MOLECULAR CONTROL OF THE δ-OPIOID RECEPTOR SIGNALING AND FUNCTIONAL SELECTIVITY BY SODIUM

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ABSTRACT

Accumulating evidence suggests a prominent role of the arrestin-dependent signaling pathway in triggering most of the deleterious side effects observed using δ-OR targeting drugs. Numerous small molecules targeting the δ-OR receptors have been developed but their pharmacological properties, including their functional selectivity, have been poorly characterized. The absence of functionally selective opioid drugs, and the lack of knowledge of the pharmacological profile and signaling properties of the δ-OR receptor, limits its therapeutic exploitation. The development of functionally selective modulator toward the canonical G protein pathway could importantly increase the therapeutic potential of this receptor while decreasing its deleterious effects. An approach to fine-tune the functional selectivity of a GPCR is by using allosteric modulators. These allosteric modulators would reduce problems associated with drugs targeting the orthosteric site by not chronically activating the receptor. The overall goal of the proposed research is to study the molecular mechanism by which sodium-channel inhibitors allosterically regulates the delta opioid receptor (δ-OR) signaling and functional selectivity. Additionally, the signaling features of the δ-OR signal transduction triggered by biased receptor activation have been investigated. A combination of approaches, including functional studies, molecular modeling and mutagenesis, were used to study the general mechanism underlying the activation and tuning of the δ-OR signal transduction behavior. Thus, this work suggests the druggability of the allosteric sodium pocket by using sodium channel inhibitors. The current research represent discovery of two different allosteric profiles for the β-arrestin recruitment and one allosteric profile for the G-protein pathway at activated DOR and would serve as scaffold for further refinement of modulators with the desired pharmacological profile.

ACKNOWLEDGEMENTS

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Last but not least, I would like to dedicate this thesis to the best mother in the world, my mother, Mastora and to the spirit of my father, Saad. I would also like to thank my siblings, particularly my brother, Nasrallah, and my sister Naima for all their support and advices. Finally, I would like to thank the government of my country, Libya, for largely contributing in my study expenses by awarding me a generous scholarship.

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ABBREVIATIONS

(A_{2A}-AR): Adenosine receptor 2A

μ-OR/MOR: Mu-opioid receptor

7TM: Seven transmembrane helices

Ago-PAM: Agonist-PAM activity

Arg/R: Arginine

ASIC3: Acid-sensing ion channel 3

Asn/N: Asparagine

Asp/D: Aspartic acid

ATP: Adenosine triphosphate

BCS: Bovine calf serum

cAMP: Cyclic adenosine monophosphate

CXCR4: C-X-C chemokine receptor type 4

Cys /C: Cysteine

D3: Dopamine receptor

DADLE: [D-Ala^2, D-Leu^5] enkephalin

DAG: Diacylglycerol

DMEM: Dulbecco's modified Eagle's medium

DOR/δ-OR: Delta-opioid receptor

ECL1, ECL2, and ECL3: Extracellular loops 1, 2 and 3

ENaCs: Epithelial sodium channels

FBS: Fetal Bovine Serum

GABA: Gamma-Aminobutyric acid

$GABA_B$: Metabotropic transmembrane receptor for gamma-aminobutyrate

GDP: Guanosine diphosphate

GIRKs: G protein-coupled inwardly-rectifying potassium channels

Glu/E: Glutamic acid

GPCRs: G-protein coupled receptors

GRKs: G-protein-coupled receptors kinases

GTP: Guanosine triphosphate

H_2 /H_1: Histamine receptor

HEK293T: Human Embryonic Kidney 293T cell line

ICL1, ICL2, and ICL3: Intracellular loops 1, 2 and 3

IP_3: Inositol -1,4,5-trisphosphate

KOR/κ-OR: (kappa) for *K*etocyclazocine receptor

Leu/L: Leucine

M2: Muscarinic receptor 2

NAM: Negative allosteric modulator

NHE1: Na-H exporter

NOP/NOR: Nociception/orphanin FQ peptide receptor

PAM: positive allosteric modulator

PAR1: Protease-activated receptor 1

PIP_2: 4,5-biphosphate

PKA: Protein kinase A

PKC: Protein kinase C

Pro/P: proline

R*: Active state of the receptor

R: Inactive state of the receptor

RLU: Relative Luminescence Unit

SAR: Structure-activity-relationship

TM: Transmembrane domains or transmembrane region

Trp/W: Tryptophan

Val/V: Valine

YFP: Yellow fluorescent protein

α1: Alpha-1 adrenergic receptor

α2: Alpha-2 adrenergic receptor

β-AR: β-adrenergic receptor

β-arr1: β-arrestin1

β-arr2: β-arrestin2

Chapter1: General Introduction and Literature Review

1.1 Preface

Delta-opioid receptors (δ-OR/ DOR) belong to class A G-protein-coupled receptors (GPCRs) family, which is targeted by more than 30% of prescribed medications. These receptors have an important role in pain management but were also found to have an antidepressive activity and a potential for the treatment of spasm associated with Parkinson's disease. However, the clinical use of δ-OR agonists is limited due to the generation of potentially life-threating side effects such as tolerance, constipation, dependence, convulsions, and seizures [1]. GPCRs transduce extracellular stimuli into intracellular outcomes through two main mechanisms; 1- G-protein dependent pathway, which facilitates a change in the concentration of an intracellular second messenger via the activation of the heterotrimeric G-protein. 2- G-protein independent mechanism where the receptor signaling is attenuated by phosphorylation and receptor internalization, both dependent on the initial recruitment of the β-arrestin protein adaptor.

GPCR activation is tightly controlled through intramolecular determinants serving as intrinsic locks or switches. Those motifs are found to be highly conserved within GPCRs, especially within the class A family. Recent studies have revealed the presence of a highly conserved cavity as an allosteric binding site for sodium ion and water molecules cluster in the middle of the 7TM bundle of most class A GPCRs including the δ-OR [2]. The suggested functional importance of the allosteric pocket in receptor activation and its exceptionally high conservation in class A GPCRs makes

it an attractive target for discovery of small molecules with unique functional and pharmacological properties, which can then be used to test our hypotheses and serve as a novel starting point for drug discovery. Although most drugs target GPCRs' orthosteric site, exploration of allosteric binding sites provides several advantages. For instance, it would allow more precise control of subtype, functional selectivity and preserves spatiotemporal profile of endogenous signaling, which holds great potential for the development of new tool compounds and candidate drugs [3, 4]. Some of the initial insights into the allosteric Na^+ pocket have been obtained with amiloride and its derivatives as non-selective modulators of GPCRs. Amiloride was discovered as a potent inhibitor of epithelial sodium channels and Na^+/H^+ exchangers [5]. Since then, a few derivatives have shown comparable potency in some receptors [6]. The stabilizing effect of amiloride suggests that broadly selective allosteric ligands targeting this pocket may have a basic utility for conformational stabilization of GPCRs for use in crystallization, and in biochemical assay development targeting orphan GPCRs. At the same time, extending highly selective orthosteric compounds to target the conserved allosteric pocket establishes a platform for the design of bitopic ligands that combine subtype selectivity with signaling properties, useful for tool compounds and therapeutic indications [7]. As our latest data reveal a key role of this Na^+ site as an "efficacy switch" in δ-OR [2], targeting it with allosteric or bitopic moieties would be useful for probing biased signaling mechanisms and potentially leads to a rational platform for biased ligand design. However, low affinity and selectivity of amiloride at GPCRs and the lack of molecular and structural information hindered the systematic study of the allosteric ligands. Here, my research project aims to use pharmacological and biochemical approaches to perform structure-activity-relationship (SAR) of small

molecule drugs targeting the sodium site with the goal to gain more information on the sodium site functionality as well as to study the molecular recognition mechanisms of these molecules. My research represents a first step toward the exploitation of this cavity for drug discovery.

1.2 Biological Significance and Functions of GPCRs

GPCRs play a critical role in human physiological processes such as immune regulation, sympathetic and parasympathetic nervous functions, metabolism, reproduction, and hormone responses. Also, they are known to have a role in taste, smell and vision [8]. Importantly, GPCRs are vital targets for several pathological processes such as cardiovascular disorders, inflammatory diseases, cancer and monogenic diseases. Furthermore, the cell membrane location of GPCRs and the variety of their tissue expression make GPCRs ideal targets for drug discovery. Consequently, 30-40% of prescribed drugs target GPCRs, and there is still a growing interest in GPCRs both within the pharmaceutical industry and academia [9, 10]. Moreover, GPCRs are activated by different ligand messengers such as calcium ions, hormones, neurotransmitters, and chemokines, as well as many GPCRs are activated by other substances such as odorants or photons [11].

1.3 Classification of GPCRs Superfamily

The human genome has more than 800 GPCRs, which are classified into four families based on sequence similarity within the seven transmembrane domains (TM) as show in **Figure 1.1**. Hence, the largest group is Rhodopsin family (class A) which comprises more than 700 members, including hormones, neurotransmitters and light receptors. Since it is the largest family, it is divided into four subgroups; the α-group,

the δ-group, the γ-group, and the β-group, also these groups are further divided into subfamilies or clusters. For example, adrenergic receptors and opioid receptors are in the γ-group and about 388 Olfactory receptors are in the δ-group [12, 13].

The second GPCR family is Secretin and Adhesion family (Class B), which consists of 24 members of Adhesion receptors and 12 Secretin receptors. All members are coupled to G-protein $G\alpha_s$ which activates adenylyl cyclase. Receptors for the parathyroid hormone, calcitonin, and some receptors for gastrointestinal peptide hormone-like glucagon and secretin are examples of some class B GPCR members [8].

The smallest GPCR family is the Glutamate family (Class C) which contains only 15 receptors. For instance, the $GABA_B$ receptor and metabotropic glutamate receptor are part of this family. All members of class C possess a large extracellular N-terminus which could be important for receptor activation and function. Finally, the Frizzled/TASZ family which is also relatively small comprises 24 members [11, 12].

On the other side, there are a lot of GPCRs with unknown endogenous ligands which are referred to as orphan GPCRs. Consequently, the physiological function of these orphan GPCRs remains still unknown, which makes them excellent candidates for the pharmaceutical industry and deorphanization by GPCRs scientists. Thus, the numbers of orphan GPCRs are now diminishing but at a very low rate [14, 15].

Figure 1.1

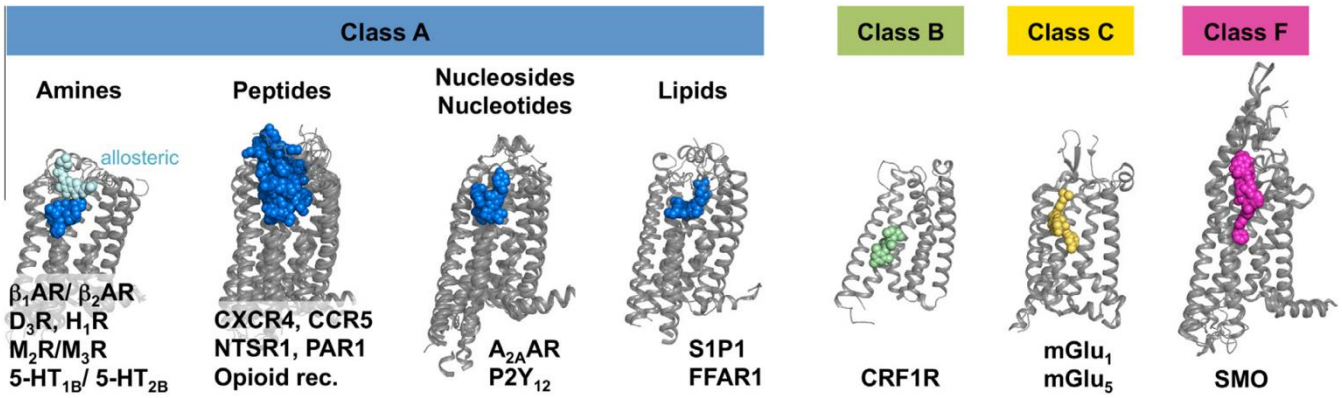

Class A
Amines
Peptides
Nucleosides
Nucleotides
Lipids
Class B
Class C
Class F
allosteric
β1AR/ β2AR
D3R, H1R
M2R/M3R
5-HT1B/ 5-HT2B
CXCR4, CCR5
NTSR1, PAR1
Opioid rec.
A2AAR
P2Y12
S1P1
FFAR1
CRF1R
mGlu1
mGlu5
SMO

Figure 1.1: Diagram of human G-protein-coupled receptor (GPCR) superfamily.

Overview of GPCR subfamily and ligand binding pocket within the helical bundle. The delta-opioid receptor, the focus of this thesis, is part of the peptide class A family [16].

1.4 Structural Features of GPCRs Superfamily

The past decade has witnessed spectacular progress within our understanding of GPCR structure and function, especially due to the progress in structural biology. We now have growing information in this field which is not only important for understanding the receptor mechanism of action and function, but is also an important key to discover new drugs which are more selective for the treatment of several diseases [17]. The first crystal structure of the rhodopsin was reported in 2000 [18], followed seven years later by the structure of the β2-adrenergic receptor [19], both bound to antagonist ligands. Since then, several other GPCRs were crystallized; for instance, the β1-adrenergic receptor [20] and A2A-adenosine receptor [21], both were solved in 2008. By 2012, the chemokine receptor CXCR4 [22], dopamine D3 receptor [23], histamine H1 receptor [24], M2 muscarinic receptor [25], kappa opioid receptor [26], and mu-opioid receptor [27] were solved with antagonist or inverse agonist ligands. These receptors were stabilized by ligand-binding and some biochemical methods, involving truncations, stabilizing mutations, binding of Fab fragments, and protein fusions [28].

As a result, these studies found similarities and diversities in GPCRs structure between the four main families, whereas all GPCRs share the same common structure that can be separated into three main regions. The extracellular part which contains the three extracellular loops that are usually symbolized as ECL1, ECL2, and ECL3 [29]. Also, the amino-terminus (N-terminus) which is responsible for the variety between the GPCRs families, it consists of a different length of amino acid sequences. It could either have a short amino acid sequence, ranging from 10 to 50 amino acids, such as in peptide and monoamine receptors, or a large amino-terminal domain which

includes about 350 to 600 amino acids, like in GPCR class C (glutamate family). Glutamate family possesses a large N-terminal domain because it has the bilobed structure (flytrap) which forms the ligand binding site based on X-ray crystallography studies. Indeed, the biggest N-terminus is found in adhesion family class B which is characterized by a common fold that seems to be important for receptor activation and ligand binding [30, 31]. The transmembrane region (TM) contains seven α-helices ranking from TM1 to TM7 (**Figure 1.2)**, and it has been known as the most conserved component of GPCRs [32]. This latter region binds and transfers the information from the external part of the cell to its intracellular region, and it is implicated in forming the different ligand binding sites [17]. Finally, the intracellular part consists of three intracellular loops coined as ICL1, ICL2, and ICL3 and the Carboxyl group (C-terminus). The C-terminus domain seems to be important in transducing the downstream signaling pathways by binding to a diversity of effectors, for instance, β-arrestins, G-protein-coupled receptor kinases, and others [33, 34].

On the other side, these similarities in GPCRs structures are conflicting with the great variety of natural ligands that transduce the GPCRs extracellular signals. This diversity of endogenous ligands is ranging from smallest substances like photons, ions, and moving to small organic molecules, then larger ligands like peptides and proteins. These ligands have a well known binding site for most of GPCRs classes. For example, most proteins and peptides bind with the N-terminus and some sequences of TM segments, while smaller ligands such as small organic molecule agonists can interact with the TM parts [35].

Figure 1.2

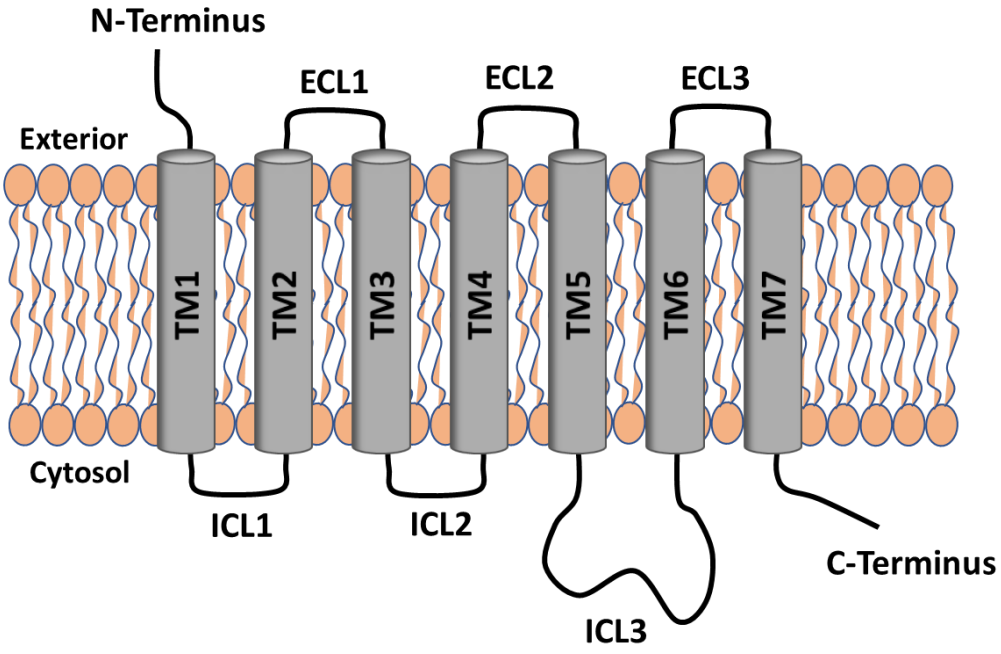

N-Terminus
ECL1
ECL2
ECL3
Exterior
TM1
TM2
TM3
TM4
TM5
TM6
TM7
Cytosol
ICL1
ICL2
ICL3
C-Terminus

Figure 1.2: GPCRs common topology.

All GPCRs share a common structure of seven transmembrane helices (7TM). These 7TM are linked together by three extracellular loops and three intracellular loops. Also, they are characterized by an extracellular N-terminus and an intracellular C-terminus.

In the light of these recent GPCRs structural studies, four motifs were found to play an important role in the receptor activation by agonists (**Figure 1.3**). The most conserved motifs are; D[E]R$^{3.50}$ Y (the superscript number which is above the Arginine residue represents numbering using the Ballesteros Weinstein nomenclature [36]) is located in helix III. In term of its function, it is a part of so-called ‘‘ionic lock’’ by forming a salt bridge with D/E$^{6.30}$ in helix VI. This ionic lock is noticeable in an inactive state of Rhodopsin family receptors blocking the G-protein binding at this intracellular region [37]. The second most conserved GPCRs motif is CW$^{6.48}$xP located in helix VI. The Trp residue of CWxP motif undergoes a conformation transition that constraints the receptor in a specific conformation. It is also known as a microswitch or toggle switch with a distinct orientation between the active and inactive state of the receptor. In addition to the above two conserved motifs, the interaction of the Asn of the NP$^{7.50}$xxY motif in helix VII with either residues located in helix VI as well as the aromatic-aromatic interaction between the Tyr of NP$^{7.50}$xxY with an aromatic residue within the intracellular helix VIII also contributed to constraint the receptor in a specific state [38].

Figure 1.3

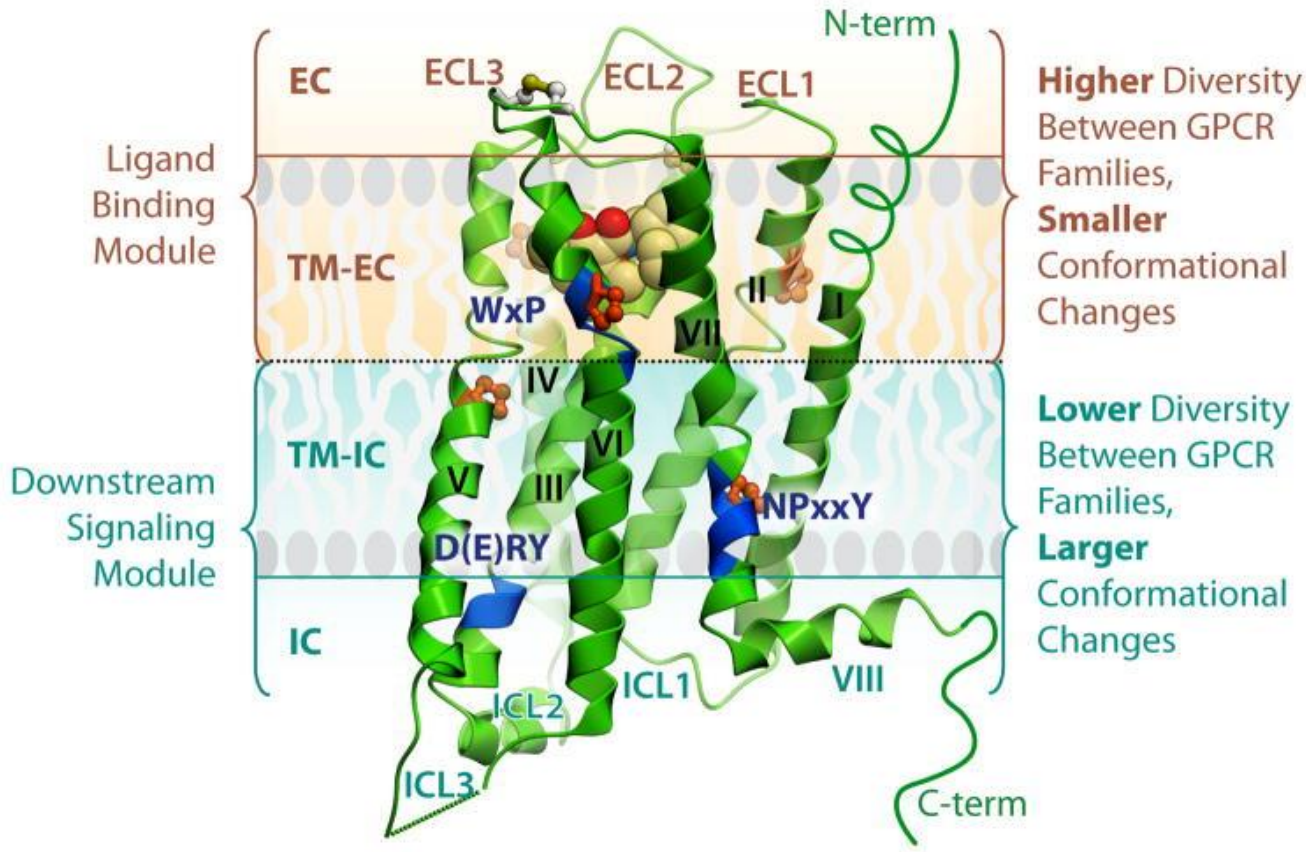

N-term
EC
ECL3
ECL2
ECL1
Ligand Binding Module
TM-EC
WxP
II
I
VII
IV
TM-IC
VI
III
NPxxY
D(E)RY
Downstream Signaling Module
IC
ICL1
VIII
ICL2
ICL3
C-term
Higher Diversity Between GPCR Families, Smaller Conformational Changes
Lower Diversity Between GPCR Families, Larger Conformational Changes

Figure 1.3: Molecular signatures of GPCRs shown on D3R (PDB ID 3PBL) crystal structure. The extracellular region (EC) contains three extracellular loops (ECL) and the N-terminal. The N-terminal domain is presented as a short domain consisting of the unstructured peptide as in a majority of the receptors in class A. The middle region consists of 7 transmembrane alpha helices and contains the most conserved GPCRs motifs D[E]$R^{3.50}$ Y, $W^{6.48}$XP, and $NP^{7.50}$xxY (shown in blue ribbon patches). Also, some of the prolines (represented in orange color) are found in 7TM region, which divides the receptor into two modules. The EC module (TM-EC with EC) is central for ligand binding and has higher diversity among GPCRs families. On the contrary, the intracellular (IC) module contains IC with some of TM regions, which contribute to downstream signaling, and has a lower diversity between GPCRs. In comparison with EC module, the IC module undergoes to large conformation changes during the receptor activation. Finally, the C terminal consists of helix VIII -when present-, and it is diverse in length between different families of GPCRs [34].

1.5 Dynamic and activation of G-protein Coupled Receptor

GPCRs are extremely dynamic macromolecules that transit between numerous conformations from the inactive state R, which can be stabilized by inverse agonist or antagonist, to active state R* which is stabilized by an agonist. The capability of the receptor to transit from the inactive to the active state in the absence of a ligand generates the constitutive or basal activity [39]. Therefore, in the absence of the ligand, the level of constitutive activity is defined by the dynamic equilibrium between the inactive R and active R* conformations of GPCR. The effect of the ligand on the functional properties and coupling of the receptor is known as efficacy. The efficacy of the ligands is responsible for altering the equilibrium between the two main GPCRs conformations [40]. The full agonist usually shifts the equilibrium towards the active state and stabilizes the R* while the inverse agonist binds to and shifts the equilibrium towards the inactive state R, which leads to a decrease in the level of basal activity. Partial agonist seems to have an affinity for both active R* and inactive state R. Its partial efficacy will thus depend on its preference for the active R* state when compared to a full agonist that solely binds to the R* state. The neutral antagonist does not affect the basal equilibrium; therefore, has no efficacy, as it binds to both active and inactive conformations where its main role is to compete with other ligands that bind to the same site [41].

The interaction of the ligand with the receptor causes large conformational changes through all regions of the receptor. The receptor will undergo a series of conformational changes associated with the transition from inactive state to intermediate-active state then from intermediate-active state to an active state [42]. It

is now clear that receptor activation cannot be anymore explained using a simple bimodal switch system (i.e., on-off).

Many structural insights were discovered using biochemical, pharmacological and structural approaches. For example, the common rotational movement of the conserved microswitches, characterizing more than 85 % of the class A GPCRs, were found to be hallmarks of receptor activation [12, 43]. The IC3 is important for G-protein coupling and this site is thus well positioned to detect agonist-induced conformation changes relevant to G protein activation. Also, the TM3, TM5, and TM6 contain important residues involved in the binding of the agonist.

Besides to the transition of the rotamer toggle switch, the disruption of the ionic lock is another molecular switch that occurs upon the receptor activation. The ionic lock is usually formed by the interaction between the Asp130 $^{3.49}$/ Arg131$^{3.50}$ from the D[E]R$^{3.50}$ Y motif which is located at the cytoplasmic end of TM3 and Glu268$^{6.30}$ at TM6, which is common in most class A GPCRs. In general, most agonists are capable of disrupting this ionic lock during the receptor activation [39]. This broken ionic lock is considered one of the common changes that happen upon receptor activation. Importantly, there is also a common rearrangement of transmembrane helices coupled with receptor activation. For instance, the movement of TM helices that are responsible for opening the helical bundle at the intracellular region to allow G-protein recruitment. This rearrangement occurs by outward movement of helices V and VI and inward movement of helix VII [17].

