR18 and GPR55, points towards the improvement and innovation of drug design techniques, allowing more accurate studies of molecular structures and of signalling dynamic of the receptors. Biased ligands, allosteric modulators and monoclonal antibodies are becoming an attractive therapeutic strategy, aiming the development of more selective and effective ligands (2,8).

In summary, our preliminary results demonstrate that, within the set of compounds tested, compound 56 is the most promising antagonist for GPR18, while compound 76 seem to be a promising molecule targeting CB2, acting as a potent agonist. In the future, more studies need to be done to understand how these compounds modulate these receptors' activity and to optimize their activity, with the goal of developing new molecules that could be used as a therapeutic approach to several diseases related with disfunctions in CB1, CB2, GPR18 and GPR55.

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