1.6 GPCR Signaling

Despite the diversity within GPCR family at the structural and functional level, they possess a similar signal transduction mechanism which involves a change in the conformation of the seven-transmembrane helix upon agonist binding that allosterically activated the bound G-protein [10, 12]. GPCR characteristically transduces the signal through activation of at least one member of the guanine nucleotide binding protein (Gα protein) family, leading to their name G protein-coupled receptor (GPCR). The intracellular signals transduced by these heptahelical proteins are themselves tightly controlled by the heterotrimer Gαβγ subunits, as well as a growing array of regulatory and accessory proteins. Once activated by an extracellular trigger, the GPCR undergoes a conformational change that allosterically activated the bound heterotrimeric G-protein leading to its dissociation. These three subunits were purified for the first time in 1971 by Martin Rodbell [44]. After the binding of the agonist, the receptor undergoes conformational changes, allowing GDP to be exchanged by GTP on the Gα subunit, and having the receptor act as a guanine nucleotide exchange factor. The activated Gα dissociates from the tightly bound Gβγ dimer [45] [46]. This dissociation is manifested by an increase or decrease in the level of second messengers like cAMP, calcium, or a change in the ionic channel functions. G-proteins are usually referred by their type of α subunits. There are 16 different Gα subunits which can be divided into four main classes based on their function **(Figure 1.4)**. 1- $G_{\alpha s}$-proteins cause the stimulation of the adenylyl cyclase, which in turn increases the production of cAMP from ATP within the cell. This induces cAMP-dependent pathways which cause activation of protein kinase A (PKA) among others.

PKA is responsible for phosphorylating several downstream targets. 2- The $G_{\alpha i/o}$ -proteins inhibit the adenylyl cyclase which leads to the reduction of cAMP within the cell. 3- The $G_{\alpha q/11}$ proteins cause the activation of the phospholipase Cβ (PLC_{β}), which is responsible of cleaving phosphatidylinositol 4,5-biphosphate (PIP_2) into two second messengers, diacylglycerol (DAG) and inositol -1,4,5-trisphosphate (IP_3). Hence, these two second messengers lead to protein kinase activation and the release of calcium, respectively. 4- The $G_{\alpha 12/13}$ protein causes the activation of Rho Guanine-Nucleotide Exchange Factors (GEFs) which are important for cell migration by regulating the actin cytoskeleton [47]. The Gβγ heterodimer can be made from the combination of 5 different Gβ subunits with one of the 12 Gγ subunits increasing the heterogeneity of heterotrimeric signaling complex. While the main role of the Gβγ dimer is to act as a guanine-dissociation inhibitor (GDI) and thus stabilizes the inactive form of the heterotrimer, it also activates its own effectors such as G-protein-coupled inwardly-rectifying potassium channels (GIRKs), phospholipase C β, and lipid kinases PI3K and many more [15]. To complete the G-protein cycle of activation, the Gα subunit possesses an intrinsic GTPase activity that hydrolyzes the GTP back to GDP leading to the reassociation with the Gβγ [48] .

Figure 1.4

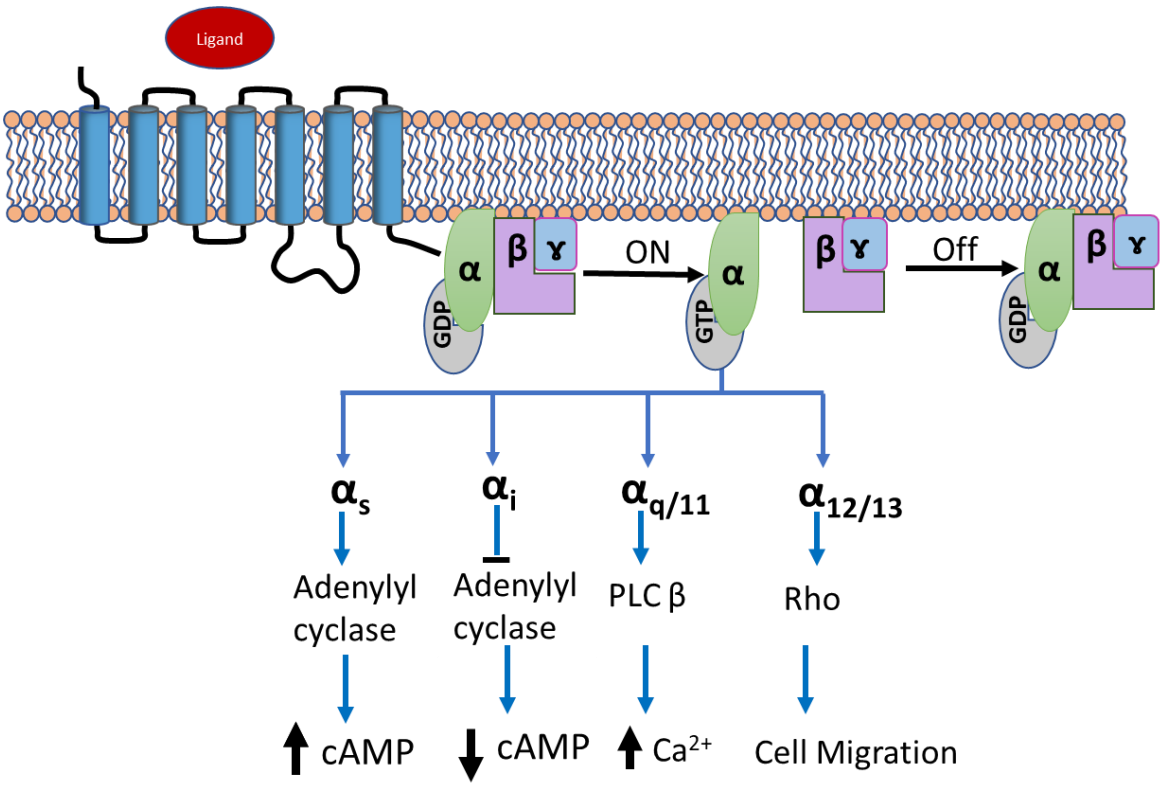

Ligand
α
β
γ
GDP
ON
GTP
Off
α_s
α_i
$\alpha_{q/11}$
$\alpha_{12/13}$
Adenylyl cyclase
Adenylyl cyclase
PLC β
Rho
cAMP
cAMP
Ca^{2+}
Cell Migration

Figure 1.4: Subtypes of Gα subunits that couple with G-protein-coupled receptors and associated effectors. The figure shows examples of the main four types of the 16 types of Gα subunits that mediate different downstream cascades by different second messengers, thus causing diversity in the physiological responses within the cell.

1.7 Regulation of Signaling and G-protein independent signaling pathways

GPCR signaling is tightly controlled in terms of amplitude and duration by different processes such as desensitization, internalization, and down-regulation. These processes are mainly mediated through the recruitment of a cytosolic protein called β-arrestin to the activated receptor. Binding of β-arrestin to the activated receptor prevents overstimulation by blocking the re-coupling with G protein. This process is called desensitization. It is still unclear whether β-arrestin is a real competitor of G-protein. While both proteins share a common binding site within the receptors, overexpression of neither β-arrestin nor G-protein seems to negatively affect the other pathway, respectively. It is thus possible that both proteins interact with a state-specific receptor that excludes the other.

The recruitment of β-arrestin to the receptor is a multistep process that is still unclear and could be different from receptor to receptor. In general, it is accepted that β-arrestin can be recruited to the non-phosphorylated receptor mostly via interaction with the intracellular loop 3 (ICL3). This interaction could mediate receptor desensitization as discussed above. When the receptor is phosphorylated at the C-terminal tail and in some receptors, at the ICL3, the polar interaction of the phosphorylated receptor and the β-arrestin induces a conformation change within the β-arrestin. This activated β-arrestin releases its C-terminal domain, which bridges the receptor complex to the clathrin endocytosis machinery. Phosphorylation of the receptor at serine and threonine residues is accomplished by different types of kinases such as G-protein-coupled receptors kinases (GRKs), protein kinase C (PKC) or protein kinase A (PKA) using a G-protein dependent or independent mechanism

[49]. After internalization, the receptor can be either degraded in the lysosomes (down-regulation) or dephosphorylated and recycled back to the cell membrane depending on the ‘’strength’’ of the interaction between the GPCR and β-arrestin (**Figure 1.5).**

To date, four different human arrestin genes have been cloned. Two of them are found in the retina (cone arrestin and visual arrestin) and are refered to as arrestin-1 and arrestin-4, respectively, while the other two are non-visual arrestins and refered to β-arrestin-1 (arrestin-2), and β-arrestin-2 (arrestin-3) which are broadly distributed in other tissues [50]. It has been reported that β-arrestin can interacts with the C-terminal tail or the ICL3 of GPCRs. For instance, a study at the DOR showed that mutation of serine or threonine within the C-terminal tail confers selectivity for β-arrestin-2 vs β-arrestin-1 without affecting the stability of the complex and the fate of the internalized receptor [51]. Another study showed that the phosphorylation controls receptor trafficking by regulating an internal brake, thus removing this brake by C-terminal truncation, allows recruitment of β-arrestin and internalization solely via the recruitment by the ICL3 [52]. In term of DOR internalization, the fate of DOR internalization can be regulated by GRK2 and receptor phosphorylation as well as the type of β-arrestin isoforms. Thus, there are two different DOR internalization pathways: 1-Internalization involving GRK2 receptor phosphorylation dependent mechanism mediated by both β-arrestin-1 and β-arrestin-2 which will lead to DOR recycling. 2-Internalization of the receptor through phosphorylation independent mechanism mediated by β-arrestin-2 lead to receptor degradation [53].

Figure 1.5

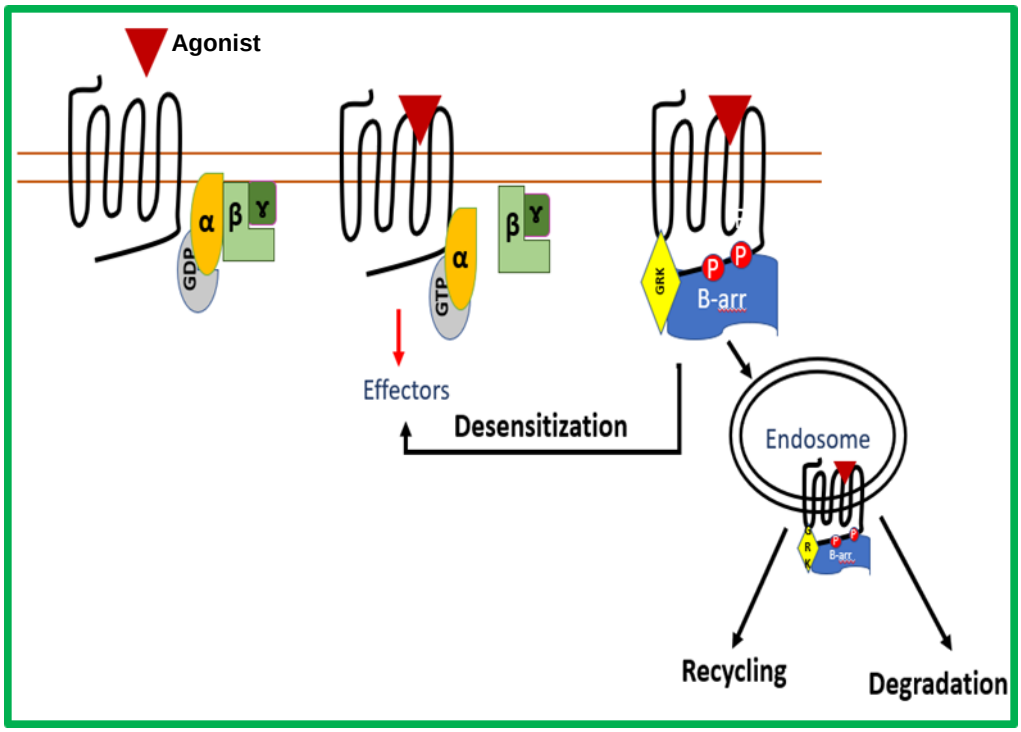

Agonist
α
β
γ
GDP
GTP
GRK
P
B-arr
Effectors
Desensitization
Endosome
Recycling
Degradation

Figure 1.5: G-protein-coupled receptor trafficking.

A very important player in GPCR regulation is the adaptor protein arrestin, once recruited at the activated receptor it terminates the signaling from G protein. Another important function of arrestin is to bridge the receptor with the clathrin-coated pits machinery leading to the receptor internalization. The internalized receptor can be degraded by fusion of the endosome with the lysosome or recycled back to the cell membrane. It is not clear what controls the fate of the internalized receptor but, the affinity of the receptor-arrestin complex seems to have an important role. This affinity is controlled by the ligand identity but also by the phosphorylation of the receptor [54].

1.8 Opioid Receptors

Pain and noxious information are important mechanisms of protection from tissue damage within the body. Pain is a multidimensional system; it involves the sensory system that dictates the intensity and amplitude of the pain, the affective system that will judge the pleasantness of the noxious information and the memory-cognitive system that will evaluate the context of the pain or the trigger to avoid similar experiences in the future. One of the most studied and important physiological pain management systems acts via the release of endogenous opioids. These neuromodulators will moderate pain (pain-relieving) through their analgesic properties but also have an important role in the cognitive and affective system [55, 56]. Endogenous opioids act through the activation of the three classical opioid receptors (μ-, κ- and δ-OR) and the related nociception/orphanin FQ peptide receptor (NOP), all the four opioid receptors are part of GPCRs family [57-60]. The μ-OR or MOR ((mu) represents ***m***orphine which is the prototypical μ-opioid receptor agonist), mediates its actions through binding of endogenous peptides endomorphin-1 and -2. Mu-opioid receptors are located in the central and peripheral nervous system, where it is mainly involved in pain management. An example of its use is to inhibit the severe post-operative pain and cancer pain, as well as it is used for acute, chronic or neuropathic pain [61].

The κ-OR or KOR (κ (kappa) for ***K***etocyclazocine which stands for the first kappa opioid receptor agonist), acts through the opioid peptides dynorphins. Kappa agonists have anti-seizure properties and anti-nociception, but their medical use for pain management is limited due to the generation of dysphoria and hallucination.

The δ-OR or DOR (δ for *deferens* since it was first discovered in mouse *vas deferens*), have enkephalins as their classical endogenous opioid peptides [62]. Also, they can bind with different exogenous agonists such as [D-Ala^2, D-Leu^5] enkephalin (DADLE) while Naltrindole and Naltrinbene are the most common DOR antagonists. Intriguingly, other studies have also reported that the co-administration of the DOR antagonist Natrindole with MOR agonists (Morphine) can reduce the liability for dependence and addiction *in vivo* caused by morphine and its derivatives [62]. Pre-clinical assays showed that DOR agonists have profound anti-inflammatory activities and could also be used for the treatment of neurological and psychiatric diseases like depression. While not as effective as μ-OR agonists, DOR agonists can be used as chronic pain relievers, with some being investigated as treatments for migraine in clinical trials [1, 63, 64].

Finally, the nociceptin/orphanin FQ receptor (NOP) was cloned in 1994 and was found to have over ~60% sequence homology with classical opioid receptors, it showed weak response to the non-selective opiate agonist etorphine with no affinity for opioid peptides and morphinan [65]. Using reverse pharmacology, its ligand was discovered in 1995 and was coined nociceptin since it acts as a pro-nociception peptide and possesses potent anti-analgesic effects, even counteracting potent pain-killer such as morphine, this effect is normally referred to as allodynia and hyperalgesia [66-68].

Opioids are among the oldest and most prescribed and abused medications [69]. Different natural alkaloids (opiates) and small molecules acting at the natural ligand binding-site have been discovered [70-72]. Opioid receptors are among the most chemically annotated GPCRs, and all four members of this subfamily have

resolved crystal structures, including the inactive and active states for the kappa and mu opioid receptors. This has revealed unexpected insights in their structures. Despite its overall similarity (>60%), the non-classical NOP was found to have important structural rearrangements at the orthosteric site consistent with its divergent ligand selectivity and the absence of morphinan-based opioid acting at this receptor [12] [73]. Morphine-related drugs are the best characterized and the only family of molecules used at the clinical level. However, their weak selectivity and the divergent pharmacology behavior toward each member of the classical opioid receptor family (μ, κ and δ-OR) lead to numerous undesired effects limiting their use [74, 75]. All four members of the opioid subfamily have two conserved cysteine residues in their first and second extracellular loops which are involved in the formation of a disulfide bridge. The highest level of diversity among the four opioid members occurs within the extracellular loops and extracellular ends of the transmembrane segments. The function of these loops is covering the binding cavity which is formed by conserved inner transmembrane helices 3, 4, 5, 6, 7 [76, 77].

All opioid receptors are coupled to the inhibitory G-protein (Gαi) subunits. Thus, activation of the receptor leads to the inhibition of adenylate cyclase activity and consequently to the decrease of cAMP levels. In term of their location in the human body, they are expressed in the brain (as in cortex, thalamus, and periaqueductal gray), spinal cord, and some of them are present in the intestinal and respiratory tract within specific immune cells [1]. Therefore, activation of these receptors in the gastrointestinal tract inhibits peristaltic action which causes constipation, considered as one of the major side effects associated with the usage of opioid agonists as treatments.

1.9 δ-Opioid Receptor (DOR)

The work presented in this thesis is mainly focused on the δ-opioid receptor (DOR) subtype. DOR agonists have shown properties that are different from other classical opioid receptors MOR and KOR agonists as they are less effective as analgesics in acute pain, while having therapeutic potential for the management of chronic and inflammatory pain [78, 79]. Additionally, DOR agonists can be useful for mood disorders like depression. In this context, *in vivo* studies conducted by Drs. Konig and Filliol showed that the genetic deletion of the gene encoding the DOR, or its endogenous ligand lead to depression and anxiety phenotypes. Thus, the emotional behavior related to opioid system manipulation seems to specifically involve DOR since KOR and MOR did not show this phenotype when tested in knockout mice [61]. Therefore, these results suggested that the DOR agonists could be used not only as positive modulators for mood disorders, but also for the treatment of chronic pain diseases such as migraine since there is a high chance of comorbidity of migraine with anxiety and depression [80]. Moreover, DOR agonists have proven efficacy in treatment of spasms associated with Parkinson's disease, and likely as immunomodulators [81]. However, the clinical use of these DOR agonists have been limited because of generation of sides effects [82].

As for most GPCRs, activation of the DOR leads to stimulation of the G-protein dependent signaling pathway or other independent pathways as mentioned above. These intracellular signaling of the DOR are tightly regulated and terminated by desensitization process. In this context, recruitment of β-arrestin at the classical opioid receptor, including the DOR, was found to be responsible for some of the unwanted

adverse effects that are associated with drugs targeting those receptors [2]. For instance, β-arrestin recruitment modulates the DOR trafficking and internalization. Consequently, some receptors recycle rapidly while other receptors recycle slowly or do not recycle at all and are degraded. As a result, chronic activation of the receptor results in reduced number of active receptors at the cell membrane which leads to decrease in the maximal response and eventually generates one of the negative side effects; tolerance [1]. The discovery of low-internalizing agonist such as ARM00390 illustrated well the role of receptor internalization in the eventual generation of tolerance toward drugs that induce DOR internalization such as SNC80 [83, 84].

Similarly, the unwanted respiratory depression produced by classical opiates is strongly diminished in β-arrestin knockout mice models. These findings suggest that DOR agonists favoring G-protein signaling pathway over β-arrestin recruitment would produce a useful analgesia with less harmful secondary effects such as constipation and respiratory depression [85, 86]. This concept is now known as functional selectivity or bias signaling [4].

1.10 Type of Ligands

1.10.1 Orthosteric modulator of GPCRs

GPCRs are activated by two main types of ligands depending on the localization of their binding site. The first type of ligand, called orthosteric ligand, interacts with the same site as of the endogenous ligand. These ligands are subdivided based on their efficacy to trigger a specific pathway compared to the endogenous ligand; full agonist, superagonist, partial agonist, and inverse agonist

(Figure 1.6). Therefore, the full agonist usually activates the target GPCR with an efficacy similar to that of the natural ligand (100% efficacy), while the partial agonist has lower efficacy (<100%). On the other side, the neutral antagonist is a competing ligand and do not possess any intrinsic activity. It normally binds and stabilizes the inactive conformation. Finally, the inverse agonist is a drug that can bind to the active and inactive state of the receptor but will stabilize the inactive conformation. It is believed that inverse agonists could stabilize the uncoupled receptor but remains controversial at the cellular level. The constitutive or basal activity is a spontaneous activation of the receptor without any ligand bound; this activity is usually happening during the dynamic equilibrium between the active and inactive state of GPCRs [87, 88].

Figure 1.6

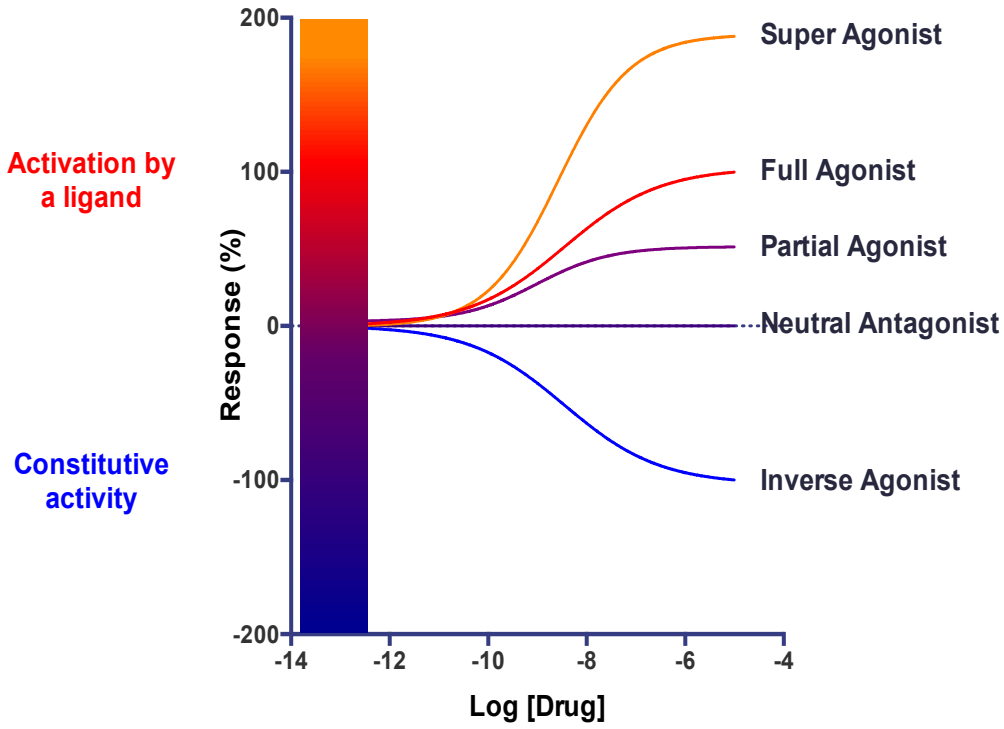

Activation by a ligand
Constitutive activity
Response (%)
200
100
0
-100
-200
-14
-12
-10
-8
-6
-4
Log [Drug]
Super Agonist
Full Agonist
Partial Agonist
Neutral Antagonist
Inverse Agonist

Figure 1.6: The dose-response curve with five types of ligands that can bind to the orthosteric site.
The red, orange and purple curves illustrate three different agonists that can activate the receptor with different levels of efficacy. The black curve represents the Neutral antagonist that does not affect the basal activity, and the blue curve shows the inverse agonist which reduces or inverses the pharmacological response produced during intrinsic activity of the receptors.

1.10.2 Allosteric modulator of GPCRs

The second type of ligands are called allosteric modulators or allosteric ligands which interact with a topographically distinct site differing from the orthosteric site, and they can be found wherever in the receptor. There are three main types of allosteric ligands presented in **Figure 1.7**. A positive allosteric modulator or PAM is a molecule that increases the signaling already activated by an agonist targeting the orthosteric site. On the other side, negative allosteric modulator or NAM will decrease the signaling triggered by the agonist while the neutral allosteric modulators will compete with NAM or PAM. Moreover, GPCRs signaling can be affected by allosteric modulators at two levels; first by modulating the affinity of the ligand binding the orthosteric site, second by affecting the efficacy of the response, or both simultaneously. Also, these allosteric modulators in the most stringent definition only have an effect when the endogenous ligand is present at the orthosteric site and are normally referred to as pure allosteric modulators [2, 89].

A new emerging concept for drug design is based on this secondary binding site effects. A successful potential drug will be capable of binding to an allosteric site and remotely alter the behavior of the natural ligands to the orthosteric site. There are many advantages of using allosteric modulators. For instance, they could reduce the negative side effects like dependence, tolerance, or overdose of medicines targeting the orthosteric site by not chronically activating the receptor. An allosteric modulator can also increase the selectivity within a specific subtype of class A GPCRs by targeting a potential divergent site. A clear example of this situation could be applied for serotonin receptor, whereas 13 subtype receptors bind the same ligand. The divergence within allosteric sites could thus be exploited to selectively target one

receptor subtype. Another advantage of allosteric modulators is to increase the druggability of non-favorable receptors such as chemokine receptors, which evolved to interact with large ligands. Further, some of the allosteric modulators can be used to design a bitopic ligand that can bind and act on both allosteric and orthosteric sites simultaneously when topographically feasible. Finally, they can be used as drug tools for GPCR's crystallization, and as radioligands for ligand-binding-assay at chemically non-annotated receptors. However, the molecular basis of allosteric sites recognition and their mechanism of action are still largely unknown.

Figure 1.7

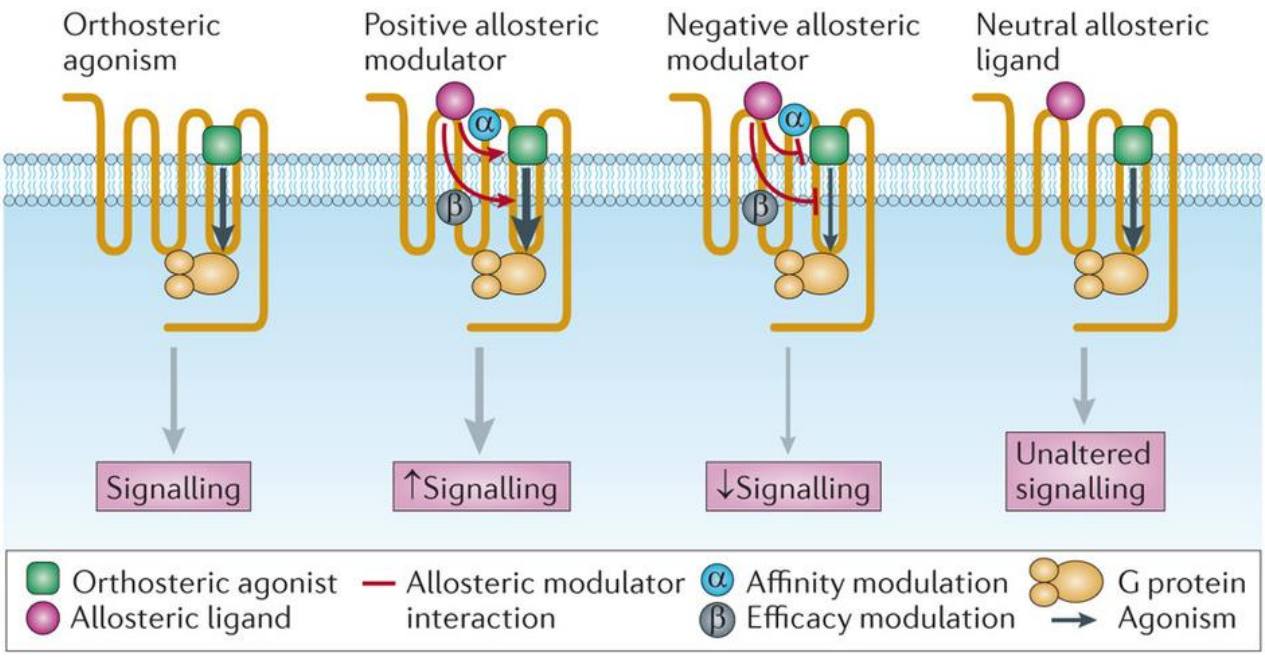

Orthosteric agonism
Positive allosteric modulator
Negative allosteric modulator
Neutral allosteric ligand
α
β
Signalling
↑Signalling
↓Signalling
Unaltered signalling
Orthosteric agonist
Allosteric ligand
Allosteric modulator interaction
Affinity modulation
Efficacy modulation
G protein
Agonism

Figure 1.7: Types of ligands interacting with GPCRs.

The two types of ligands that can activate GPCRs are; 1- The orthosteric ligand, which is represented in green color. It is a ligand that binds to the receptor at the same site than the endogenous ligand; 2- The second type of ligand is called allosteric modulator, it is shown in pink color and can interact with a distinct site wherever within the receptor. The allosteric modulator will affect the affinity of the natural agonist and the efficacy of the signaling induced; positive allosteric modulator can increase affinity and/or efficacy while the negative allosteric modulator will decrease the signaling triggered by the natural ligand. Allosteric molecules that cannot change the efficacy or affinity are called neutral allosteric ligands [88].

1.11 Allosteric Sodium cavity in DOR

Advance in protein biochemistry and in crystallography were important; these studies have led to uncovering the high-resolution crystal structures of all members of opioid subfamily receptors including the delta opioid receptor. This opens the door to understand their structure and mechanism of action. Also, it could help answering some questions related to their therapeutic functions; as well identifying the signaling framework which controls their severe side effects. The first crystal structure of mouse DOR bound to the antagonist naltrindole was resolved at 3.4Å resolution and was published in 2012. It shows a high homology in the transmembrane structure and within the lower parts of the binding pocket where morphinan ligands interact when compared to MOR and KOR receptors. The upper part of binding pocket is dissimilar among the three classical opioid receptors [90].

Subsequently, the crystal structure of human DOR with amino-terminal b_{562}RIL(BRIL) fusion protein was identified in a complex with Naltrindole at high resolution 1.8 Å in 2014. Unfortunately, the DOR complex with an agonist has not been resolved to date. As antagonists and inverse agonists stabilize the less dynamic inactive steady state, it is easier to get the crystal [30]. Overall, the structure of human DOR is very similar to mouse DOR crystal structure, but the atomic details of the native intracellular loop 3 (ICL3) suggest a stable closed conformation of ICL3 in the inactive state that was well defined in this structure[2] [91].

The recent high-resolution crystal structure of the human DOR described by Drs. Fenalti and Giguere, *et al (2014)*. highlighted the presence of a sodium ion and water clusters within a 16 amino acids cavity in the middle of the 7TM helical bundle of DOR [2] as shown in **Figure 1.8**. However, the first high-resolution crystal structure that spoted a sodium ion in an allosteric site was in adenosine receptor A_{2A}AR [7]. Two years later, this similar sodium coordination was found in the adrenergic receptor (β_1 AR)[92], and a protease-activated receptor PAR1[93]. Thus, it is now believed that the cavity housing the sodium ion could be present in more than 95% class A GPCR based on the conserved amino acids lining the cavity [46]. Moreover, this sodium cavity is formed by the side chain of 16 amino acid residues, of which 15 residues are conserved in all class A branches, while structural studies revealed an important disparity in the sodium cavity structure which seems to affect their ligand selectivity and their function. In human DOR, the oxygen atom of side chain Asn131$^{3.35}$ coordinates directly with the sodium ion, and the nitrogen atom forms a hydrogen bond to Asp128$^{3.32}$ and a salt bridge with nitrogen group of naltrindole bound to the orthosteric site. Thus, these interactions between Asn131$^{3.35}$, Asp128$^{3.32}$, and the sodium ion suggest the connectivity between the allosteric site and the orthosteric binding site in the inactive state of the receptor. Consequently, this allosteric pocket could be critical for modulation of signaling and ligand binding.

Figure 1.8

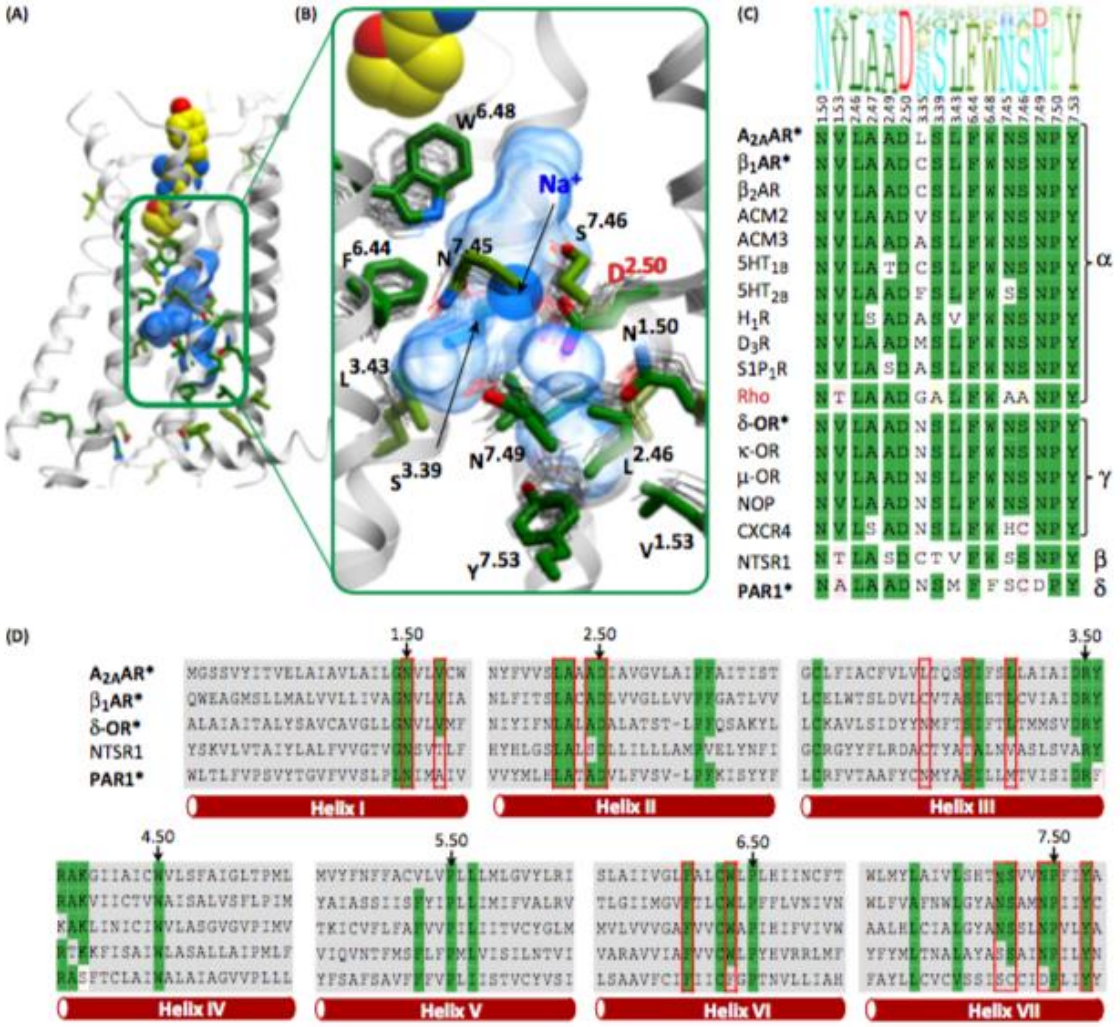

(A)
(B)
(C)
(D)
W6.48
Na+
S7.46
F6.44
N7.45
D2.50
N1.50
L3.43
S3.39
N7.49
L2.46
Y7.53
V1.53
A2AAR*
β1AR*
β2AR
ACM2
ACM3
5HT1B
5HT2B
H1R
D3R
S1P1R
Rho
δ-OR*
κ-OR
μ-OR
NOP
CXCR4
NTSR1
PAR1*
α
γ
β
δ
1.50
2.50
3.50
4.50
5.50
6.50
7.50
Helix I
Helix II
Helix III
Helix IV
Helix V
Helix VI
Helix VII

Figure 1.8: Structural and sequence conservation of the Na+ and water pocket in G-protein-coupled receptors.
(A) A2A adenosine receptor ($A_{2A}AR$) crystal structure, viewing residues with greater than 50% conservation in all non-olfactory class A GPCRs as branches with green carbons. **(B)** A zoom of the dominant allosteric pocket (transparent blue surface). **(C)** Sequence conservation of the 16 residues forming the sodium cavity. **(D)** Tables Show identity sequences of all four subtypes group of class A receptors. Red boxes emphasized residues forming the Na^+ pocket and their positions related to the 34 highly conserved amino acids in green [46].

However, there are some unique features of DOR sodium cavity which distinguished it from other sodium sites in Class A GPCRs. The sodium ion interacts with conserved residues which are arranged into two shells; the first coordination shell of the sodium ion in the allosteric site is formed by five oxygen atoms, three of them from Asp95$^{2.50}$, Ser135$^{3.39}$, and Asn131$^{3.35}$ side chains and the other two oxygen atoms of water molecules. The second coordination shell consists of side chains of three amino acid residues Trp274$^{6.48}$, Asn310^{7,45}, and Asn314$^{7.49}$ with two further water molecules in contact with the first shell. Also, the Aspartic acid residue in position 2.50 (Asp$^{2.50}$) is essential for binding the sodium in this site because it forms a strong salt bridge with the positively charged sodium ion. Remarkably, these conserved residues of the sodium cavity are within the most conserved motifs in class A GPCRs that have critical roles in GPCRs activation; CW$^{6.48}$xP in helix VI and NP $^{7.49}$xxY in helix VII[2] (**Figure 1.9).**

Figure 1.9

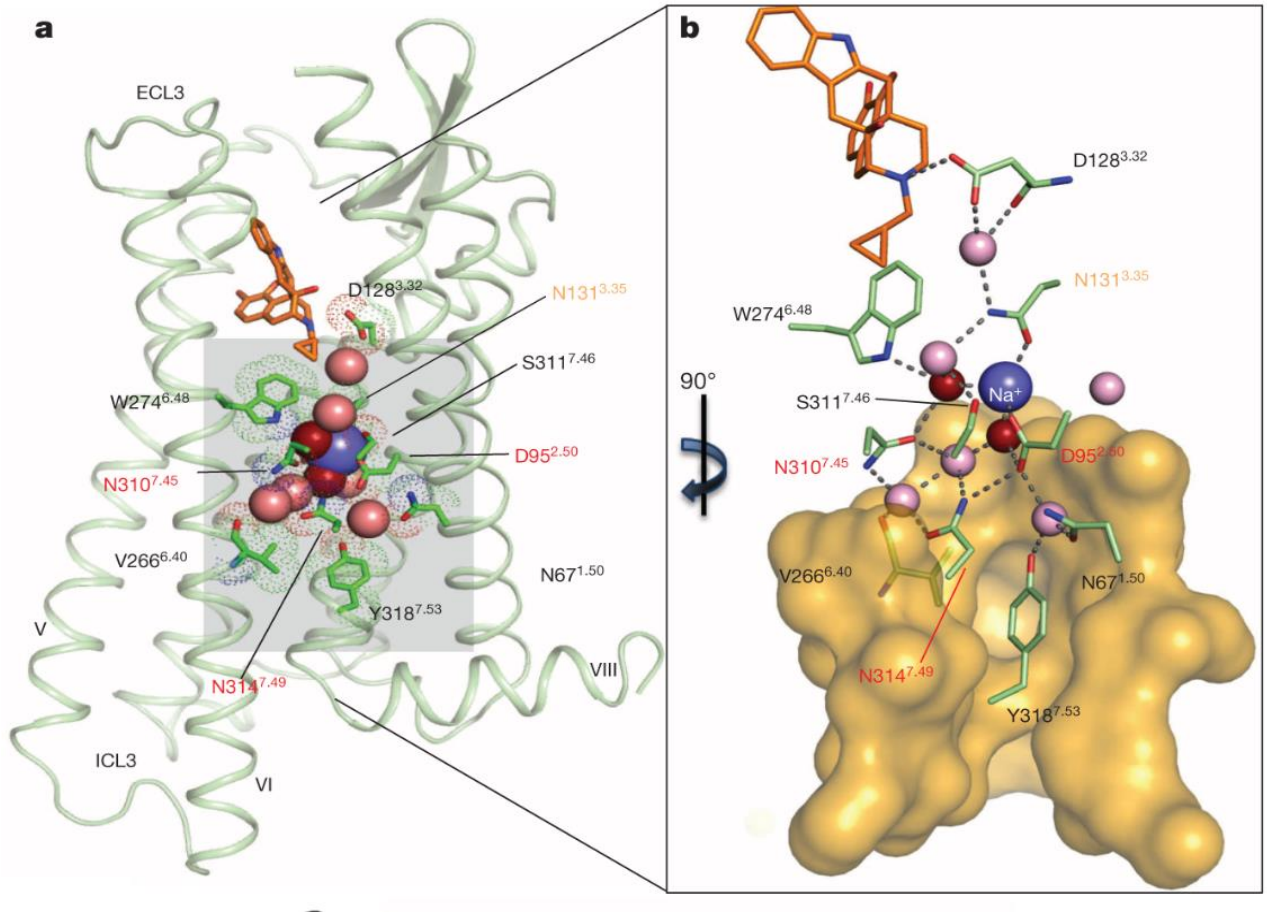

a
ECL3
D128$^{3.32}$
N131$^{3.35}$
S311$^{7.46}$
W274$^{6.48}$
D95$^{2.50}$
N310$^{7.45}$
V266$^{6.40}$
N67$^{1.50}$
Y318$^{7.53}$
V
VIII
N314$^{7.49}$
ICL3
VI
b
D128$^{3.32}$
N131$^{3.35}$
W274$^{6.48}$
90°
S311$^{7.46}$
Na^{+}
N310$^{7.45}$
D95$^{2.50}$
V266$^{6.40}$
N67$^{1.50}$
N314$^{7.49}$
Y318$^{7.53}$

Figure 1.9: Crystal structure of the δ-OR in complex with the antagonist Naltrindole. Naltrindole is presented as orange branches and the green sticks represent residues directly coordinating the sodium ion (violet ball). Red balls represent water involved in a direct H-bond with Na^+ and pink balls indirect H-bond. **(b)** Rotation and close-up view of the sodium cavity highlighting critical amino acids for sodium coordination (D2.50, N3.35, N7.35, N7.49) [2].

The size of the sodium cavity can accommodate small molecules of about 200-300 Daltons in the inactive state conformation [2]. Despite this new information about the atomic structure of sodium cavity, the functional role and allosteric effect of sodium ion in GPCRs are still poorly understood. Interestingly, the effect of sodium ions on ligand binding at certain GPCRs and especially at opioid receptors was observed over forty years ago. It was reported that the presence of high concentration of sodium increases antagonist binding affinity to the opioid receptors while decreasing or has no significant effect on the affinity of the agonist. Therefore, the researchers were using (- or + Na^+) to discriminate whether a ligand was an agonist or an antagonist [94]. This finding suggested that the sodium ion stabilized the inactive state of opioid receptors and was described to act likely as an allosteric modulator. Later on, the same allosteric effect of sodium ion was observed for another six different GPCRs, Neurotensin NTSR1, Dopamine D2R, β2-AR, Adenosine A_{2A}AR, and Protease Activated PAR-1 [46]. All of these studies were not conducted using functional assays. A more thorough investigation of sodium site was done by mutagenesis studies which shed light on the important aspartic acid residue $Asp^{2.50}$ in helix II, where mutating it to alanine abolishes the allosteric effect of sodium ion. This finding supported the idea that sodium ion acts via specific site within the seven transmembrane helical bundles and could be described as an allosteric modulator of GPCRs [95]. Even though the discovery of sodium-mediated allosterism was observed four decades ago, the functional role of this interaction remains to date elusive. Taking advantage of having a high-resolution crystal structure of sodium-bound GPCRs, we believe this information will be critical for further functional characterization.

1.12 Amiloride and its derivatives as DOR allosteric modulators

The suggested functional importance of the allosteric pocket in receptor activation, and its exceptionally high conservation in GPCRs makes it an attractive target for discovery of small molecules with unique functional and pharmacological properties, which can then be used to test our hypotheses and serves as a novel starting point for drug discovery. Although most drugs target GPCR orthosteric sites, exploitation of allosteric-binding sites provides a number of advantages. It allows more precise control of subtype and functional selectivity and preserves spatiotemporal profile of endogenous signaling, which holds a great potential for the development of new tool compounds and candidate drugs [4, 96-100]. Some of the initial insights into the allosteric Na^+ pocket have been obtained with amiloride and its derivatives as non-selective modulators of GPCRs. Amiloride is a potassium-sparing diuretic drug, it works by directly blocking the epithelial sodium channels (ENaCs) and Acid-sensing ion channel 3 (ASIC3). Its mechanism of action is by the reduction of the potassium excretion and inhibition of sodium reabsorption in the distal tubule which leads to loss of sodium and water from the body [101]. It is also used for the treatment of edema associated with hepatic cirrhosis, and in the treatment of heart failure by blocking Na^+/H^+ exchangers-1 which leads to decreases re-perfusion injury in ischemic attacks [101].

Some reports have proposed that the diuretic amiloride could exert allosteric modulation on different GPCRs with modest affinity and potency (>10uM) [102]. Since then, a few derivatives have shown comparable potency in several receptors [6, 95, 103-107] It has been proposed that the guanidium group, which is found in all

amiloride derivatives, could bind with the carboxylate group of $Asp^{2.50}$ residue, which is believed to coordinate the Na^+ ion. Also, Na^+ affects the affinity of ligands interacting with numerous of GPCRs at very high concentration [102]. However, a review from the literature reveals a lot of controversial results. It was observed in most cases that amiloride increases agonist affinity which is conflictual with the actual Na+ effect. Moreover, the effect of Na^+ as a competitor of amiloride was not observed most of the time. It has been shown, for some receptors, that mutation of the $Asp^{2.50}$ reduces the affinity of amiloride. However, it must be noted that in most of the cases, the affinity for the radioligand and/or the agonist used for displacement were also highly reduced, raising the concern about the validity of such results. The weak affinity of amiloride used so far makes those experiments challenging and could explain the controversial results as at >100 μM, amiloride can easily act as a chaotropic agent through its guanidine moiety.

However, no study addressed the effect of amiloride as an allosteric modulator of GPCR signaling using functional assays. Furthermore, the proximity of this cavity to the orthosteric site and the ability of some small molecules and small ions to bind both binding sites, opens the possibility to create bitopic ligands that can act on both sites simultaneously [108].

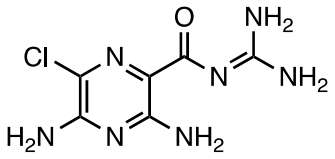
These results suggest that the cavity housing the sodium is a potential druggable allosteric site that can be used to develop novel allosteric modulators for the δ-OR and other receptors.

Functional importance of the allosteric sodium site in several class A GPCRs has recently emerged, there remains little understanding of specific functional

features of this pocket which are needed to effectively exploit it as a ligand design target. Previous studies were primarily concentrated with Na^+ allosteric effects on binding of orthosteric ligands, while its role in receptor signaling mechanisms remains largely unknown. In our preliminary studies, we have demonstrated allosteric activity of some amiloride derivatives at some new GPCRs, including opioid and orphan receptors. The δ-OR was by far the most pharmacologically affected receptor and was thus selected for further studies. To gain insight into their structure-activity relationship (SAR), we thus selected the δ-OR and performed in-deep pharmacological characterization as well as a screening of other sodium-channel inhibitors structurally related to amiloride. To date, the small molecules MIA, HMA and Zoniporide are the allosteric modulators that showed the strongest allosteric effect and were selected for our research project. The chemical structures of these small molecules are shown in **Figure 1.10.** It has been found that these small molecules can modulate the signaling pathways of delta-opioid receptors when activated by an agonist that targets the orthosteric site. MIA and HMA have been designed as high-affinity Na^+ channel inhibitors compared to the parent molecule amiloride. It is important to mention that amiloride itself has no activity at the δ-OR at a non-toxic concentration allowed in the functional assays. Zoniporide is a selective inhibitor of Na^+-H^+ exporter (NHE1) and is so far, the first non-amiloride compound that, we believe, targets the sodium site.

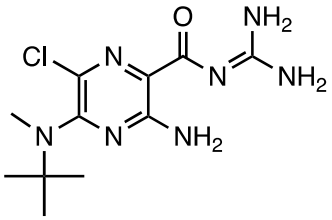
Figure 1.10

Amiloride



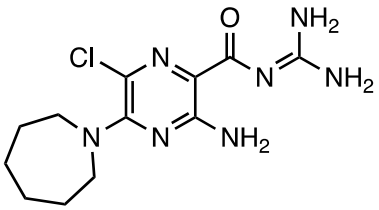
MIA

5-(N-Methyl-N-isobutyl) amiloride



HMA

5-(N,N-Hexamethylene) amiloride



Zoniporide

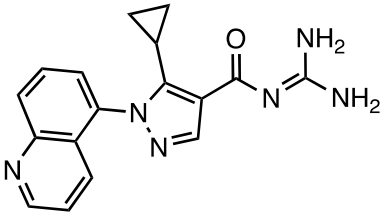


Figure 1.10: Chemical structures of the three allosteric modulators of δ-OR characterized in this project. MIA and HMA are amiloride derivatives while Zoniporide is a small molecule inhibitor containing the guanidium group but not the amiloride scaffold. All of them are sodium channel inhibitors. Amiloride is shown to provide the basic amiloride scaffold structure.

1.13 Rationale

Our actual degree of knowledge concerning the function and molecular determinant regulating sodium-mediated allosterism is limited. We believe that the allosteric site plays a fundamental role at the functional selectivity level. The development of allosteric modulators targeting the sodium pocket could be exploited to modulate and fine-tune GPCRs signal transduction. With the discovery of the Na^+ pocket within GPCRs, it was then hypothesized that amiloride and its derivatives could regulate GPCRs functions through interaction with this pocket.

From mutagenesis studies, it was found that mutation of the conserved sodium-coordinate residue D2.50 to asparagine or alanine leads to a decrease in the affinity of amiloride binding to sodium site. However, these findings are not supported by any functional data. Moreover, mutation of the D2.50 has strong effect on ligand interaction raising concern about the validity of the data presented so far. Most evidence published so far on the interaction of amiloride with the sodium pocket were performed *in vitro* with radioligand experiments using a high concentration of amiloride (>100 μM), a concentration found to be toxic for cells and consequently, preventing any functional characterization. To circumvent this problem, our lab has undertaken to find any similar compounds with activity below the toxic concentration to perform functional assays and study their recognition and activities in cell based-assay. A GPCR-ome parallel approach was used to identify the best GPCR targets to study the amiloride-dependent allosterism. This parallel approach identified six out of 350 receptors differently modulated by the sodium-channel inhibitors tested, BB3, CXCR7, NPY5R, DOR, MOR and NOP. It has to be noted that 3 out 4 opioid receptors came

out from the screening. Only KOR was found to be refractory to Na+-channel inhibitors tested despite its high structural homology with the DOR and MOR. The project presented here is focusing on the activity of the best Na+-channel inhibitors found in that screening at the DOR. Thus, the small molecules 5-(N-Methyl-N-isobutyl) amiloride (MIA), 5- (N, N-Hexamethylene) amiloride (HMA), and Zoniporide are the main allosteric modulators that are the focus of this research. The overall goal of the proposed research is to elucidate the molecular mechanism by which sodium regulates the delta opioid receptor (δ-OR) signaling and functional selectivity by using those potential allosteric modulators to remotely control the Na+ pocket.

1.14 Hypothesis and Objectives

We thus hypothesized that the sodium cavity could be used as a druggable target site for the design of allosteric modulators.

To assess this hypothesis, we will exploit a multidisciplinary approach using structural, biochemical, and pharmacological assays to study Na+-channel inhibitors as new chemotype drugs targeting the sodium cavity.

Main Objectives

The proposed study will help establish new pharmacological rules for the rationale design of new drugs for opioid receptors both as tool components, and potential drug candidates that distinguish functional properties.

Specific research aims are:

Specific Objective 1: Identify and functionally characterize new ligand chemotypes targeting the sodium allosteric site by looking at activity receptor functionality using the arrestin-recruitment assay Tango and cAMP biosensor GloSensor.

Specific Objective 2: Perform the structural-activity-relationship (SAR) at the receptor level by performing point-mutation within the Na+ cavity and characterize Na+-inhibitor activity at those mutants.

1.15 Significance

The delta opioid receptor has proven its potential as a drug target for the treatment of pain-associated diseases, depression, and symptoms associated with Parkinson's disease and possibly as an immunomodulatory. Actual δ-OR targeting drugs, as well as other opioid receptors, trigger potentially life-threatening adverse effects. The absence of functionally selective opioid drugs or modulators and the lack of understanding of the molecular and biological pharmacology of this receptor limit importantly its therapeutic exploitation. The development of allosteric δ-OR modulators would overcome the caveats generated by orthosteric-targeting drugs, as well as the difficulty in creating or designing a selective drug for the δ-OR with functional selectivity. It has been proposed that allosteric modulators would reduce undesired side effects associated with drugs targeting the orthosteric site and increase the degree of selectivity of drugs by acting bias for one of two GPCR signaling mechanisms.

Chapter 2: Materials and Methods

2.1 Materials

General Reagents and kits

- Hank's Balanced Salt Solution (HBSS), 1X pH 7.4 (Life Technologies, Paisley).
- BCA kit, Promega.
- Poly-L-Lysine (PLL), Sigma.
- Bright-Glo reagent, Promega.
- D-luciferin, Gold Biotechnology.

Tissue culture reagents

- Dulbecco′s Modified Eagle′s Medium (DMEM), Corning
- Bovine calf serum (BCS), Corning
- Fetal Bovine serum (FBS), Wisent
- Hygromycin, Sigma
- 0.4% Trypan Blue stain, Thermo Fisher Scientific
- Puromycin, KSE Scientific
- Blasticidin S HCl, Gold Biotechnology,
- 100X Antibiotic Antimycotic solution, Wisent Inc
- 100X Penicillin and Streptomycin, Wisent Inc
- Ampicillin, Bio Basic Canada Inc

Ligands, drugs, and allosteric modulators

- 5-(N-Methyl-N-isobutyl) amiloride (MIA), Millipore Sigma

- 5- (N, N-Hexamethylene) amiloride (HMA), Millipore Sigma
- Zoniporide Hydrochloride hydrate (Zoniporide), Sigma,
- D-Ala^2, D-Leu^5 – Enkephalin- (DADLE), Bachem
- BW373U86, Tocris Bioscience
- Met-Enkephalin, American Peptide
- Leu-Enkephalin, American Peptide
- Naltrindole Hydrochloride, Tocris Bioscience
- Naltriben Mesylate, Tocris Bioscience
- 3-Iodobenzoylnaltrexamide (IBNTXA), Purdue Pharma
- Diprenorphine, Sigma
- (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*, *N*-diethylbenzamide (SNC80), Torcis Bioscience
- 1-(3′,4′-Dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride (Isoproterenol), Sigma

<u>Radioligands</u>

- ^{3}H-DADLE, ARC (American Radiolabeled chemicals Inc)
- ^{3}H- Naltrindole, ARC (American Radiolabeled chemicals Inc)

2.2 Methods

2.2.1 Cell Culture

Human Embryonic Kidney cells (HEK293T) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 5% bovine calf serum (BCS) and 100 µg/ml of penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. HTLA cells (provided by R.Axel) which are HEK293T stably expressing human β-arrestin fused to Tobacco Etch Virus (TEV) protease and luciferase reporter gene were maintained in DMEM supplemented with 5% FBS, 5% BCS, 100 µg/ml of penicillin and streptomycin, 2.5 µg/ml of puromycin and 50 µg/ml of hygromycin. HTLA cells stably expressing DOR-T, the delta opioid receptor (DOR) with TEV cleavage site following by Tetracycline-Controlled Transcriptional Activation (TTA) sequence, were cultured in the same media than HTLA cells supplemented with 5 µg/ml of Blasticidin.

2.2.2 Transfection

Cells were transiently transfected with different constructs of pcDNA3.1 vector with the coding DNA (cDNA) of the receptor of interest (DOR wild-type or mutants) and cDNA which encodes the yellow fluorescent protein (YFP) to follow the transfection efficiency. Calcium phosphate precipitation method was used as described by Jordan and al. in 1996 [109]. In brief, HTLA and HEK293 cells are subcultured in 15 cm dishes at a density of $12x10^6$ cells and $15x10^6$ cells, respectively in 25ml of media to reach a 50% confluency the day of the transfection. On the following day, for each 15 cm dish a total of 1 ml mixture (900 µl TE 0.1X, 100 µl 2.5 M $CaCl_2$, and 18 µg of plasmid of interest (DOR-T, DOR or mutants) with 2 µg of YFP

are mixed by vortexing. An equal volume of HBS 2X (Hepes buffer solution 2X, pH 7.05) is added to the mixture and gently mixed. After one minute incubation at room temperature, the whole solution is added dropwise to the cells. Cells are then incubated at 37C^{o} for 16-18 hours and detached using trypsin 0.05%, counted and seeded to the appropriate plate depending on the experiment.

2.2.3 Functional assays

There are numbers of assays which are available to test the activity of the drug in functional models. These assays are called functional assays, which consist of any biological system that can produce a physiological response or biological product as a response to the drug stimulation. Hence, the biological system can be membrane preparation, cells or even the whole tissues. These assays can detect molecules that cause production or blocking of any biological or physiological response after stimulation with drugs. The functional experiments are sensitive to detect any changes in the efficacy or potency produced after stimulation with drugs.

2.2.3.1 Tango Assay for β-arrestin recruitment

Tango assay has been used to study β-arrestin recruitment of activated δ-opioid receptors [110]. Furthermore, Tango assay has the advantage of having a readout specific to the target receptor (no effect at endogenous receptors can be detected), proximal to the site of receptor activation and independent of G-protein (**Figure 2.1**). The assay was performed using a modification of original Tango assay [111-113]. Briefly, for our research, we used stable HTLA cells expressing the δ-OR. Also, HTLA transiently transfected by using calcium precipitation method as described above were used for the mutants of DOR. The next day following the transfection or

not (if using stable cells), the cells are seeded on PLL (poly-L-Lysine)-coated 384-well clear bottom cell culture plate and starved overnight in DMEM 1% dialyzed FBS (20,000-22,000 cells/well in a total volume of 45 μl per well). The following day, cells are first incubated with 20 μl/well of the allosteric modulators at one final concentration of 10 μM or at different concentrations (0 to 10 μM) for a Schild analysis in DMEM 1% dialyzed FBS. The allosteric modulators were incubated for 15 minutes before adding a serial dilution of 16 concentrations of an agonist (D-alanine D-Leucine-enkephalin (DADLE) or BW373U86) or an antagonist (Naltrindole) selective for the DOR. Serial dilution of the ligands are prepared using an assay buffer (20 mM HEPES, 1X Hanks' balanced salt solution (HBSS), pH 7.40) at 4X, then it is added to the cells at 20 μl/well for 16 to 20 hours of incubation [114]. On the last day, media was removed and 20 μl /well of BrightGlo reagent is added, after 5-10 minutes of incubation at room temperature in the dark place, the plates are read by a luminescence reader (MicroBeta). Data were subjected and fitted using the nonlinear least-squares regression analysis using the sigmoidal dose–response function provided by GraphPad Prism. Data of four independent experiments (n=4) performed in quadruplicate are presented as relative luminescence unit (RLU).

Figure 2.1

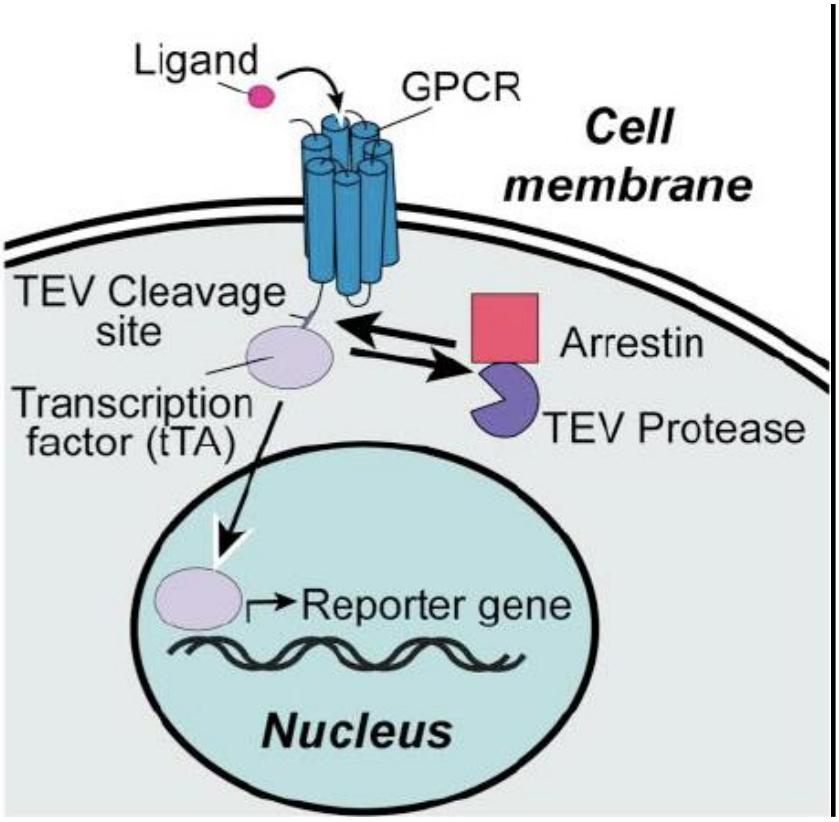

Ligand
GPCR
Cell membrane
TEV Cleavage site
Arrestin
Transcription factor (tTA)
TEV Protease
Reporter gene
Nucleus

Figure 2.1: Design and principle of the Tango Assay.

HTLA cells are HEK293T that stably expresses the fusion protein β-Arrestin2-TEV protease and the reporter gene luciferase controlled by the tetracycline-operon ($TetO_7$). The receptor is expressed as a fusion protein containing the TEV cleavage site followed by the tetracycline-transactivator protein tTA which is created by a fusion of the tetracycline repressor (TetR) with the activation domain of VP16. When the receptor is activated, the β-Arrestin2-TEV is recruited to the activated DOR receptor; the TEV protease cleaves the TEV cleavage site leading to the release and translocation of the transcription factor (tTA) into the nucleus, which allowed the transcription of the luciferase. Following 16 hours of incubation, cells are lysed with a lysis buffer containing the Luciferin (Luciferase substrate) and stabilizer following by luminescence read on a microplate reader [115].

2.2.3.2 Glosensor Assay for $G\alpha_i$-protein Activation

This functional assay is used to quantify the Gαi pathway of GPCRs by measuring the cAMP levels within live cells. The assay is modified from the commercial Glosensor cAMP assay (Promega) as described [116] in **Figure 2.2**. HEK293T are transiently co-transfected with cDNA encoding the DOR wild-type or mutant, and with the Glosensor plasmid encoding the gene of split-luciferase-based cAMP biosensor by using the calcium phosphate precipitation method. Twenty hours later, cells were transferred to PLL-coated 384 wells plate (18000 cells/well) in DMEM 1% dialyzed FBS and incubated overnight. The media was removed on the next day from the plate, and the cells were incubated with the tested allosteric modulators and 0.8 mg/ml of D-Luciferin in the assay buffer (20 mM HEPES, 1X HBSS, PH 7.4) for 45 minutes at room temperature. DOR agonists or antagonists were then added to the cells at a 3X concentration for a serial dilution of 16 points (final concentration of 0 to 10 μM) for a 15 minutes incubation period. Finally, isoproterenol was added at a final concentration of 400 nM to increase the endogenous level of cAMP, allowing the quantification of the decreasing levels of cAMP induced by the activation of Gαi signaling. The isoproterenol increases the cAMP levels by the activation of the $G\alpha_s$ protein through the β2-Adrenergic receptors endogenously expressed in HEK293 cells. Sodium channel inhibitors were tested against the β_2-AR and no effect were observed (data not shown).

Figure 2.2

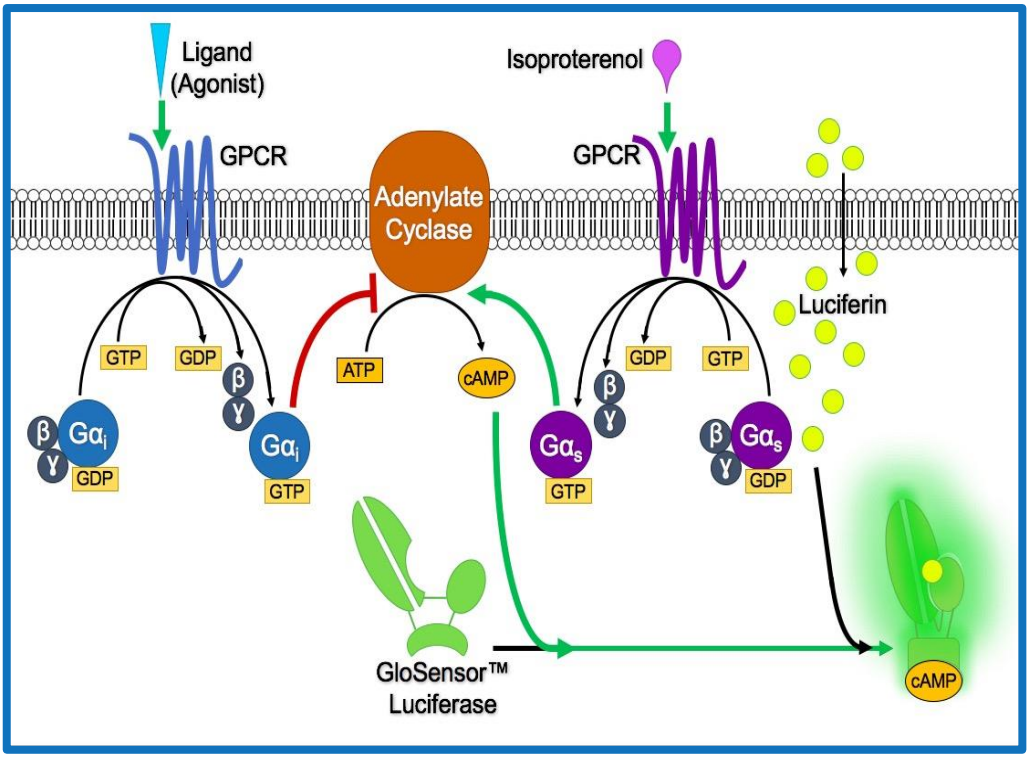

Ligand
(Agonist)
GPCR
Adenylate
Cyclase
Isoproterenol
GPCR
Luciferin
GTP
GDP
ATP
cAMP
GDP
GTP
Gαi
GDP
Gαi
GTP
Gαs
GTP
Gαs
GDP
GloSensor™
Luciferase
cAMP

Figure 2.2: Principle of Glosensor Assay.

Activation of a Gαs- or Gαi-coupled GPCRs leads to changes in cAMP levels within the cells. The Glosensor is a biosensor assay that was developed based on complementation of the split luciferase once cAMP interacts with the cAMP-binding domain of protein kinase A. Upon cAMP binding, the firefly luciferase undergoes a conformational change and becomes functional to interact with its substrate (D-Luciferin) leading to increase in luminence which is quantified by using MicroBeta luminescence reader.

2.2.4. Binding Experiments

2.2.4.1 Cell Membrane Preparations

HEK293T cells stably expressing DOR were grown to 80% confluency in 15 cm dishes. Then, cells were washed and scraped with PBS and spun down for 3 minutes at 0.5 x g speed. Then, PBS was decanted and cells were resuspended in 8 ml per 15 cm culture dish of hypotonic cold lysis buffer (50 mM Tris-HCl pH 7.4). Then the lysate solution was incubated for 10 minutes on ice and centrifuged at 30,000 x g for 25 minutes. After decanting the supernatant, the pellet was resuspended in 4 ml per 15 cm plate of cold binding buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4). Using a Polytron, membranes were homogenized 4-5 times for 2-3 seconds each. Finally, the protein quantification was done using a BCA kit from Promega. For all binding experiments, 25 to 40 µg of membrane in 75 µl of binding buffer per well of 96 well plate was used [117].

2.2.4.2 Saturation binding assay

Saturation binding assay is usually performed to measure the binding affinity which is defined as the equilibrium dissociation constant (K_D) of the selected radioligand. The assay was conducted in 96-well plates with a final volume of 125 µl/well. Twenty-five µl of ^{3}H-DADLE is added to each well of a 96 well plate with a serial dilution from 10 nM to 0.08 nM, followed by addition of 25 µl of antagonist naltrindole at a final concentration of 10 µM for nonspecific binding or 25 µl of binding buffer (total binding). Finally, 75 µl containing 25 to 40 µg of membrane protein was added and incubated for two hours in the dark at room temperature. Then, the reaction

was stopped by vacuum filtration through glass fiber filters, which was previously socked in cold washing buffer containing 0.3% polyethylenimine (PEI) and wash three times with cold washing buffer (50 mM Tris HCl, pH 7.40). Filter was dried on hot plate for 2 minutes, and a melt-on scintillation sheet (Melt Lex A) was melted on the top of the filter. Finally, the quenched radioactivity was mesured by using a luminescence reader (MicroBeta). The K_D value was calculated from specific binding (total binding - nonspecific binding) using non-linear fit with one site – specific binding analyzing on GraphPad Prism.

2.2.4.3 Radioligand displacement assay

Radioligand binding assay is also referred to as a competition assay or displacement assay; it is performed to determine the binding affinity which is the inhibition constant of drug ($Ki=IC50/(1+([L]/K_D)$). The assay procedure is similar to the saturation assay described above. It is performed with a final volume of 125 µl/well in a suitable binding buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4). The concentration of the hot ligand ^{3}H-DADLE or ^{3}H-Naltrindole is at a concentration close to their respective K_D which were calculated previously by using the saturation binding assay. Thus, 25 µl of the hot ligand was added in every well (final concentration of 1 nM for ^{3}H-DADLE or 2 nM for ^{3}H-naltrindole), followed by the addition of 25µl of cold ligand (DADLE or naltrindol as a reference) in serial dilutions starting with 10 µM to 0.1 µM. In the case of the cold ligands MIA, HMA, and Zoniporide, they were added in serial dilution, starting with a high concentration of 31.5 µM due to weak binding affinity . The reaction was started by adding 40 µg of the cell membrane in 75 µl of binding buffer in each well. Following 2 hours of incubation period at room temperature in the

dark, the rest of procedure is the same as the saturation assay which was previously described [117]. The data were analyzed with GraphPad Prism using binding competitive -one-site fit Ki.

Chapter 3: Results

3.1 Molecular Control of δ-Opioid Receptor Functionally by Sodium

In 2014, a study was published in Nature magazine by Dr. Giguere and his colleagues that has revealed the presence of a highly conserved sodium cavity serving as a binding site for sodium ion and water molecules cluster in the middle of a 7TM bundle of delta- opioid receptors [1]. Later studies have also reported the presence a similar sodium pocket in more than 95% of class A GPCRs [2]. This pocket was identified as a binding site for sodium ion and amiloride derivatives. The functional and biochemical analysis of the sodium-dependent allosteric site in the DOR and A_{2A}AR receptors established a critical role for this polar pocket in the modulation of receptor signaling pathways and pharmacological function in most of class A GPCRs. These studies also highlighted that the sodium ion stabilizes the inactive state of the receptor. It appears that the binding of the sodium ion to the sodium cavity seems to modulate the biased signaling for DOR because dissociation of sodium ion favors the β-arrestin signaling pathway. Importantly, it is well known that the activation of β-arrestin signaling is responsible for generating unwanted and adverse side effects that are associated with drugs used to target opioid receptors, including DOR. Thus, we suggest using this cavity as an allosteric site to modulate the receptor's function towards a therapeutic effect and away from the adverse side effects by activating specific signaling pathways over others. Our lab has recently characterized promiscuous sodium channel inhibitors, amiloride-derivatives (MIA, HMA), and the non-amiloride small molecule Zoniporide as potent allosteric modulators. We suggest

that these Na^+ inhibitors target the sodium cavity of DOR and modulate the receptor's function positively or negatively. These findings lead to the hypothesis that this cavity could serve as a target allosteric site to design a novel generation of drugs with reduced side effects and increased therapeutic potential. This research project aims to use functional experiments, binding assays, and perform structure-activity-relationship (SAR) studies of the allosteric modulator (Na+ inhibitors) on DOR. For this reason, this study will provide a broad understanding to help identify and characterize these new ligands at their functional selectivity level. We believe that a better understanding of the mechanism of sodium-mediated functional selectivity and a molecular determinant of sodium-mediated allosterism will help generate new opioid drugs with selected therapeutic properties.

3.2 Amiloride-Derivatives and Zoniporide as DOR Allosteric Modulators

It is well known that GPCRs are not only activated by orthosteric ligands but also are affected by another group of drugs or endogenous ligands that target a topographically distinct site called an allosteric binding site. The drugs or endogenous ligands that bind to the allosteric site are called allosteric modulators. These modulators can regulate the receptor's function, acting as positive, negative, or neutral allosteric modulators. Allosteric modulators have been identified for several GPCRs including dopamine receptors; adenosine; histamine; muscarinic; serotonin; chemokine; and class C GPCRs receptors. In fact, they are more selective than the orthosteric ligands, and they have little conserved inherent activity, which makes them excellent therapeutic candidates with less adverse effects [17]. Some high-resolution crystal structures of many GPCRs revealed a sodium ion bound in a conserved

sodium pocket (allosteric site). The size of the cavity housing the sodium ion can accommodate small molecules suitable for binding with amiloride-derivatives and other small molecules [102]. It has also been suggested that these amiloride analogs could compete with sodium ions to bind at the sodium cavity [118] and modulating the orthosteric ligand binding positively or negatively, thus amiloride derivatives and some small molecules could act as allosteric modulators.

Early studies showed that sodium cavity tolerance of amiloride to derivatization at both ends of the aroylguanidine scaffold, including bulky R-group such as in benzamil and the R2 group as in HMA in the binding model [46]. It is also described that amiloride and HMA have allosteric modulator effects in several GPCRs including; dopamine receptors, the α_2 adrenergic receptor, and the gonadotropin-releasing hormone receptor [102]. The allosteric effect of amiloride and its analog HMA have been widely studied for adenosine receptors; these showed that HMA increased the affinity of its antagonist ^{3}H-ZM-241,385 compared with the amiloride in binding studies. These studies shed further light on the investigation of other amiloride derivatives which might be more potent from their parent's effects [119]. In general, the proposed binding of amiloride and its derivatives to the sodium cavity happens because of the interaction between the positively charged quaternary ammonium group - present in most channel blockers - with the negatively charged carboxylate of sodium ion site residue Asp2.50 that is present in all allosteric sodium pockets. In this current study, the three small molecules MIA, HMA, and Zoniporide showed the strongest allosterism, so they were selected for our research project. The chemical structures of these small molecules are shown in **Figure 1.10** (chapter 1). HMA and

MIA are the amiloride derivatives while Zoniporide is a selective inhibitor of Na-H exporter (NHE1) and is, so far, the first non-amiloride compound that, we believe, targets the sodium site and can modulate the signaling at DOR.

3.3 Results

3.3.1 Discovery of Two Different Allosteric Profiles for the β-arrestin recruitment and One Allosteric Profile for the G-protein pathway at Activated DOR

Two functional experiments were performed to examine the allosteric effects of the best three sodium-channel inhibitors previously discovered in a broad panel screening (MIA, HMA, and Zoniporide) at activated DOR. The first functional assay that we optimized, called Tango assay, has excellent sensitivity for the arrestin-recruitment pathway. In this experiment, the HTLA cells expressing DOR were pre-incubated for fifteen minutes in the absence of allosteric modulators (buffer) - which is shown as a blue curve, and in the presence of three small molecules (MIA, HMA, and Zoniporide) following stimulation with increasing concentrations of the DOR agonist DADLE ([D-Ala^2, D-Leu^5]-Enkephalin). DADLE is a synthetic opioid peptide with analgesic properties similar to Leu-Enkephalin [120]. We use it in most of our experiments as a reference since its pharmacological effect is like the endogenous DOR agonists. As shown in **Figure 3.1**, we observed two allosteric profiles for β-arrestin recruitment at activated DOR, which is a positive allosteric modulator (PAM), and agonist-PAM (Ago-PAM) activities. The red and green curves illustrate the significant Ago-PAM activity of MIA and HMA respectively. The Ago-PAM effect of MIA and HMA are shown in the absence of the DOR agonist DADLE at (-12.5) drug concentration on the graph, since

a zero level of drug concentration cannot be used on a logarithmic scale. The PAM effect is observed by the increase in DADLE efficacy in the presence of MIA and HMA. The non-amiloride molecule Zoniporide - which is represented by a violet curve - has only a positive allosteric modulator (PAM) effect, and no intrinsic agonist activity (Ago-PAM) was observed.

Figure 3.1

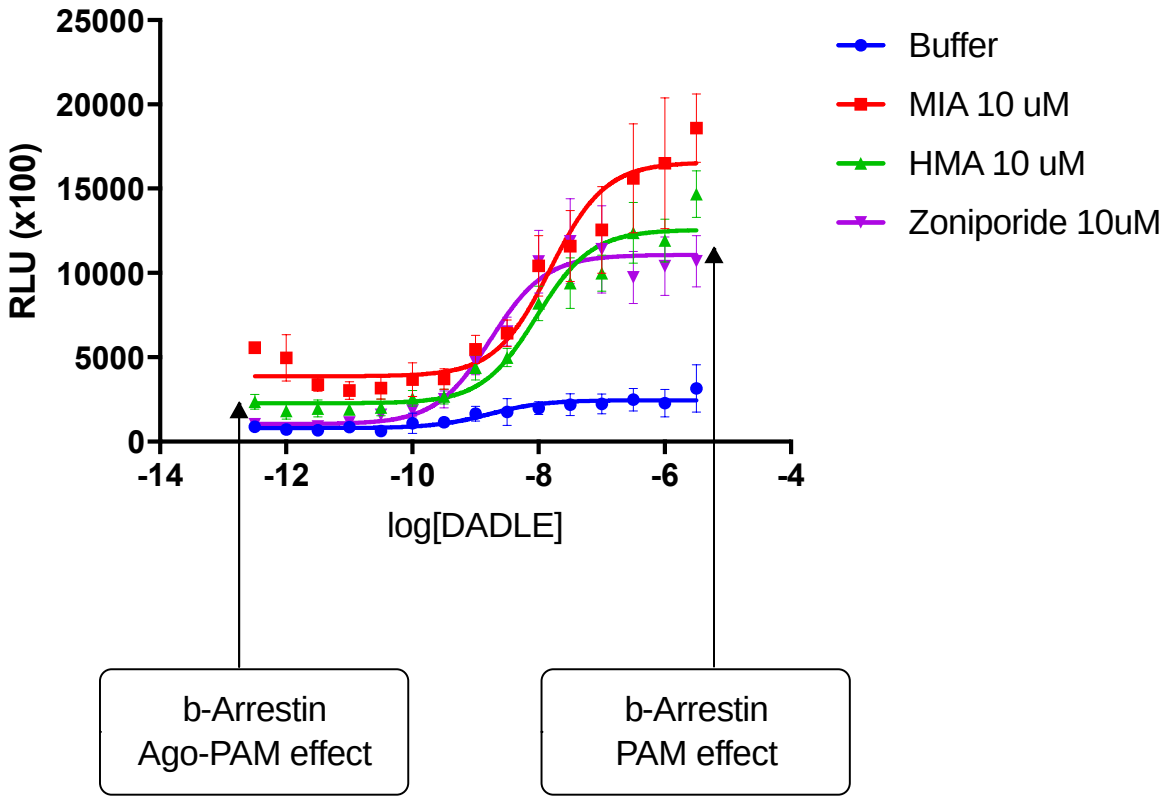

25000
20000
15000
10000
5000
0
RLU (x100)
-14
-12
-10
-8
-6
-4
log[DADLE]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10uM
b-Arrestin
Ago-PAM effect
b-Arrestin
PAM effect

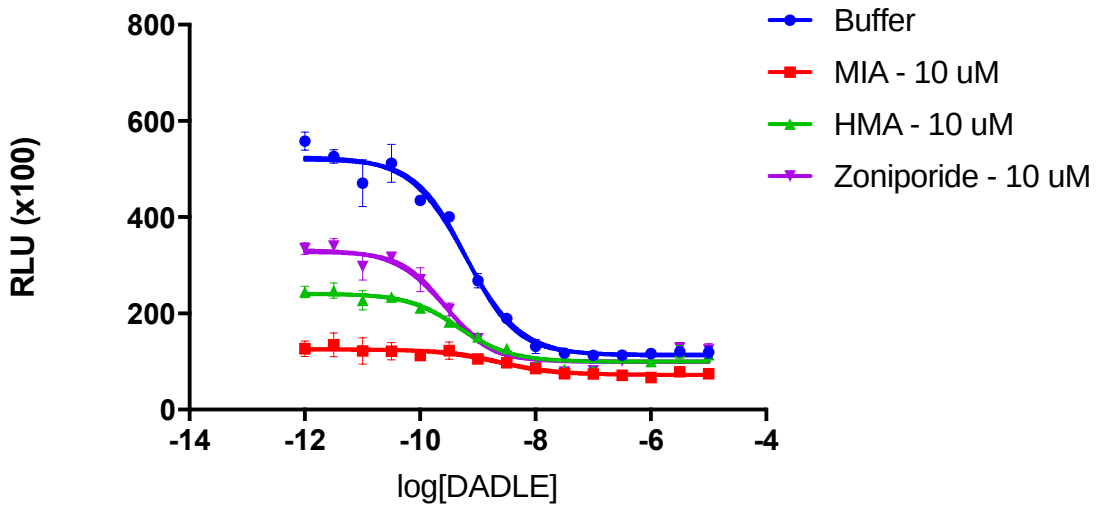
Figure 3.1: **The allosteric modulators (AM) have intrinsic agonist and/or PAM activities for the β-arrestin recruitment.** HTLA DOR-T stable cells were pre-incubated in the absence of allosteric modulators (buffer) which is represented as a blue curve or in the presence of three allosteric modulators followed by stimulation with increasing amounts of a DOR agonist DADLE. MIA and HMA have a significant Ago-PAM activity that is shown by red and green curves, respectively. Zoniporide which is represented by a purple curve has positive allosteric modulator (PAM) activity only. The PAM activity, which represents increases of the DADLE efficacy, was observed with the three allosteric modulators, where MIA had the strongest PAM effect. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

The second functional experiment was performed to test the druggability of the sodium allosteric site at DOR with the three sodium channel inhibitors in the Glosensor assay (cAMP assay). This assay is used to measure the inhibition of cAMP signaling pathway induced by the activation of the Gαi protein at DOR. Thus, HEK293T cells were co-transfected with the wild-type DOR and Glosensor plasmids and were treated as previously described in the Materials and Methods section (Chapter 2). The three small molecules MIA, HMA, and Zoniporide, were tested at fixed concentration of 10 µM followed by stimulation with decreasing concentrations of synthetic ligand DADLE starting with 10 µM. It has been well known that DADLE binds to the orthosteric binding site and activates the Gαi protein pathway. Thus, the activation of Gαi signaling leads to a decrease in cAMP levels within the cell. However, in order to observe the decrease induced by activation of Gαi, and consequently inhibition of the adenylate cyclase, basal level of cAMP has to be raised via activation of the adenylate cyclase. As a result, isoproterenol was added, before reading of the 384 plates, to increase the cAMP levels within the cells via the endogenously expressed β_2-adrenergic receptor (coupled with Gαs).

As shown in **Figure 3.2**, the effect of DADLE alone (without the Na^+ inhibitors) was observed as a buffer (the blue curve) that shows a significant decrease in cAMP levels within the cells. The Ago-PAM effect was observed with the three Na^+ inhibitors at a drug concentration labeled as (-12) which is in the absence of DADLE, whereas MIA shows the strongest effect (the red curve) among HMA and Zoniporide which are illustrated by the green and purple curves respectively. The PAM activity is observed by the increased efficacy (decrease cAMP) in the presence of DADLE. G-protein activation is limiting to measure PAM activity as the assay is easily saturable. In the

case of a Gαi assay, it is impossible to reduce cAMP level further to the effect of full agonist.

Figure 3.2



	Bottom	Top	LogEC50	EC50
Buffer	520.4	114.9	-9.203	6.261e-010
MIA - 10 uM	124.8	73.2	-8.617	2.414e-009
HMA - 10 uM	241	100.3	-9.323	4.753e-010
Zoniporide - 10 uM	331.9	99.31	-9.574	2.664e-010

Figure 3.2: **All allosteric modulators have Ago-PAM activity for the cAMP signaling pathway at activated DOR**. HEK293T cells were transiently transfected with DOR and Glosensor cDNA and then pre-incubated in the absence of allosteric modulators (buffer) which is represented as a blue curve or in the presence of three allosteric modulators followed by stimulation with DOR agonist DADLE. MIA has a significant Ago-PAM activity that is shown by a red curve while the green and purple curves represent the Ago-PAM activity for HMA and Zoniporide, respectively. The PAM effect of all the small molecules was not observied. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

3.3.2 The Allosteric Effect of Na^+ Inhibitors with Endogenous DOR Agonists

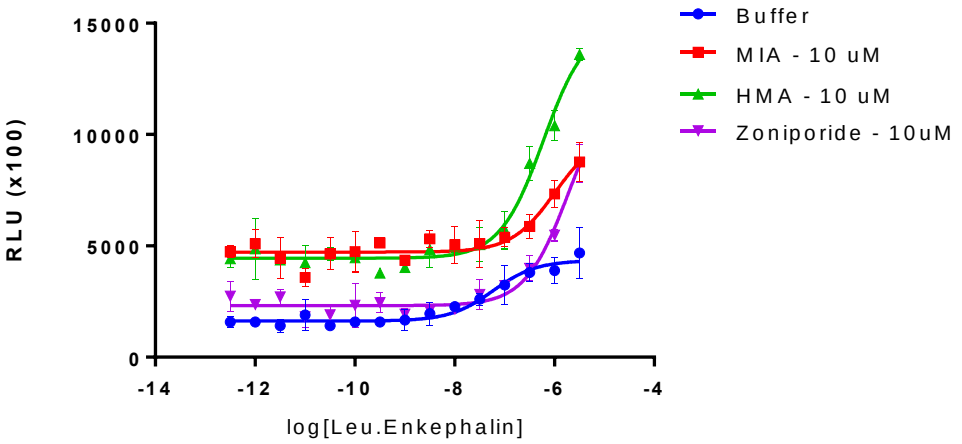
While DADLE is a peptidomimetic of the endogenous ligand Leu-enkephalin, we wanted to confirm that the effect observed with our allosteric modulators is also observed with the endogenous enkephalins. The two functional experiments, Tango and Glosensor assays, were performed in the presence of two DOR endogenous ligands Met-enkephalin and Leu-enkephalin**.** In the case of the Tango experiment, the HTLA cells were stably transfected with DOR and were pre-incubated in the presence or absence (Buffer) of MIA, HMA, and Zoniporide at the non-toxic concentrations of 10µM. After 15 minutes, the cells were stimulated with the DOR endogenous ligand tested. As shown in **Figure 3.3 (A, B),** the blue curve (buffer) indicates the effect of the DOR endogenous agonist Leu-enkephalin (A) or Met-enkephalin (B) on β-arrestin recruitment alone without the allosteric modulators. After stimulating the cells with the Na+ inhibitors, the allosteric effect of MIA, HMA, and Zoniporide with the two DOR endogenous agonists was similar to that was observed with the synthetic DOR agonist DADLE. MIA and HMA showed the strongest agonist-PAM effect in the absence of the endogenous agonists at drug concentration (-12.5), which is effectively a zero concentration of the drug. Also, the positive allosteric modulation (PAM) of both amiloride-derivatives was observed by increasing DADLE efficacy. As expected, Zoniporide - which is represented by a violet curve - has a PAM effect without any observable Ago-PAM activity.

In another set of experiments, the allosteric effect of our compounds was studied on the G-protein activation signaling pathway at DOR stimulated by the two-

endogenous peptides (Glosensor assay). From the graph in **Figure 3.4 (A, B)**, the Ago-PAM effect was observed with MIA, HMA, and Zoniporide (red, green, and violet respectively), but was strongest with MIA and HMA. These results are very similar to those observed with the synthetic ligand DADLE except for the potency, which is reduced with the endogenous ligand. DADLE has the advantage to be protected from proteolysis and is way less expensive than the natural peptide ligands Leu- and Met-enkephalin. Thus, we have decided to only use DADLE as the prototypic ligand for the rest of the studies.

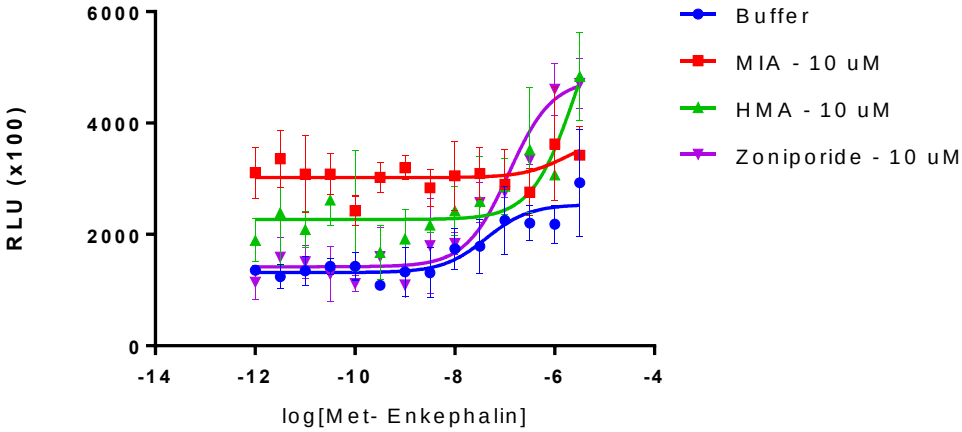
Figure 3.3

A



	Bottom	Top	LogEC50	EC50
Buffer	1626	4347	-7.231	5.874e-008
MIA - 10 uM	4723	10040	-5.994	1.014e-006
HMA - 10 uM	4446	15008	-6.223	5.985e-007
Zoniporide - 10uM	2317	12429	-5.719	1.909e-006

B

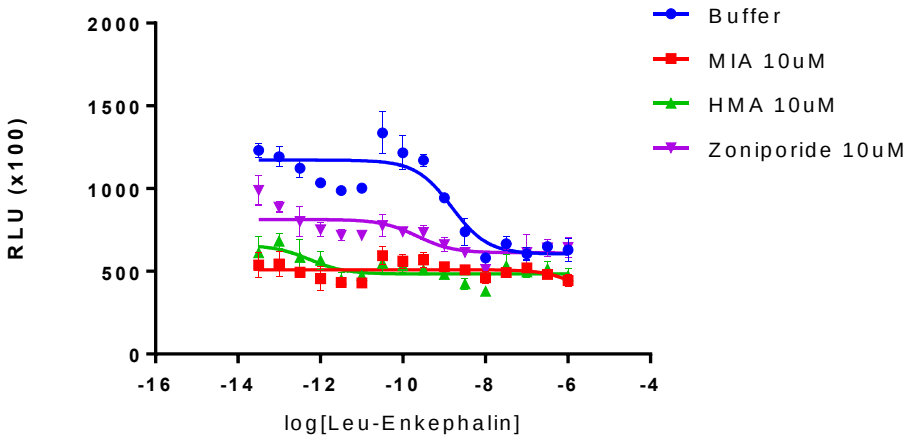


	Bottom	Top	LogEC50	EC50
Buffer	1315	2538	-7.376	4.209e-008
MIA - 10 uM	3020	3762	-5.769	1.703e-006
HMA - 10 uM	2268	6739	-5.593	2.554e-006
Zoniporide - 10 uM	1419	4787	-6.915	1.216e-007

Figure 3.3: Allosteric effect of MIA, HMA, and Zoniporide with DOR endogenous agonists at the β-arrestin recruitment. HTLA DOR-T stable was pre-incubated in the absence of allosteric modulators (buffer) which is presented as a blue curve or in the presence of MIA, HMA, and Zoniporide then stimulated with the two DOR endogenous ligands, Leu- and Met-Enkephalin (figure A and B respectively). MIA and HMA have both significant agonist-PAM and PAM allosteric activity that is shown by the red and green curves. However, Zoniporide, which is represented by a purple curve, has a positive allosteric modulator (PAM) activity only. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

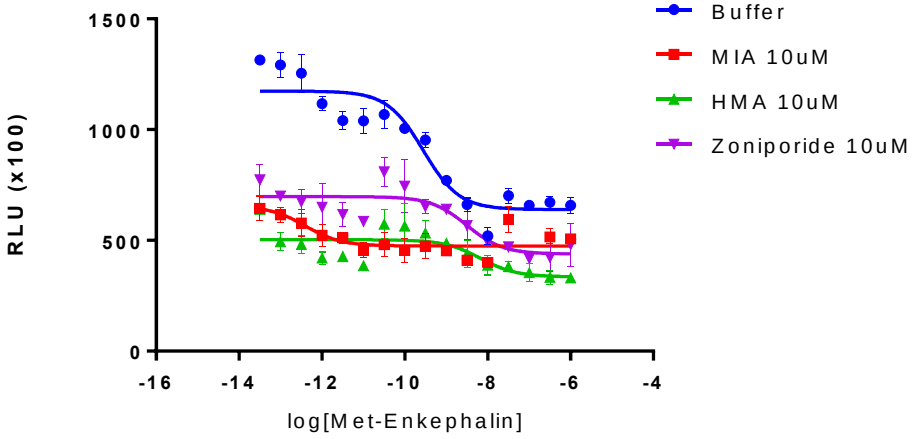
Figure 3.4

A



	Bottom	Top	LogEC50	EC50
Buffer	1172	604.1	-8.819	1.518e-009
MIA 10uM	509.7	212.4	-5.455	3.510e-006
HMA 10uM	658.7	483.5	-12.26	5.489e-013
Zoniporide 10uM	813.0	611.6	-9.656	2.209e-010

B



	Bottom	Top	LogEC50	EC50
Buffer	1174	639.3	-9.556	2.783e-010
MIA 10uM	655.4	474.9	-12.40	3.979e-013
HMA 10uM	502.9	335.5	-8.174	6.693e-009
Zoniporide 10uM	697.7	438.6	-8.502	3.146e-009

Figure 3.4: Allosteric effect of Na+ inhibitors with DOR endogenous agonists for the cAMP signaling pathway. HEK 293T cells were transiently transfected with the DOR and either pre-incubated with MIA, HMA, Zoniporide or not, which is shown as a buffer. The cells are then stimulated with the two DOR endogenous ligands, Leu-enkephalin (A) or Met-enkephalin (B). The effect of the three allosteric modulators was the same with both ligands where MIA, HMA, and Zoniporide have a significant agonist-PAM activity that is shown by red, green, and violet curves, respectively. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

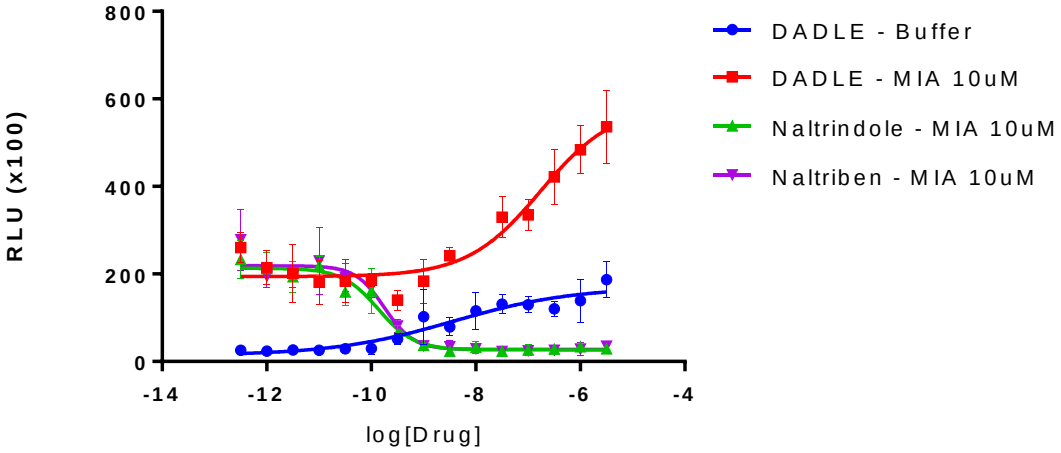
3.3.3 Opioid Receptor Antagonist/Weak Inverse Agonist Reverse the Agonist-PAM Activity by MIA and HMA.

To validate the intrinsic agonist activity (Ago-PAM) that was observed by MIA and HMA in Tango experiments at DOR, the selective opioid receptor antagonists, Naltrindole, and Naltriben, were used. The HTLA cells were treated as described above in Tango assay (chapter 2). As usual, the DOR prototypic agonist (DADLE) was used as a reference (buffer), which indicates the effect of DADLE alone without MIA or HMA. As shown in **Figure 3.5 (A, B)**, the Ago-PAM effect of both MIA (A) and HMA (B) is shown as red curves. The two DOR antagonists with weak inverse agonist activity, Naltrindole, and Naltriben, were added after the pre-incubation with MIA or with HMA. As expected, the Ago-PAM activity of MIA and HMA were entirely reversed by Naltrindole (the green curve) or Naltriben (the purple curve).

These results suggest that both amiloride derivatives MIA and HMA stabilize a partially active state of the receptor which can be antagonized non-competitively by Naltrindole and Naltriben. These results strongly agree with the literature that has shown that the two-opioid antagonists, naltrinole and naltriben, stabilize the inactive state of the receptor. These data also support the direct effect of the drug at the receptor and exclude any action on the assay used.

Figure 3.5

A


800
600
400
200
0
RLU (x100)
-14
-12
-10
-8
-6
-4
log[Drug]
DADLE - Buffer
DADLE - MIA 10uM
Naltrindole - MIA 10uM
Naltriben - MIA 10uM

B

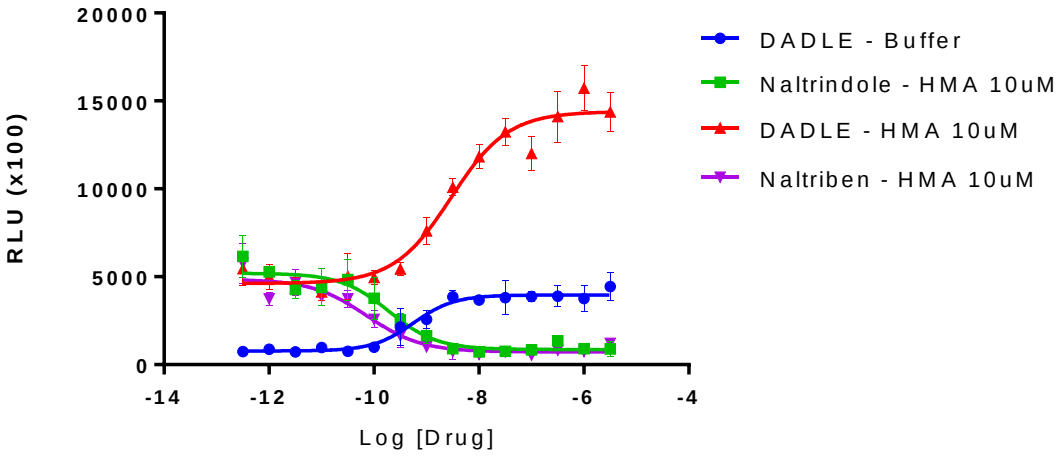

20000
15000
10000
5000
0
RLU (x100)
-14
-12
-10
-8
-6
-4
Log [Drug]
DADLE - Buffer
Naltrindole - HMA 10uM
DADLE - HMA 10uM
Naltriben - HMA 10uM

Figure 3.5: MIA and HMA agonist-PAM activity stabilizes a partially active state conformation of the receptor. The agonist effect generated by MIA (A) and HMA (B) in the absence of DADLE is completely reversed by Naltrindole (green curve) or Naltriben (purple curve). The inhibition of the agonist-PAM effect of MIA and HMA by selective antagonists (weak inverse agonist) indicates the stabilization of the partially active state and the specific activation of the receptor. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

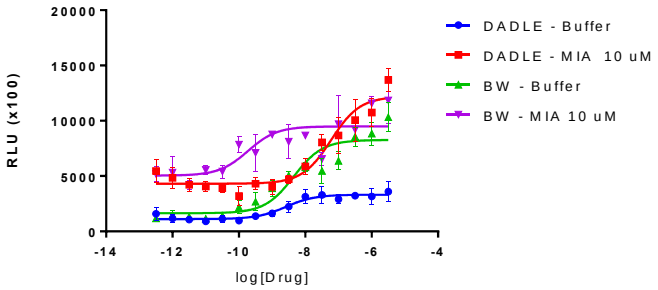
3.3.4 Probe Dependence of Na^+ Inhibitors at Different DOR Agonists for the β-arrestin Recruitment

Allosteric modulators possess different properties. For instance, the effect of an allosteric modulator is inherently limited and has to be saturable. Moreover, it is quite well accepted that the allosteric activity characterizes the interaction pair of ligands – the same allosteric ligand can modulate different orthosteric ligands to different extents. This phenomenon is called probe dependence [89]. To test this probe dependency for the β-arrestin recruitment at activated DOR; the three Na+ inhibitors were tested on HTLA DOR-T stable cells and then stimulated with several DOR agonists chemotypes; BW373U86, IBNTXA, SNC80, and diprenorphine. Dose-response curves of DADLE are shown with all DOR agonists, and they are used as references for the allosterism mediated by the sodium cavity, which is illustrated as a buffer with or without the three allosteric modulators. The data presented in **Figure 3.6 (A, B, C)** shows the effect of DADLE (buffer) without Na^+ inhibitors. The Ago-PAM activity of MIA and HMA. Also, the PAM effect of all our compounds are shown as red curves in the present of DADLE at the three graphs. These data were obtained by plating the HTLA DOR-T stable cell line in the 384 plates, where half the plate was stimulated with DADLE and the other half with BW after 15 minutes of pre-incubation with MIA, HMA, or Zoniporide. An important increase in the efficacy of BW373U86 was observed in the absence of allosteric modulators (BW-Buffer) compared with DADLE (DADLE-Buffer). This difference in efficacy of BW373U86 vs DADLE is not published but has been repetitively observed in our lab. BW373U86 is thus a super-agonist for the arrestin pathway [121]. In term of allosterism, MIA (A) and HMA (B) -

which are illustrated by violet curves for both - show the Ago-PAM activity with BW373U86 which is similar to the Ago-PAM effect that was demonstrated with DADLE previously. Importantly, both amiloride derivatives showed a significant increase in the potency of BW373U86 (the violet curves) observed by up to 3 log left-shifted curves with no change in the efficacy as observed in **Figure 3.6 (A and B)**. As BW373U86 is a super-agonist, we do not observe the PAM activity of MIA and HMA as observed with the reference DADLE. For the non-amiloride small molecule Zoniporide, neither the Ago-PAM nor PAM activity of Zoniporide was observed in the presence of BW373U86; however, there was a one logged left-shift increase in the potency as seen in **Figure 3.6 (C)**.

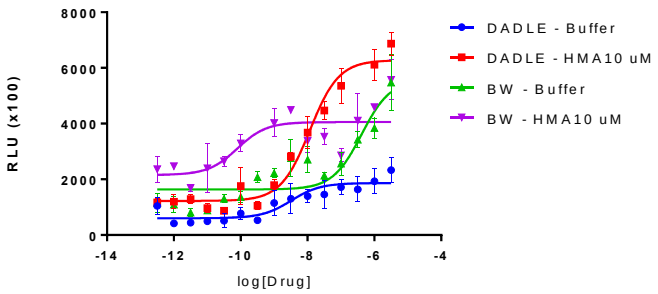
Figure 3.6

A



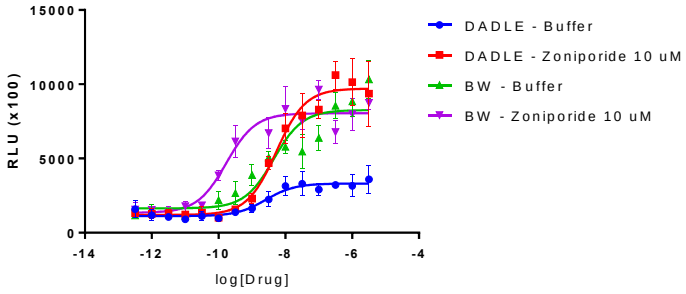
	Bottom	Top	LogEC50	EC50
DADLE - Buffer	1113	3314	-8.588	2.585e-009
DADLE - MIA 10uM	4311	12189	-7.223	5.982e-008
BW - Buffer	1638	8265	-8.350	4.471e-009
BW - MIA 10 uM	5039	9490	-9.728	1.870e-010

B



	Bottom	Top	LogEC50	EC50
DADLE - Buffer	604.2	1866	-8.489	3.243e-009
DADLE - HMA10uM	1224	6278	-7.933	1.168e-008
BW - Buffer	1635	5590	-6.382	4.153e-007
BW - HMA10uM	2155	4056	-10.10	7.957e-011

C



	Bottom	Top	LogEC50	EC50
DADLE - Buffer	1113	3314	-8.588	2.585e-009
DADLE - Zoniporide 10uM	1193	9714	-8.272	5.348e-009
BW - Buffer	1638	8265	-8.350	4.471e-009
BW - Zoniporide 10uM	1341	8044	-9.767	1.710e-010

Figure 3.6: MIA, HMA and Zoniporide increase potency of the superagonist BW373U86. Dose-response curves of DADLE with (red curve) and without (blue curve) the three allosteric modulators are shown with all DOR agonists being tested and are used as a reference. The allosteric effects on BW373U86 is absent for all of our allosteric modulators (BW-Buffer) which shown as green curves while those with the allosteric modulators as purple curves (graphs A, B, and C).

(A, B)- We found that MIA(A) and HMA(B) increase the potency of BW373U86 as observed by the 1 to 3 log left-shifted curve. There is no change in the efficacy because the PAM effect was not observed with both amiloride derivatives. Also, the Agonist-PAM activity of MIA and HMA was detected in the absence of superagonist BW373U86 at a drug concentration (-12.5) effectively zero.

(C)- The PAM effect of Zoniporide was not detected with BW373U86; only the potency was increased by one logged left-shift.

This is one experiment performed in quadruplicate that represent at least three repeated experiments.

Furthermore, the probe dependency of the Na^+ inhibitors, MIA, HMA, and Zoniporide was tested with another DOR agonist (IBNTXA) by performing a Tango assay experiment similarly to BW373U86. IBNTXA (3-iodobenzoyl naltrexamide) is known as a non-selective opioid agonist. It has a powerful painkiller activity without producing adverse effects such as constipation and respiratory depression but induces dysphoria by its action on the KOR [122]. IBNTXA is also a super-agonist as BW373U86, this activity is believed to be exerted through its long-lasting interaction with the receptor (low K_{off}). IBNTXA was found to react similarly to DADLE with only efficacy being modulated. Also, the Ago-PAM activity of MIA and HMA (violet curves) was observed with IBNTXA as shown in **Figures 3.7 (A and B)**. We do not, however, observe the PAM activity with Zoniporide at IBNTXA as demonstrated in **Figure 3.7 (C)**.

Figure 3.7

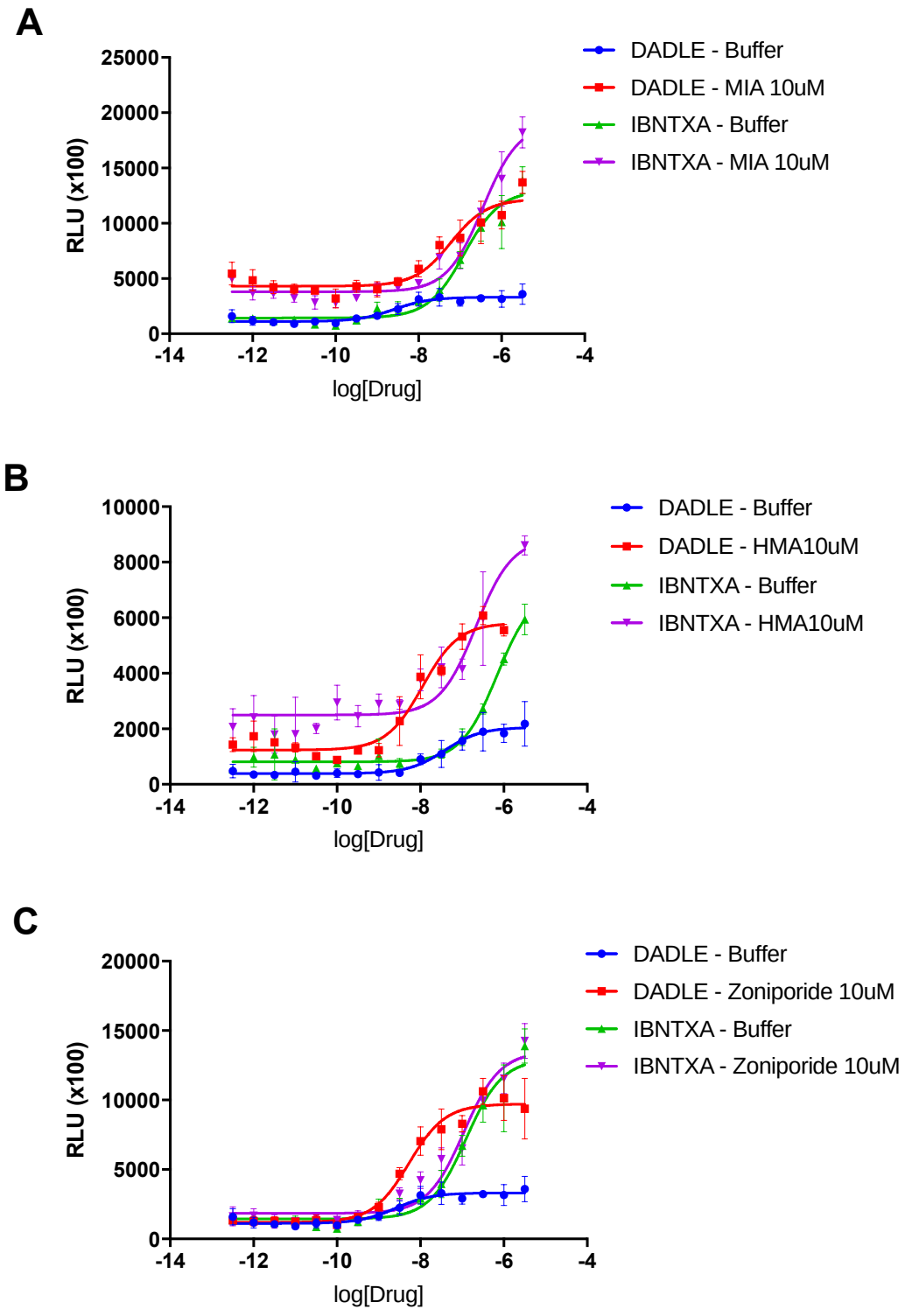

A
RLU (x100)
log[Drug]
DADLE - Buffer
DADLE - MIA 10uM
IBNTXA - Buffer
IBNTXA - MIA 10uM
B
RLU (x100)
log[Drug]
DADLE - Buffer
DADLE - HMA10uM
IBNTXA - Buffer
IBNTXA - HMA10uM
C
RLU (x100)
log[Drug]
DADLE - Buffer
DADLE - Zoniporide 10uM
IBNTXA - Buffer
IBNTXA - Zoniporide 10uM

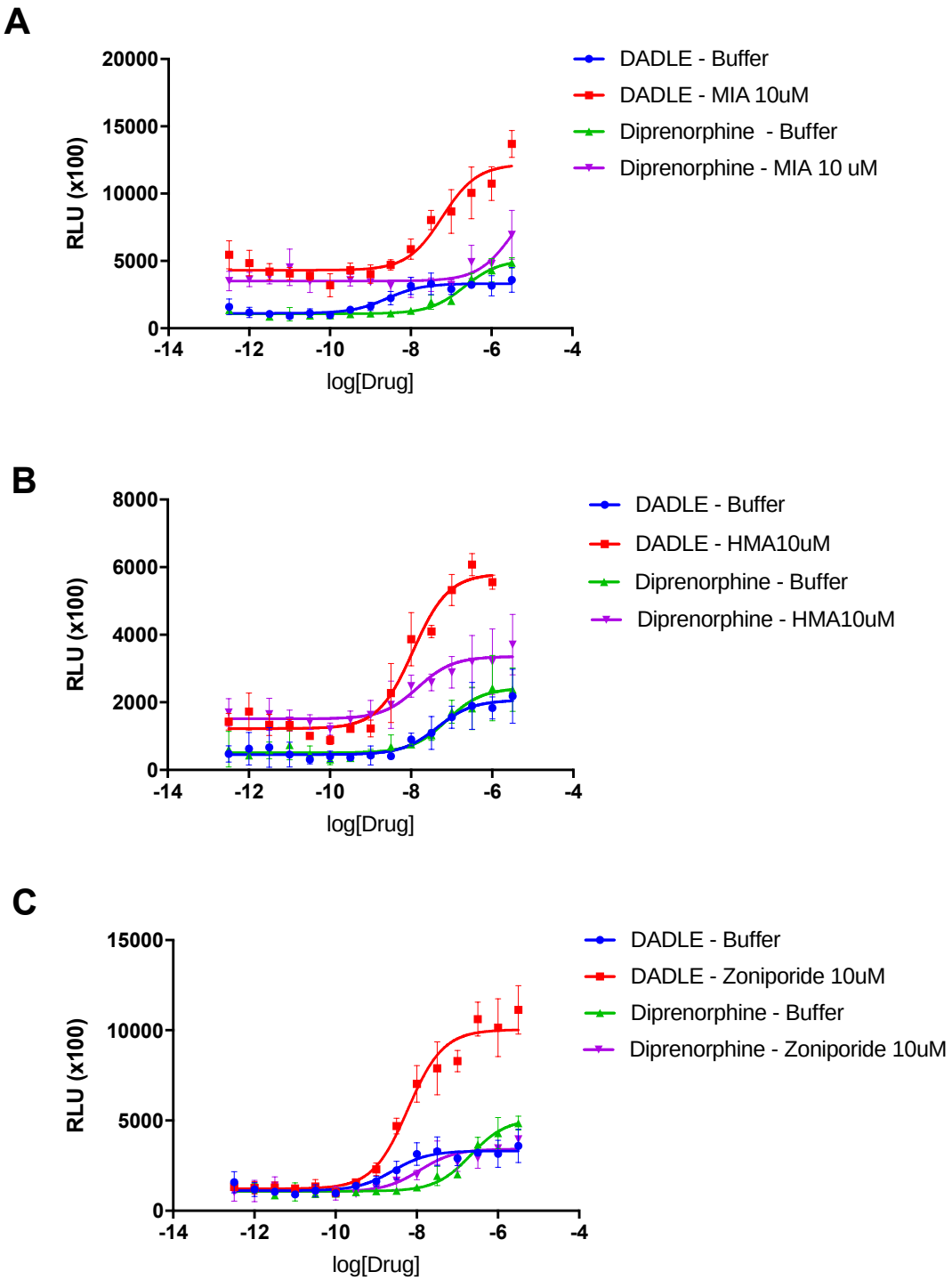
Figure 3.7: Agonist dependency of the three allosteric modulators with IBNTXA. The same Agonist-PAM effect and PAM activity of MIA (A), and HMA (B) was observed in the presence of DOR non-selective IBNTXA agonist as DADLE. There is no allosterism detected with Zoniporide at DOR activated by IBNTXA as shown in figure 3.7 (C).
This is one experiment performed in quadruplicate that represent at least three repeated experiments.

The probe dependence of Na^+ inhibitors at another DOR agonist was verified with the same set of functional experiments. Therefore, the allosteric activity of Na^+ inhibitors was also tested at diprenorphine, which is one of the most potent narcotic analgesics. In fact, diprenorphine is known as the most potent commercially available mixed-opioid [123]. This drug is only approved for veterinary use and is not used in humans because the safe dose for a human is so small that it would be difficult to control. For this reason, there is an extreme risk of overdose leading to fatal respiratory depression [124]. In **Figure 3.8 (A-C)**, the dose-response curve of DADLE was shown in all the three graphs to compare the DOR prototype DADLE and diprenorphine. The Ago-PAM activity of MIA **(A)** and HMA **(B)** was observed with diprenorphine while no critical PAM activity was detected. However, Zoniporide **(C)** does not have any allosteric activity at DOR stimulated with the super potent diprenorphine.

The important, and so far, unique increase the potency of BW373U86 by MIA, HMA, and Zoniporide lead us to test a very similar compound named SNC-80. Numerous compounds with similar scaffolds were generated long ago based on BW373U86. The SNC80 was found to be the most active nonpeptidic compound synthesized from BW373U86 and shows a significant selectivity profile to the DOR [125]. The preclinical studies have emphasized the pharmacological use of the SNC80 to reduce chronic pain, but it has also been found to induce a mild epileptic seizure in rodents [126]. **In Figure 3.9**, the dose-response curve of DOR agonist SNC80 (Buffer) represents the effect of SNC80 alone. The allosteric activity of MIA, HMA, and Zoniporide was tested with the same Tango assay protocol. We observe one log left-shifted curve (increased potency) with HMA and Zoniporide for SNC80,

which was less than what we had seen with BW373U86. The Ago-PAM activity of MIA and HMA was still present with no PAM activity detected with DOR activated by SNC80.

Figure 3.8


A
20000
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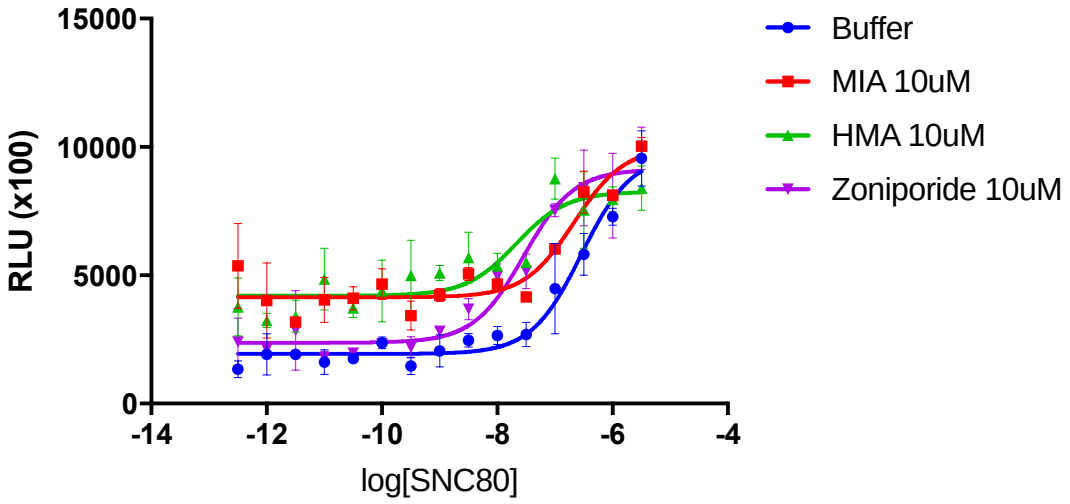
10000
5000
0
RLU (x100)
-14
-12
-10
-8
-6
-4
log[Drug]
DADLE - Buffer
DADLE - MIA 10uM
Diprenorphine - Buffer
Diprenorphine - MIA 10 uM
B
8000
6000
4000
2000
0
RLU (x100)
-14
-12
-10
-8
-6
-4
log[Drug]
DADLE - Buffer
DADLE - HMA10uM
Diprenorphine - Buffer
Diprenorphine - HMA10uM
C
15000
10000
5000
0
RLU (x100)
-14
-12
-10
-8
-6
-4
log[Drug]
DADLE - Buffer
DADLE - Zoniporide 10uM
Diprenorphine - Buffer
Diprenorphine - Zoniporide 10uM

Figure 3.8: Agonist dependency of MIA, HMA, and Zoniporide with diprenorphine. The super potent diprenorphine was tested with the three allosteric modulators for β-arrestin recruitment**.** The Ago-PAM activity of MIA (A) and HMA (B) was observed with diprenorphine while no significant PAM activity was noticed. Zoniporide (C) has no positive allosteric activity with the diprenorphine at activated DOR. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

Figure 3.9



	Bottom	Top	LogEC50	EC50
Buffer	1938	9698	-6.54	2.884e-007
MIA 10uM	4144	10001	-6.663	2.171e-007
HMA 10uM	4208	8255	-7.67	2.14e-008
Zoniporide 10uM	2372	9135	-7.54	2.885e-008

Figure 3.9: Agonist dependency of MIA, HMA, and Zoniporide at DOR selective agonist SNC80. The closest BW373U86 analog is SNC80, the allosteric activity of Na+ inhibitors was identified in the presence of the agonist SNC80 for arrestin recruitment. The Ago-PAM activity of MIA and HMA was observed with SNC80 which like what is we have observed with DADLE and BW373U86 previously. Also, we have found that HMA and Zoniporide produce a moderate increase in potency with a maximum of one log left-shifted which is represented as the green and purple curves, respectively. The PAM activity of all our novel small molecules was not significant. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

3.3.5 Evaluation of the quality of the interaction between Na^+-inhibitors and DOR by doing the Schild plot analysis.

Schild plot analysis (not to confuse with Schild regression) is a pharmacological method used to characterize the interrelation between two drugs acting at the same receptor. It consists of an experiment in which a fixed concentration of one ligand is used along with a graded concentration of the other ligand. Schild plot can be used *in vitro* in binding experiment and thus gives an indirect measure of the affinity of a ligand toward another ligand-receptor complex. It can also be used in a functional assay where the effect at the efficacy and potency can be observed. In that case, it is particularly useful to evaluate the quality of the interaction via the effect of the efficacy. For instance, in the presence of a pure antagonist, the dose-response curve of an agonist will be shifted to the right without affecting the efficacy as the pure antagonism will solely compete for the binding of the agonist, thus not affecting the quality of the interaction but rather only the potency by the reduction of the number of active sites. The Schild plot, when performed at equilibrium, can be used to estimate the affinity constant K_B of this ligand.

Schild plot analysis can also be used to study allosteric modulators or non-competitive inhibitor. In that case, the effect of an allosteric modulator on the efficacy and potency of an orthosteric agonist can be estimated. However, the change of affinity will be cooperatively modulated (negatively or positively) by a factor α (αK_B) while the efficacy (E) will be cooperatively modulated by a factor β (βK_E). Under perfect circumstances, these constants can be estimated using a derivative of the Gaddum equation:

Log (DR-1) = Log[B]-Log K_B

KB is the concentration that binds to half of the receptor
DR is the dose ratios which is usually calculated from EC50
[B] is the concentration of antagonist

In the present study, the KB value represents the approximate concentration of allosteric modulators that gives 50% of the maximum allosteric effect at activated DORs. To estimate the KB value for the Na^+ inhibitors, the DOR receptors stably expressed HTLA cells were pre-incubated with increasing concentrations of small molecules MIA, HMA or Zoniporide and then stimulated with different DOR agonists: DADLE, BW, and SNC80. The Tango assay was used to quantify β-arrestin recruitment at activated receptors by exploiting the G-protein independent amplification system. The dose-curve response of DOR agonists; DADLE (A), BW373U86 (B) or SNC80 (C) with allosteric modulators are shown in **Figure 3.10 (A-C)**. As expected, we found that the efficacy of DADLE was increased with increasing concentrations of HMA, MIA or Zoniporide at a range of 0.1 µM to 10 µM. The highest increase in the efficacy was obtained at 10 µM without any cell toxicity. In the case of BW373U86 and SNC80, there is an increase in the potency, observed by the left shifted curves, in addition to the increase in the efficacy, with increasing concentrations of allosteric modulators. Also, this type of experiment would provide information about the optimal concentration of Na^+ inhibitors that should be used without any toxicity by calculating the K_B value.

Figure 3.10

A

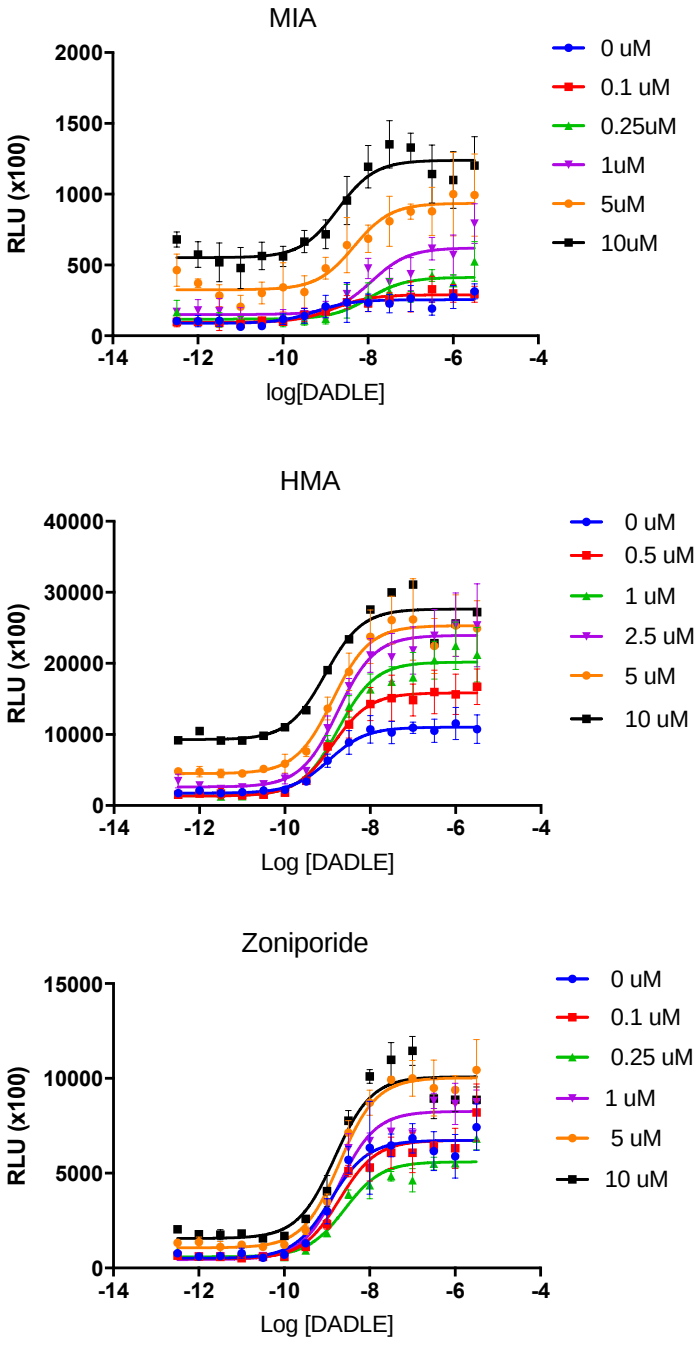

MIA
RLU (x100)
2000
1500
1000
500
0
-14
-12
-10
-8
-6
-4
log[DADLE]
0 uM
0.1 uM
0.25uM
1uM
5uM
10uM
HMA
RLU (x100)
40000
30000
20000
10000
0
Log [DADLE]
0 uM
0.5 uM
1 uM
2.5 uM
5 uM
10 uM
Zoniporide
RLU (x100)
15000
10000
5000
0
Log [DADLE]
0 uM
0.1 uM
0.25 uM
1 uM
5 uM
10 uM

Figure 3.10

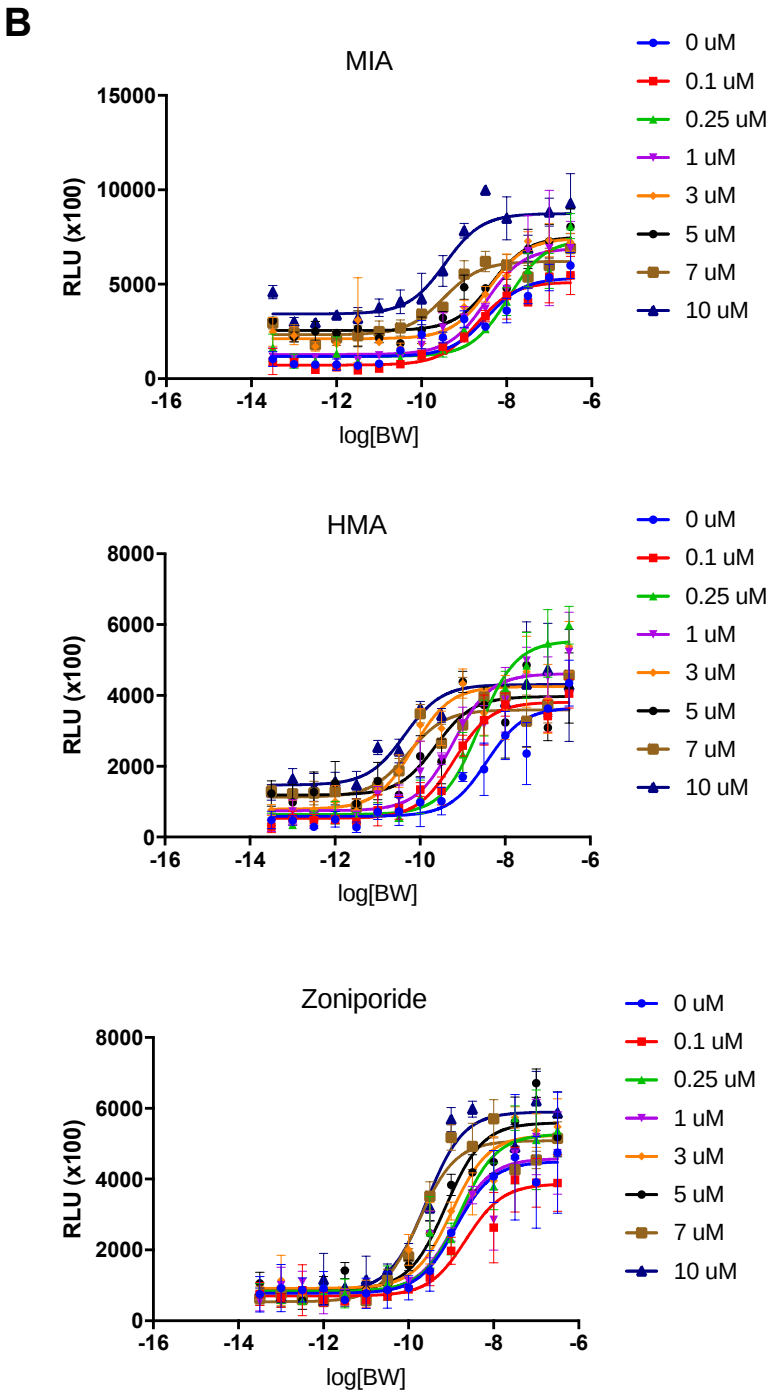
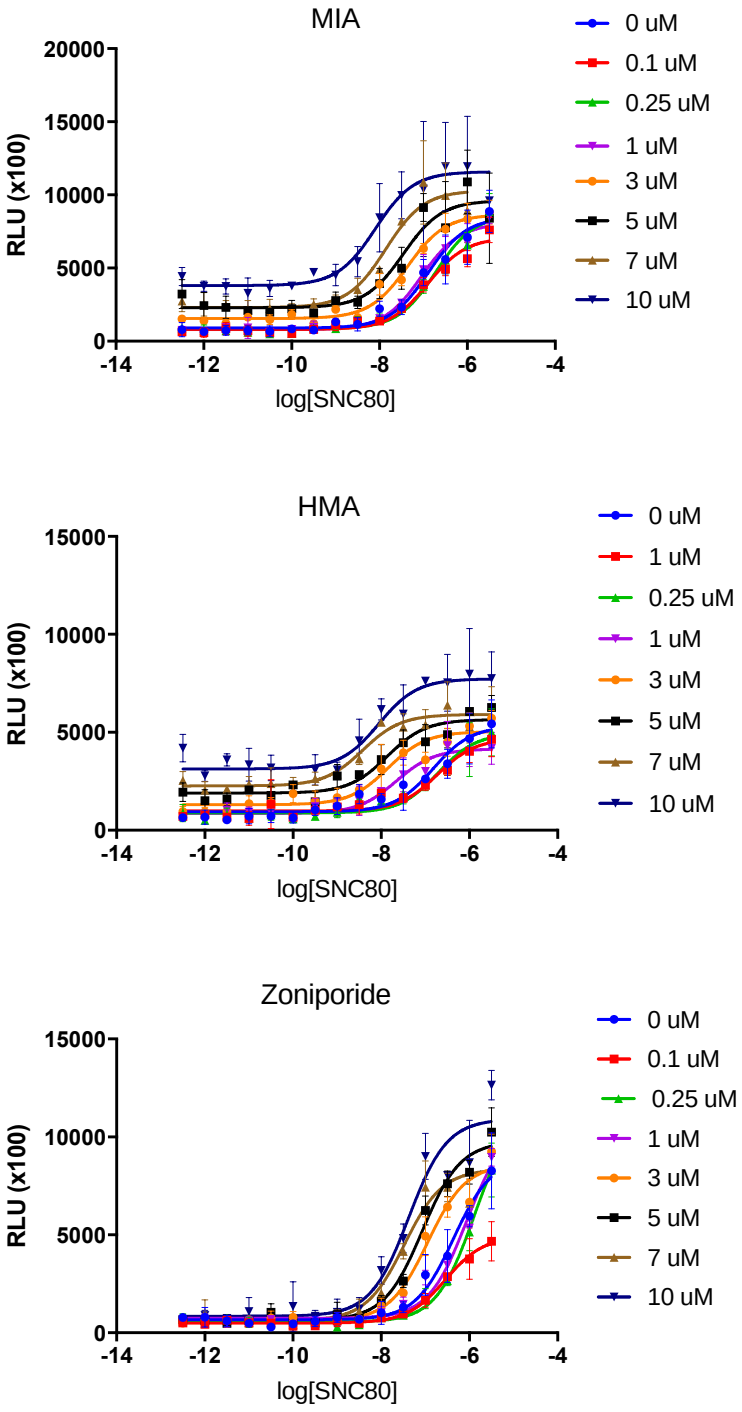

B
MIA
RLU (x100)
15000
10000
5000
0
-16
-14
-12
-10
-8
-6
log[BW]
0 uM
0.1 uM
0.25 uM
1 uM
3 uM
5 uM
7 uM
10 uM
HMA
RLU (x100)
8000
6000
4000
2000
0
log[BW]
Zoniporide
RLU (x100)
log[BW]

Figure 3.10

C


MIA
RLU (x100)
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10000
5000
0
-14
-12
-10
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-6
-4
log[SNC80]
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0.1 uM
0.25 uM
1 uM
3 uM
5 uM
7 uM
10 uM
HMA
RLU (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
log[SNC80]
0 uM
1 uM
0.25 uM
1 uM
3 uM
5 uM
7 uM
10 uM
Zoniporide
RLU (x100)
15000
10000
5000
0
-14
-12
-10
-8
-6
-4
log[SNC80]
0 uM
0.1 uM
0.25 uM
1 uM
3 uM
5 uM
7 uM
10 uM

Figure 3.10: Schild analysis of the allosteric modulators with DADLE, BW373U86, and SNC80 on β-arrestin recruitment at DOR. HTLA DOR-T cells were pre-incubated with increasing concentrations of allosteric modulators MIA, HMA or Zoniporide in the range of 0.1 µM to 10 µM. Then, the cells were stimulated with increasing concentrations of the different DOR agonists DADLE **(A)**, BW373U86 **(B)** or SNC80 **(C)**. Increasing concentration of the allosteric modulator leads to an increase in the efficacy of the activated receptor without any observable toxicity. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

3.3.6 Structure-Activity-Relationship (SAR) Study of Allosteric Sodium Cavity for DOR

Over the past four decades, the physiological concentration of sodium ion was found to alter the opioid ligand binding and signaling by reducing the agonist affinity. The molecular basis for the allosteric effect of sodium on opioid receptors function was mysterious. Therefore, the unknown mechanism by which the sodium ion stabilizes the inactive state of the opioid receptor in binding mode makes crystallization of many receptors including the δ-opioid receptor at 1.8Å high resolution essential. These studies allow us to identify the critical amino acid residues that are essential for the allosteric effects of sodium and our Na+ inhibitors on delta- opioid receptor. Thus, from these crystallization studies, the four amino acids residues found to coordinate the sodium ion directly in human DOR are N131, D95, N310, and S135. These residues were found to regulate the receptor function at DOR. For instance, mutation of D95, N310, and S135 were found to reverse antagonist to agonist. This efficacy switch is believed to be caused by the breaking of this internal ionic lock and reduce the energy barrier towards the active conformation. Moreover, the N131 mutant leads to a lock-on receptor that is constitutively active for the arrestin recruitment but can't be reversed by antagonist nor inverse agonist ([2] and unpublished data). About 15 residues lined the Na^+ cavity, whereas 5-8 residues, depending on the receptor, are involved to form a network of H-bonds or ionic interactions. Those 15 residues are very well conserved in class A GPCRs (>95 %), highlighting their structural importance. It is well established in the GPCR field that mutations of highly conserved amino acids, most of the time, change the receptor activity on its own. However,

without the crystal structure of the receptor bound to the ligand of interest, biochemical studies remain the tool of choice but have to be taken with care.

Thus, mutagenesis studies of these residues lining the cavity could be used to identify the critical amino acid residues that are essential for the interaction and function of our compound tools. To perform the structure-activity-relationship (SAR) study; site-directed mutagenesis was carried out where single point mutation of the three critical amino acid residues in the sodium pocket (D95, N67, and S135) was mutated to alanine (D95A, N67A, S135A). Moreover, their influence on agonist, antagonist, and allosterism by sodium inhibitors was explored. We decided to test whether those amino acid residues could regulate the activity of our three allosteric modulators; MIA, HMA, and Zoniporide targeting the sodium cavity. For this, HTLA cells were transfected with wild-type δ-opioid receptors, and DOR mutants. On the next day, cells were stimulated with MIA, HMA, or Zoniporide followed by stimulation with DOR agonist DADLE or opioid antagonist Naltrindole (revert to agonist with some mutants). The dose-response curve of DADLE or Naltrindole is presented in **Figure 3.11 (A)** with DOR-T wild type as a reference and was presented before. The blue curves (buffer) represent the effect of DADLE or Naltrindole in the absence of the allosteric modulators where as the allosteric activity (Ago-PAM, PAM) of our molecules were present as indicated. The same experimental approach was used to test the allosterism of MIA, HMA and Zoniporide at the three DOR-T mutants which are shown in **Figure 3.11 (B-D)**. **Figure 3.11(B)** shows the result for the mutant S135A. We observed a complete loss of DADLE efficacy as shown by a flat curve (buffer, blue curve). Interestingly, we still observed the agonist-PAM activity of MIA

and HMA, as well as the PAM activity of MIA, HMA and Zoniporide. This later result supports strongly the allosteric modulation of our compounds as the DADLE by itself has no efficacy, but in the presence of our modulators, we observe a dose-response curve. **Figure 3.11 (B)** shows the same experiment but with Naltrindole instead of DADLE. This Na^+-site mutant converts the antagonist Naltrindole into an agonist as shown as a dose-response curve for the buffer (blue curve). This is not surprising as it was observed previously for the other Na^+ site mutants as published [2]. We also observed the agonist-PAM activity of MIA and HMA as for the left panel. Interestingly, none of the 3 modulators have PAM activity with Naltrindole. This indicates that even if we convert the Naltrindole to potent agonist with this mutant, it does not behave completely as an agonist.

In the case of D95A, the Agonist-PAM activity of MIA and HMA is lost while the PAM effect of Na^+ inhibitors is barely present with DADLE. For the antagonist Naltrindole convert to agonist as described before, we did not observe any PAM activity at the efficacy level but did observe an important left-shifted curve caused by an increase in the potency of the Naltrindole **Figure 3.11 (C)**.

N67A was the third mutant studied. This mutant was tested for its very well-known critical function for GPCR activation but also for its possible salt-bridge formation with D95(2.50) once the receptor is activated. As shown in **Figure 3.11(D),** the N67A mutant completely abolishes receptor activation with the agonist DADLE. We also noticed that there are no agonist-PAM or PAM activities for all three allosteric modulators (AM) with DADLE. Surprisingly, Naltrindole also became an agonist as observed with the two previous Na^+-coordinating mutants (D95A and S135A).

Moreover, the Ago-PAM activity for amiloride derivatives was fully lost while the PAM activity was still present with all AM. Thus, we predicted that N67 could be implicated in the interaction with those Na^+ inhibitors to the sodium pocket.

Figure 3.11

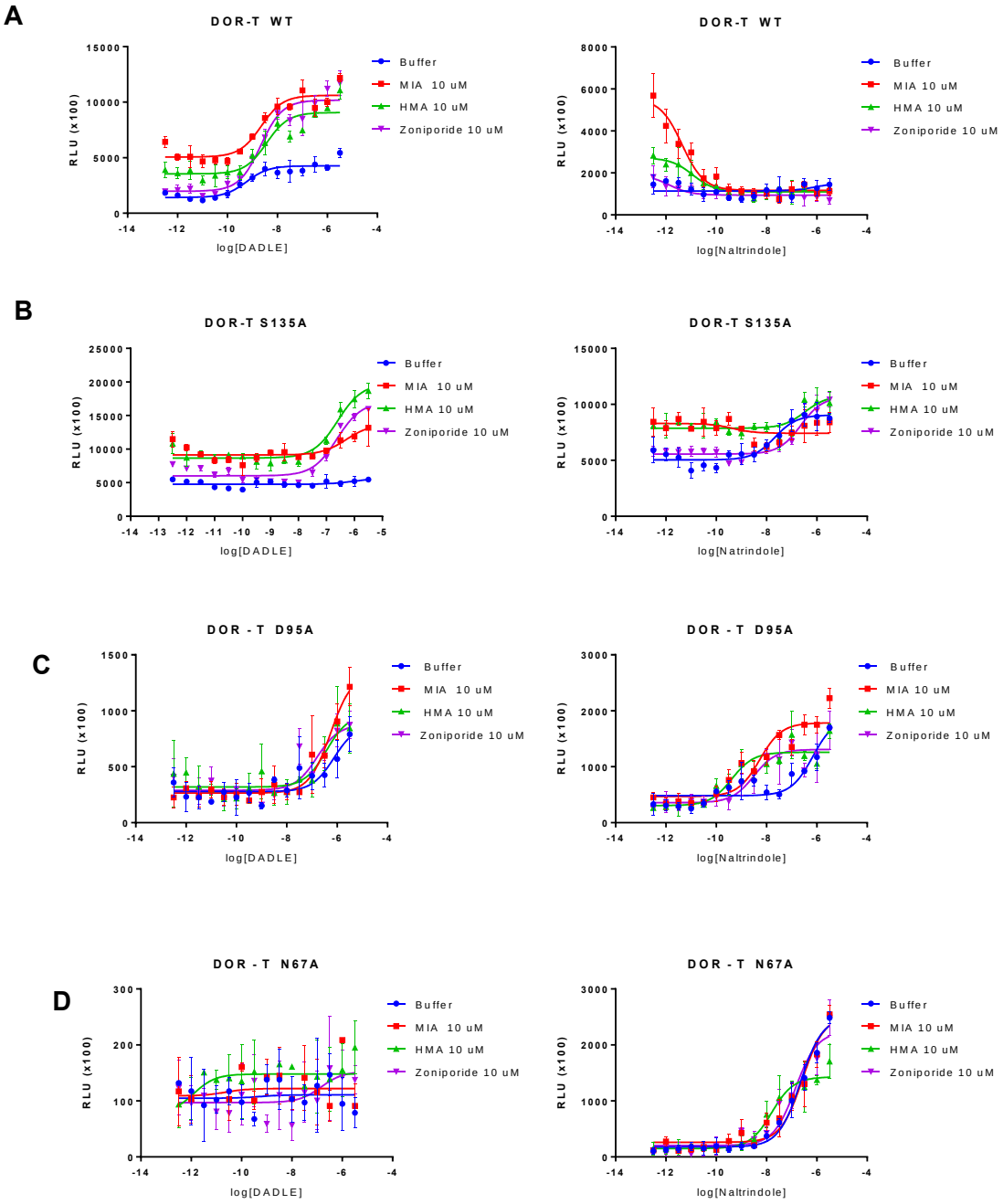

A
DOR-T WT
RLU (x100)
log[DADLE]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10 uM
DOR-T WT
RLU (x100)
log[Naltrindole]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10 uM
B
DOR-T S135A
RLU (x100)
log[DADLE]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10 uM
DOR-T S135A
RLU (x100)
log[Natrindole]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10 uM
C
DOR - T D95A
RLU (x100)
log[DADLE]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10 uM
DOR - T D95A
RLU (x100)
log[Naltrindole]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10 uM
D
DOR - T N67A
RLU (x100)
log[DADLE]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10 uM
DOR - T N67A
RLU (x100)
log[Naltrindole]
Buffer
MIA 10 uM
HMA 10 uM
Zoniporide 10 uM

Figure 3.11: Structure-activity-relationship at the DOR for the three allosteric modulators. Mutagenesis of three amino acids located within or near the sodium pocket were performed and β-arrestin recruitment was measured for all three allosteric modulators in the presence of a dose-response curve of the agonist DADLE and the antagonist naltrindole. (A) DOR WT, (B) S135A, (C) D95A, (D) N67A. This is one experiment performed in quadruplicate that represent at least three repeated experiments.

3.3.7 Evaluation of the affinity of the interaction between Na^+ inhibitors and DOR.

3.3.7.1 Binding experiment; Saturation experiment to quantify the K_D

In our quest to better characterize our three allosteric modulators, we performed another set of experiments, known as binding experiments, to evaluate the affinity of the interaction between these allosteric modulators and DOR. First, saturation binding assay was carried out to determine the equilibrium dissociation constant (K_D) of the used radioligand ^{3}H-DADLE. HEK293T cells stably expressing DOR wild-type were used to prepare crude membranes as described in chapter 2. Briefly, the radioligand ^{3}H-DADLE was used in serial dilution to determine the total binding (Bmax) whereas a fixed concentration of antagonist Naltrindole 10µM was used to define the non-specific binding. Membranes were incubated for 2 hours at room temperature in the dark place and terminated by rapid vacuum filtration onto chilled 0.3% PEI-soaked GF/A filters followed by three quick washes with cold washing buffer. Solid scintillant Meltilex A is then melted onto the dry filter and the radioactivity was counted by using scintillation reader (MicroBeta). In **Figure 3.12 (A),** the **X-**axis represents the increasing concentration of hot ligand (^{3}H-DADLE), and the **Y**-axis shows the count per minute (CPM). The plateau obtained at higher concentration indicates the saturation of the total number of sites and is essential to calculate the K_D following the law of mass action K_D = Koff/Kon =[L][R]/[LR] whereas L is the ligand and R is the receptor. In **Figure 3.12 (B),** the specific binding is obtained by subtraction of the non-specific binding from the total binding. High concentration of Naltrindole to compete with all possible DADLE binding site. The remaining count are the non-specific interaction of the radioligand with the crudes membranes. Data were plotted with the

one-site nonlinear regression parameter using Graph Pad Prism software. The K_D was estimated at 1.6 nM for DADLE that falls within the normal range for this ligand.

Figure 3.12

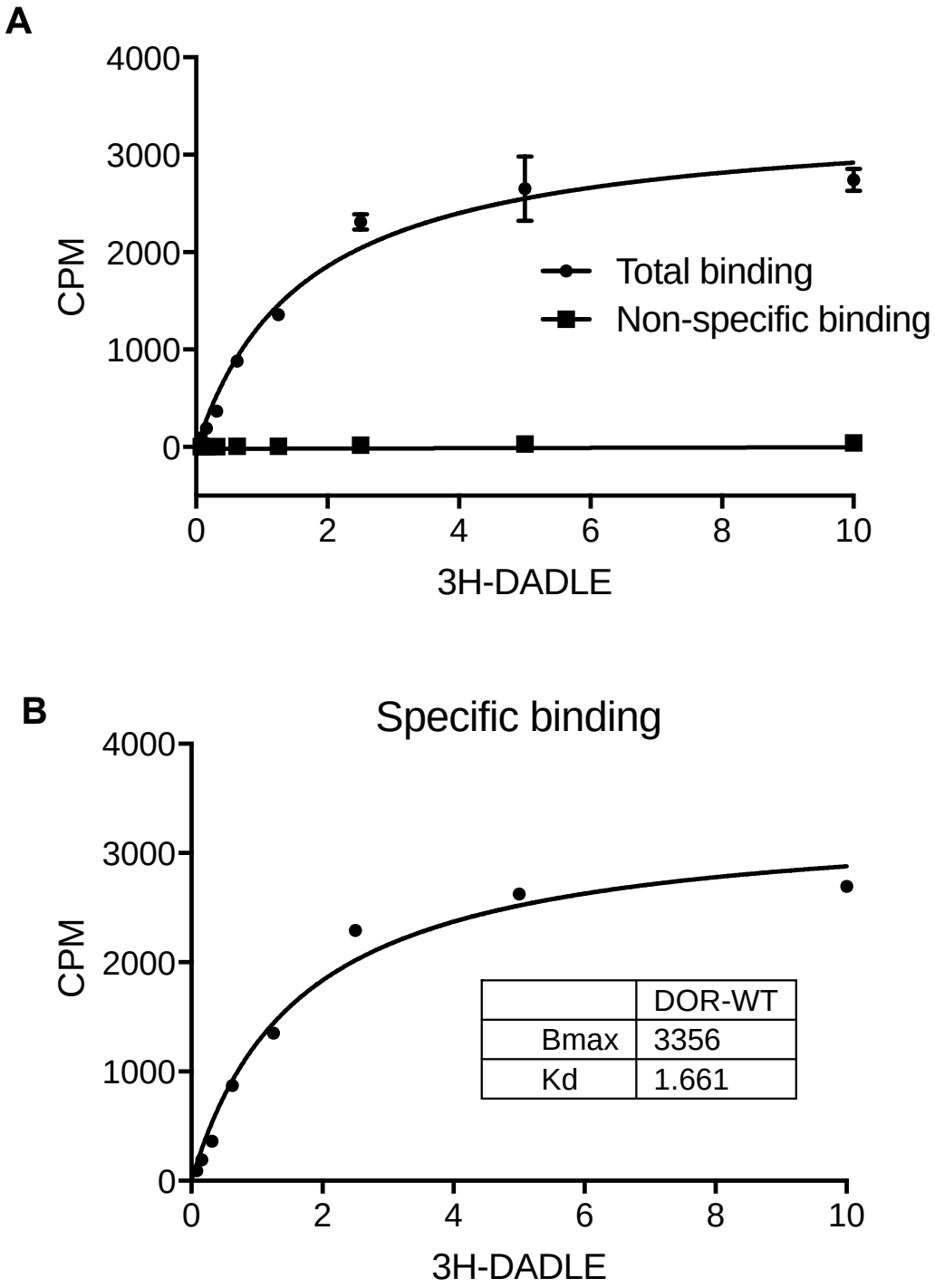

A
4000
3000
2000
1000
0
CPM
Total binding
Non-specific binding
0
2
4
6
8
10
3H-DADLE
B
Specific binding
4000
3000
2000
1000
0
CPM
DOR-WT
Bmax
3356
Kd
1.661
0
2
4
6
8
10
3H-DADLE

Figure 3.12: Saturation binding experiment to evaluate the K_D of ^{3}H-DADLE at DOR. Saturation binding experiment was performed on DOR expressing membranes to quantify the 3H-DADLE K_D. Increasing concentration of ^{3}H-DADLE was incubated with (non-specific binding) and without (total binding) 10 μM Naltrindole (A). Specific binding can be estimated by subtraction of non-specific binding from total binding (B). Data were plotted using one-site nonlinear regression to calculate the K_D.

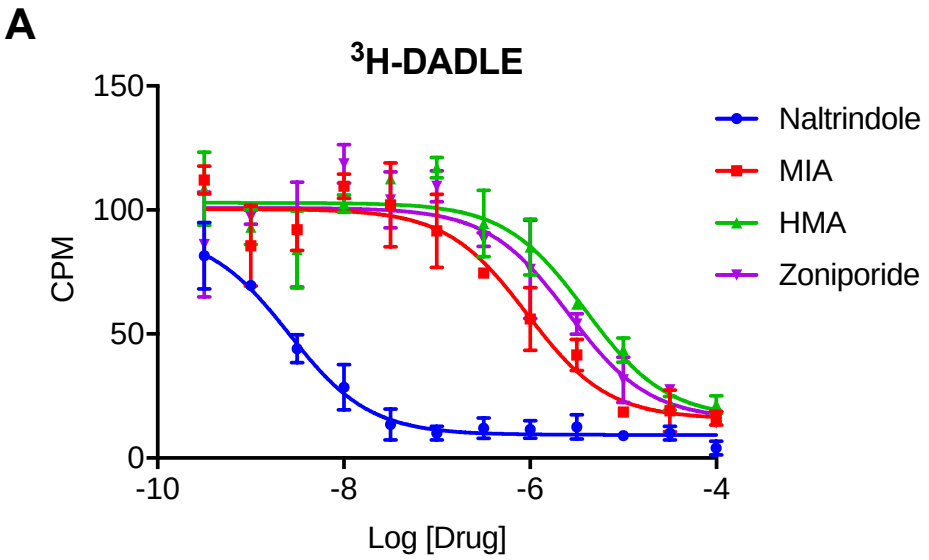
3.3.7.2 Binding experiment; Radioligand displacement with Na^+ channel inhibitors at DOR

Consequently, we decided to evaluate the binding affinity of these three allosteric modulators to the δ-opioid receptor. The saturation assay performed gave us the K_D value of the radioligand-ligand DADLE or Naltrindole (data not shown), which is essential to estimate the constant of inhibition (Ki) of an inhibitor following this formula: $K_i = IC50/1+([L1]/K_D)$ where IC50 is the inhibitory concentration that reduces by 50% the binding of the radioligand, KD is the dissociation constant of the radioligand, and [L1] is the concentration of the radioligand used. Ideally, allosteric modulators should be used in an allosteric mode in the binding experiment as performed for functional assay. However, our allosteric modulator does not change the potency and consequently the affinity of the agonist. Moreover, the allosteric mode in binding normally works solely with pure allosteric modulator – i.e allosteric modulator with no intrinsic activity – that is rarely the case. Preliminary experiments confirmed that the allosteric mode was not functional for this set of modulators, and thus we tested them in regular displacement assay. In such case, the allosteric modulator displaces the radioligand in a non-competitive manner. The displacement was thus performed using 1 nM ^{3}H-DADLE of radioligand with increasing concentrations of MIA, HMA, Zoniporide and Naltrindole as a positive control were tested.

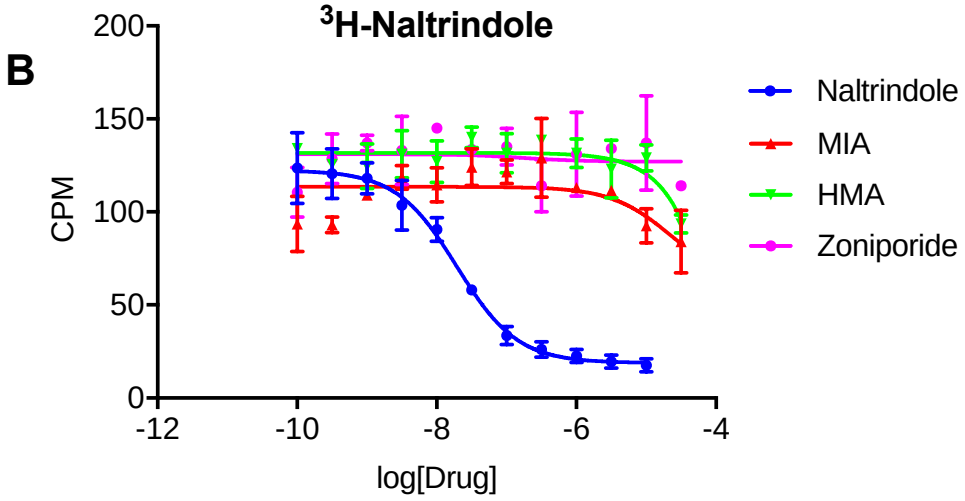
As seen in **Figure 3.13**, all three modulators displace the agonist radioligand with apparent K_i ranging from 500 nM to 1300 nM. It has to be noted that this does not represent neither a K_i nor a K_B and is sometime called apparent or observe rate

constant (K_{obs}). This value gives us an idea of the affinity and is a useful tool to compare the molecule of interest to a reference compound.

Figure 3.13 (A)



	Naltrindole	MIA	HMA	Zoniporide
Ki	1.285e-009	4.650e-007	1.970e-006	1.293e-006



	Naltrindole
Ki	9.7e-009

Figure 3.13: Radioligand displacement with Na^+ inhibitor at DOR

Radioligand binding experiment was performed using 1nM ^{3}H-DADLE as radioligand and displacement performed with all three modulators as well as with the antagonist Naltrindole as a reference. The K_i of Naltrindole was calculated at 1.3 nM whereas the apparent K_{obs} of MIA, HMA, and Zoniporide are ranging from 0.5μM to 1.3 μM.

Chapter 4: Discussion and Conclusion

4.1 Discussion

G-protein-coupled receptors (GPCRs) are protein receptors that have a distinctive arrangement of seven transmembrane helices and can transmit a large variety of extracellular stimuli into intracellular outcomes. Signal transduction triggered by GPCRs activation is mainly manifested by the canonical G-protein signaling and by the accessory protein β-arrestin. The human genome has more than 800 GPCRs, which are targeted by about half of the prescribed drugs making this type of receptor the most successful druggable target. Opioid receptors are one of the subfamilies under the γ-group within class A GPCRs. Opiates are widely used for the treatment of chronic pain, and addictive disorders. Given the difficulties associated with opioid therapy such as overdose, tolerance, addiction, respiratory depression and constipation, there is a need for safer analgesics. In the current research project, the focus has been on the δ-opioid receptors (DOR) which is one of the three classical opioid receptors with the μ- and κ-OR. Interestingly, DOR also showed great potential as an anti-depressor, and for the treatment of symptoms associated with involuntary movements in Parkinson's disease. However, the clinical use of DOR agonists is limited due to the generation of potentially life-threatening side effects. However, recent studies have revealed the presence of a sodium pocket in more than 95% of class A GPCRs including DOR. This sodium cavity was discovered as a binding site for sodium ion and water molecules. The high-resolution crystal structures of some GPCRs allow for designing some critical point mutations which disrupt the sodium

interaction within the sodium pocket. The results from these studies confirm that the binding of sodium ion stabilizes the inactive state of the receptor. During receptor activation by an agonist, the cavity undergoes important structural changes leading to sodium dissociation. The disruption of this intramolecular ionic lock was found to be critical for the β-arrestin recruitment with minor or less function toward G-protein activation. Therefore, the suggested functional importance of the sodium cavity as an allosteric site in receptor activation, and its exceptionally high conservation in GPCRs make it an attractive target for discovery of small molecules with unique functional and pharmacological properties. These properties can then be used to test our hypotheses and serve as a novel starting point for drug discovery. Although most drugs target GPCR orthosteric sites, exploration of allosteric binding sites provides several advantages, as it allows more precise control of subtype, functional selectivity and preserves the spatiotemporal profile of endogenous signaling, which holds great potential for the development of new tool compounds and candidate drugs [3] [4].

However, some of the initial insights into the allosteric Na+ pocket have been obtained with amiloride and its derivatives as non-selective allosteric modulators of GPCRs. Amiloride was discovered as a potent inhibitor of epithelial sodium channels and Na+/H+ exchangers [5], while its allosteric effect on GPCRs was of modest potencies (EC50>1μM). Since then, a few derivatives have shown comparable potency in several receptors [6, 95, 103-107]. The stabilizing effect of amiloride suggests that broadly selective allosteric ligands targeting this pocket may have a basic utility for conformational stabilization of GPCRs for use in crystallization and biochemical assay development targeting orphan GPCRs. At the same time,

extending highly selective orthosteric compounds to target the conserved allosteric pocket establishes a platform for the design of bitopic ligands that combine subtype selectivity with signaling properties that are useful for tool compounds and therapeutic indications. They can also serve as tools for studying cross-selectivity within the class A family and therefore establish a new path for developing drugs with ideal polypharmacology properties.

From early studies, sodium ion and amiloride show opposite allosteric modulation behavior when they bind to the allosteric site in several GPCRs. The studies conducted since 1998 showed the allosteric effect of amiloride in few GPCRs. Importantly, the effect of amiloride was found to be Na^+ sensitive, but the link between the Na^+ and amiloride was not known yet. However, all these evidences were from *In Vitro* binding studies using high concentration (>100µM) amiloride or derivatives. At this concentration, amiloride and its derivatives are toxic to cells. Furthermore, it is known from the crystal structure studies, that the sodium ion is coordinated by conserved side chains of D52 $^{2.50}$ and S91$^{3.39}$. Consistent with mutagenesis studies, in which the conserved sodium-coordination aspartic acid residue at position 2.50 mutated to alanine abolishes the allosteric effect of both sodium ion and amiloride in the binding experiments. However, these mutations also dramatically reduce orthosteric ligand affinity. In such case, the validity of those experiments is questionable. While ligand binding is a powerful biochemical tool, it relies on radioligand interaction. If the radioligand affinity is affected, the interpretation of the data for potential inhibitor is difficult to interpret. In this case, complementary studies have to be performed such as functional characterization. However, the low affinity of amiloride and its derivatives did not allow this approach due to toxicity. The

development of parallel screening tool such as the Presto-Tango assay, as open the door to the interrogation of a large set of GPCR simultaneously. Using the approach, Dr. Giguere found that few GPCRs where modulated by amiloride-derivatives at a non-toxic concentration (<10μM). The primary screening revels that DOR, MOR, and NOP opioid receptors were modulated to different extents and selectivity by amiloride-derivatives but surprisingly, the KOR was non-affected despite high structure and sequence homology with DOR and MOR.

As a result, once the high-resolution crystal structure of DOR was identified in 2014, we made the link between Na^+, amiloride, and D2.50. We hypothesized that the sodium pocket at DOR could act as an allosteric site for Na+ inhibitors which could be used as an allosteric modulator to modulate the functional selectivity of DOR.

Here, the current research project aims to use pharmacological and biochemical approaches to perform functional assays, binding experiments, and structure-activity-relationship studies (SAR) to test the druggability of sodium cavity and characterize the allosteric activity of sodium channel inhibitors at DOR. The best sodium channel inhibitors that were chosen from a panel for the present thesis are MIA, HMA, and Zoniporide. We believe that these small molecules target the sodium cavity and modulate the functional selectivity at activated DOR. However, MIA and HMA are amiloride derivatives, and they have been designed as high-affinity Na+ channel inhibitors compared to the parent molecule amiloride. It is important to mention that, amiloride itself has no activity at the DOR at a non-toxic concentration. Zoniporide is a selective inhibitor of Na^+-H^+ exporter (NHE1) and the first non-amiloride compound that, we believed, target the sodium cavity and can modulate the

functional selectivity of DOR. The chemical structures of these small molecules are shown in **Figure 1.10** chapter 1.

A representative experiment in **Figure 3.1** shows the effect of the three Na+-inhibitors; MIA, HMA, and Zoniporide on β-arrestin signaling pathway at activated DOR. The β-arrestin recruitment pathway activated by the synthetic DOR agonist DADLE (the blue curve), was assessed in the presence of a fixed concentration (10 μM) of the allosteric modulators MIA (the red curve), HMA (the green curve) and Zoniporide (the purple curve). These three allosteric compounds showed positive allosteric modulation (PAM) with DADLE as observed by the increasing efficacy if compare to DADLE alone. MIA and HMA have partial agonist activity in the absence of DADLE at a -12.5 concentration (which represents a zero-drug concentration), and this intrinsic activity is called Ago-PAM activity. This Ago-PAM activity is not observed with Zoniporide. Such intrinsic activity is often observed with allosteric modulators, especially as high concentration.

G-protein pathway (Glosensor assay) is another functional assay that we used to study the druggability of sodium site with Na^+ inhibitors on Gαi signaling pathway at activated DOR. The assay was used to see whether these Na^+ inhibitors have the same allosteric effect on the G-protein signaling as with β-arrestin recruitment pathway. In this experiment, the three Na^+ inhibitors MIA, HMA, and Zoniporide were tested on HEK293T cells which transiently express the DOR and Glosensor plasmids. These cells were pre-incubated with luciferin, followed the three small molecules and then stimulated with the DOR agonist DADLE as shown in **Figure 3.2.** After analyzing the data, similar ago-PAM activity of MIA and HMA was found for G-protein signaling

pathway as with the β-arrestin recruitment. The agonist effect of MIA (ago-PAM) was stronger than the ago-PAM activity observed with HMA. Also, we observed an agonist-PAM activity with Zoniporide, an activity absent in the β-arrestin recruitment.

However, the two-different allosteric profile; Ago-PAM and PAM allosteric activities that are produced by the Na^+ inhibitors on β-arrestin recruitment, were obtained not only in the presence of synthetic DOR agonist DADLE but also were obtained with the DOR endogenous agonists. We conclude that by repeating the same two functional experiments in the presence of DOR endogenous ligands, Leu-enkephalin and Met-enkephalin instead of DADLE. In **Figure 3.3 (A, B)**, the Ago-PAM and PAM activity of MIA, and HMA was observed on β-arrestin recruitment induced by Leu-enkephalin **(A)** or by Met-enkephalin **(B)**, which is similar to the allosteric effects of MIA and HMA produced with DADLE **(Figure 3.1)**. Zoniporide showed PAM effect only with both DOR endogenous agonists. In parallel with β-arrestin recruitment experiment, the allosteric effect of the three small molecules was also tested for cAMP signaling pathway (Glosensor assay) at activated DOR as seen in **Figure 3.4 (A, B).** In this experiment, the Ago-PAM effect was observed not only with MIA and HMA but also with Zoniporide as observed with the peptidomimetic DADLE.

To rule out any non-specific effect of the amiloride derivatives MIA and HMA that could explain the agonist activity observed, we tested whether we could reverse this agonist-PAM activity using the selective opioid receptor antagonists Naltrindole and Naltriben. As shown in **Figure 3.5**, the dose-response curve of DOR antagonist Naltrindole or Naltriben completely reversed the agonist activity of MIA and HMA. This result suggests that both Na^+ inhibitor MIA and HMA does activate the receptor and

this activation can be reserved by stabilizing the inactive conformation state of the receptor using an antagonist since it is known that Naltrindole, and Naltriben, favor and stabilize the inactive conformational state of the receptor.

The probe dependence of our compounds was evaluated on the β-arrestin recruitment at activated DOR by carrying out a set of Tango experiments. The allosteric effect of three small molecules was tested on HTLA DORT stable cells. Then the cells were stimulated with different DOR agonists including chemotype, BW373U86, IBNTXA, diprenorphine, and SNC80. Dose-curve responses of DADLE is shown with all DOR agonists, and it is used as a reference for the allosterism mediated by the sodium cavity which is illustrated as a buffer without (blue curve) or with the three allosteric modulators. In **Figure 3.6**, MIA (A) and HMA (B) show an important increase in the potency observed by an up to 3 log left-shifted curves. BW373U86 and its derivative are known superagonists for the β-arrestin recruitment and this could explain why this chemotype induce stronger side effects compared to most other DOR agonist *in vivo*. The binding mode of these compouds are believed to be different than morphinan and peptide agonists. Some computational modeling has been done but very few biochemical data were generated to support the model proposed. It is believed that BW373U86 could bind higher in the binding pocket with some overlap with the morphinan binding site and could involve interaction with the ECL2. Our lab is actually investigating the role of the ECL2 in the superagonist activity of this compound. However, we believe that BW373U86 probably reaches the maximum efficacy of the receptor that cannot be further activated by MIA, HMA or Zoniporide. The efficacy reached by BW373U86 is about the same as the DADLE with

the allosteric modulators. SNC80 is another DOR agonist, which was tested in the presence of Na^+ inhibitors on β-arrestin recruitment. The reason behind choosing this small molecule was the significantly increased potency of BW373U86 by MIA, HMA, and Zoniporide since the SNC80 is a very similar compound derived from BW373U86 and is an enantiomer of the planar BW373U86. In **Figure 3.9**, we observe a one log left-shifted curve (increased potency) for SNC80 with HMA and Zoniporide. This increase in potency was induced by some of the allosteric modulators in the present of SNC80 but was not strong as with BW373U86.

The combination of BW373U86 with the three allosteric modulators, make them the best tool compounds for crystallization trials that are underway. In the case of the non-selective opioid receptor agonist IBNTXA, it has an activity similar to DADLE as shown in **Figure 3.7 (A-C)** with the exception that the PAM activity was not observed with Zoniporide at IBNTXA.

Moreover, the probe dependency of Na^+ inhibitors was tested at the super potent analgesic drug, diprenorphine. The two amiloride derivatives were found to produce the same Ago-PAM effect while no significant PAM activity was detected in the presence of diprenorphine as shown in **Figure 3.8 (A, B).** Interestingly, the PAM allosteric activity of Zoniporide was not observed with this super potent drug as shown in **Figure 3.8 (C)**. However, diprenorphine, a morphinan, is known to bind the receptor differently than other morphinan opioids. The exact mechanism of interaction of diprenorphine is unknown, but it has been proposed that diprenorphine could bind the receptor with different recognition mode for the inactive and active receptor. Moreover, diprenorphine do not require the Asp3.32, a residue essential for the

interaction of all others opiates despite the fact that the Asp3.32 is required for its agonist activity. Those difference in the binding mode could explain the absence of allosterism and further investigation are underway in the lab.

To further characterize the binding of our allosteric modulators with the receptor, a set of Schild analysis experiments were performed with different DOR agonists. The experiment was carried out on β-arrestin recruitment (Tango assay), where the HTLA DOR-T stable cells were pre-incubated with increased concentrations of the Na^+ inhibitors; MIA, HMA or Zoniporide. The lowest concentration was 0.1 µM, and the highest concentration was 10 µM. Then the cells were stimulated with different DOR agonists DADLE (A), BW373U86 (B) or SNC80 (C) as observed in **Figure 3.10 (A-C)**. After analyzing the data, we found the efficacy of DADLE was increased by increasing the concentration of HMA and MIA with no toxicity observed. Zoniporide causes an increase of DADLE efficacy at the three highest concentrations only (1 µM, 5 µM, and 10 µM). Interestingly, the potency of BW373U86 and SNC80 was increased along with the increase in the efficacy, which was associated with an increased concentration of the allosteric modulators. Therefore, Schild analysis supports our previous data that these small molecules are not competing with DOR agonists and they bind with an allosteric site since there is an additive activity observed. Also, the allosteric constant K_B will be calculated by further repeating and optimizing this experiment, which will give us an idea of the exact concentration of Na^+ inhibitors that gives 50% of a maximum allosteric response. Our lab is working with biophysics to adapt the actual mathematical model. Indeed, the actual allosterism model is built for pure allosteric modulator with no

intrinsic activity. Given that our compounds MIA and HMA have intrinsic agonist-PAM activity, the actual formula does not fit our data.

Therefore, in the frame of the present study, to prove the allosteric activity of these small molecules and to confirm their binding to the sodium pocket, structural-activity-relationship (SAR) was performed. Since the high-resolution crystal structure of δ-opioid receptors was solved, it became easier to study and identify the critical amino acids residues that coordinate the sodium ion within the cavity. As a result, the last step in the present project was to identify the critical amino acid residues necessary for binding and activation of these small molecules within the allosteric sodium site. Also, the determination of essential amino acid residues within the sodium pocket could open the door to design better allosteric modulators targeting the sodium cavity.

For the above reasons, the site-directed mutagenesis was carried out to perform the SAR study. The Tango assay was performed on wild-type HTLA DOR-T stable cells as well on HTLA transiently transfected with DOR and mutants. The three mutants of critical amino acid residues were transfected are D95A, N67A, and S135A. The allosteric activity of three Na^+ inhibitors MIA, HMA and Zoniporide were tested on these mutants in the presence of DADLE, or Naltrindole. The dose-curve of DADLE and Naltrindole was used as a reference on wild-type DOR as shown in **Figure 3.11(A).** After stimulation, cells expressing the mutant S135A or D95A were stimulated with the modulators in the presence of DADLE. The partial agonist activity (Ago-PAM) produced by MIA and HMA is still observed with S135A, but not with D95A. The PAM effect of all the three compounds still appears in the presence of

DADLE for S135A and D95A while the efficacy of DADLE is lost with S135A. The presence of PAM activity of all three modulators with the S135A mutant strongly support the PAM activity as DADLE alone as no efficacy on this mutant.

As Naltrindole was found to reverse the ago-PAM activity of MIA and HMA at the wild-type receptor, we performed the same experiment at the mutants. As observed in **Figure 3.11 (B and C),** Naltrindole became an agonist for both mutants and consequently was not able to reverse the Ago-PAM activity of MIA and HMA for both mutants. Thus, the S135 residue can affect the efficacy of DADLE and Naltrindole which is called the efficacy switch but does not affect the allosteric activity induced by our allosteric modulators. Consequently, it is not involved in the interaction of these small molecules with the allosteric sodium site. In the case of D95A, there was a two log shift increase in the potency of Naltrindole in the presence of the Na^+ inhibitors. Thus, it has been suggested that the D95 residue is important for some activities of the small molecules to the allosteric site but not critical for the interaction.

Each highly conserved residue in Class A GPCRs designated X.50 have functional significance. Both Asn1.50 (N67 in DOR) and Asp2.50 (D95 in DOR) were previously found to be important for sodium allosterism. Arg3.50 is part of the conserved DRY motif important for receptor activation, Trp4.50 arguably stabilize the packing of TM2, 3,4 and Pro5.50, Pro6.50 and Pro7.50 introduce kinks into these helices and likely allow dynamic movement toward the intracellular side.

From the high-resolution structure, the N1.50 was found to be part of a water network linking $Y318^{7.53}$, $D95^{1.50}$ and the Na^+ ion. Disruption of this water network has been proposed to be an important feature of receptor control by Na^+.

The mutation of N67 to Alanine (N67A) was able to abolish the receptor activation and the allosteric activity of all the three Na+ inhibitors with the agonist DADLE entirely. As for D95A and S135A, Naltrindole with N67A is also converted to a low potency agonist. No Ago-PAM nor PAM activities was observed with all three derivatives **(Figure 3.11 D)**. For now, it is difficult to conclude whether or not N67 is involved in the interaction with our modulators. It is possible that N67A mutant disrupts receptor function to a point that our modulators can't activate the receptor. In an ideal world, we would like to have a mutant with no or weak effect at DADLE and Naltrindole while disrupting our modulators activities. Our quest is still undergoing, and we will now focus on amino acids deeper in the pocket that will probably disrupt the water network more than directly displacing the Na^+ ions as previously hypothesized.

Finally, we looked at the direct effect of our modulators on the receptors using radioligand binding experiments. Given that our modulators do not affect the affinity of DADLE, we were not expecting to have any major effect in binding. The first step was to characterize our membrane preparation and calculate the K_D of our radioligands using a saturation experiment. As it is often the case with allosteric modulators, our three modulators were found to displace the radioligand by themselves. Thus, we used displacement experiment using increasing concentration of our modulators to displace a fixed concentration of the radioligand ^{3}H-DADLE. As shown in **Figure 3.13 (A)**, all three allosteric modulators; MIA (red curve), HMA (green curve), and Zoniporide (purple curve) were able to displace the radioligand ^{3}H-DADLE. We believe that the displacement occurs via a non-competitive manner. As shown previously, the mutant S135A has no efficacy toward DADLE but gain efficacy in the presence of our modulators. Thus, both must bind to the receptor

simultaneously to get this effect. Moreover, at 10μM, all three modulators displace completely the bound ^{3}H-DADLE, whereas in, functional assay, we do observe PAM effect. Again, such observation is not unique for our modulators. For instance, the only allosteric modulator on the market for the DOR, called BMS-986187, was also found to displace the radioligand ^{3}H-diprenorphine [127], and similarly, the BMS-986122 do the same at MOR [128]. In both case, these modulators also display allosterism in a functional assay. The effect of BMS-986187 was also confirmed in our lab using ^{3}H-DADLE whereas full displacement is observed similarly to our modulators. In the case of BMS-986187, a left shift is also observed. Thus, at the same time, the BMS-986187 increases the affinity of the radioligand, but also displaces it. It should be noted that our allosteric modulators as well as the BMS-986187, only displace the radioligand agonist and not the antagonist ^{3}H-naltrindole.

The most rational explanations for such mechanism are that the allosteric modulator 1- changes the kinetic of interaction or 2- stabilizes a partially active state that is not favorable for the interaction with the agonist. Our lab is actually addressing those two hypotheses. First, we are trying to setup kinetic binding experiment. Once functional, we will calculate the K_{on} and K_{off} of ^{3}H-DADLE in the presence and absence of our modulators. This experiment was found to be technically challenging as the equilibrium is reached within 10 minutes. We are thus exploring a live kinetic assay using imaging proximity assay.

For the second hypothesis, this is more challenging to address. Receptor crystallization is underway by our collaborator Dr. Aashish Manglik. However, the fact that the receptor can’t be stabilized by both prototypic agonist and our modulators

makes this approach more difficult. We are also actually exploring approaches to identify ligands that could interact simultaneously with our modulators, in such case, partial agonist could be good candidates, but we have limited number of available partial agonist for opioid receptors. In this case, we believe the DADLE to displace the equilibrium toward the active conformation, and our modulators could then interact with the receptor and stabilize an intermediary state.

4.2 Conclusion

GPCRs form the major protein family of membrane receptors through approximately 800 GPCRs in the human genome. Also, it demonstrates the most significant family of drug targets, used for nearly half of the medical drugs. The work presented in this thesis concentrated on the δ-opioid receptor which is a member of the opioid receptors family. Delta-opioid receptor is a potential target for pain relief drugs, but its activation produces deleterious and potentially life-threatening side effects. Recent advances on δ-OR structure and function led to the discovery of a cavity housing a sodium ion and water molecules. Our work has permitted us to find some Na^+-channel inhibitors with allosteric and/or agonistic activity (Ago-PAM) at the DOR which are MIA and HMA (amiloride derivatives). Also, Zoniporide which is Na^+-H^+ exporter inhibitor and the first non-amiloride small molecule, was found to have positive allosteric modulator (PAM) at activated DOR. We hypothesized that the sodium pocket could act as an allosteric site for Na^+-channel inhibitors and can serve as a druggable target to develop novel drugs that can modulate the functional selectivity of the receptor towards therapeutic effects and away from harmful side effects. Primarily, the aim of this project was to identify and functionally characterize

these new ligands chemotypes (sodium channel inhibitors) targeting the sodium cavity at DOR. Thus, Tango and Glosensor assays were set up to test these Na^+ inhibitors in the presence of prototypic DOR agonist DADLE, the superagonist BW373U86, endogenous DOR agonists (Met-Enkephalin and Leu-Enkephalin), DOR antagonist Naltrinole, and other DOR agonists. From these results, we identified two main types of allosterism profiles at arrestin recruitment which are agonist-PAM (MIA, HMA) and PAM (MIA, HMA and Zoniporide).

For the G-protein pathway, there is an agonist-PAM and PAM effect with all three small molecules. The intrinsic agonist-PAM activity produced by MIA and HMA can be reversed by the opioid antagonists Naltrindole or Naltriben. Moreover, we observed a certain level of probe dependence where the endogenous opioids are reacting similarly to the peptidomimetic DADLE, but differently than other chemotypes such as BW373U86. However, the increasing concentration of the allosteric modulators leads to an increase in their activity at activated DOR which was investigated by doing Schild analysis experiments. Optimizing this experiment and the mathematical model will allow the calculation of the K_B value for Na^+ inhibitors.

The structure-activity-relationship (SAR) was carried out to identify the critical amino-acid residues essential for binding of Na+ inhibitors to the sodium cavity. SAR studies showed that N67 amino acid residues that could be important for binding our small molecules to sodium allosteric site while D95 and S135 residues highlighted the important role of an allosteric sodium site as an “efficacy switch.” Indeed, it was not enough that these Na+ inhibitors can increase the efficacy with DADLE, or their potency as with BW373U86. To evaluate the affinity of the Na^+ inhibitors at the DOR, binding experiment was performed. The binding results demonstrate that the three

compounds MIA, HMA, and Zoniporide displace the radioligand via a non-competitive mechanism that is not clear yet but observed with other allosteric modulators. Finding the direct correlation between the binding results and the functional data is still under investigation in the lab. Also, more SAR studies need to be done to identify the critical residues that are essential for the binding and/or activation of the Na+ inhibitors to the allosteric sodium cavity.

This work suggests the druggability of the allosteric sodium cavity. However, more work is required to better understand their recognition and activation mechanisms. Further investigation will provide minimal pharmacological rules for the design of more active allosteric modulator targeting the Na^+ site.

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Curriculum Vitae

Nuria Blgacim

Educational Qualifications

Master of Science - Biochemistry *05/2016 to 06/2018*
Faculty of Medicine
Department of Biochemistry, Microbiology, and Immunology, Ottawa University, Ottawa, ON
Study field: Development of Neuroscience, Molecular Biology, Drug Discovery, and Comprehensive Pharmacology.

Extensive English Programs
Six months of intensive academic English courses at Algonquin College, Ottawa, ON. *01/2015 to 06/2015*

Four months academic English courses in Culture Work Institute, Carleton University, Ottawa, ON. *07/2015 to 10/2015*

Bachelor of Drug Technology *09/2006 to 06/2010*
Department of Drug Technology,
Faculty of Medical Technology, Derna, Libya.
Study field: Pharmacology, Pharmaceutics, Medicinal Chemistry, Biochemistry, Botany, Quality control, and Drug Industry.

Secondary School of Medical Specialist *09/2002 to 05/2006*
Graduated with overall grade 87.27% - GPA in the transcript.

Derna, Libya.

Experience

- Laboratory Technician/ Demonstrator *09/2010 to 11/2014*
  Faculty of Medical Technology, Department of
  Drug Technology, Faculty of Medical Technology, Derna, Libya
  Assisted with teaching and acted as a Lab Monitor in various subjects including:
  Pharmacology, Medicinal Chemistry, Organic Chemistry Quality Control, and Pharmaceutics.

- Teacher's Assistant *01/2017 to 05/2017*
  Principles of Chemistry (CHM1311 assisting Dr.
  Kathy Focsaneau) Faculty of Science,
  University of Ottawa, Ottawa, ON

- Third Year Proctor *03/2018 t0 04/2018*
  Acute Care Medicine (CLI3110), and Human
  Anatomy and Physiology (ANP1106C)
  Department of Cellular and Molecular Medicine
  Faculty of Medicine, University of Ottawa, Ottawa, ON

Academic Achievements

- Conducted research while earning a Bachelor of Drug Technology,
  Faculty of Medical Technology, Derna, Libya.
  Under the direction of Professor Kutiba Alzanad, I conducted research into the following: Isolation, Structural, Elucidation and Biological Activity of the Flavonoids from "Junipers Excelsa."

- First Honors Bachelor's Degree in Drug Technology, Faculty of Medical Technology, Derna, Libya.

- Presented the undergraduate research project poster at the First Conference of Medical Technology, Derna, Libya, 2010.

- Contributed to the achievement of molecular control of the Mu opioid receptor (MOR) signaling and functional selectivity by targeting the sodium cavity study, which culminated in a poster presentation at the 18th GPCR (G-protein coupled receptor) Retreat, in Ottawa, October 2017.

- Under the direction of Dr. Patrick Giguere, I am currently working on a project focusing on Molecular Control of Delta Opioid Receptor (DOR) signaling and functional selectivity by sodium.

Academic Skills

- Paper Chromatography
- Silica Gel Chromatography for both type, column and thin layer
- Various skills in the preparation of pharmaceutical solutions
- Computer skills; Graph Pad Prism 6, PowerPoint, MS Office, vast experience using the simulation software for the pharmacology laboratory, and extensive knowledge of PubMed
- Proven teaching skills, including a talent for clearly explaining scientific facts and methods
- Volunteered at Vincent Massey Elementary School, Ottawa, ON
- Ability to work under pressure and consistently meet deadlines
- Following procedure and taking note carefully.
- Titration and dilution
- Plasmid Prep (Maxi Prep)
- TANGO assay
- GLOSENSOR assay
- Displacement Binding assay
- Saturation binding assay

Languages

First Language: Arabic.

Second Language: English.

Professional References

Available on Request