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DISCOVERY OF MOR SELECTIVE, REVERSIBLE OPIOID ANTAGONIST FOR POTENTIAL USE IN TREATMENT OF DRUG DEPENDENCE

A thesis submitted in partial fulfillment of the requirements of Master of Science at Virginia Commonwealth University.

By

Abdulmajeed M. Jali

B. S. King Khalid University 2011

Director: Dana E. Selley, Ph. D.

Department of Pharmacology and Toxicology

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First, all praises and thanks be to Allah. Without his many blessings and graces this study would not have been possible.

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List of Abberviation

ACTH	Adrenocorticotropic hormone
ADase	Adenine deaminase
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CREB	cAMP response element-binding protein
CTAP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2
CTOP	Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2
DAMGO	[D-Ala^2 ,N-$MePhe^4$, Gly^5-ol] enkephalin
DMEM	Dulbecco's Modified Eagle Medium
DPDPE	[D-$Pen^{2,5}$]Enkephalin, [D-Pen^2,D-Pen^5]Enkephalin
EL	Extracellular loop
E_{max}	maximal efficacy
ERK	Extracellular signal-regulated kinases
FBS	fetal bovine serum
GABA	gamma-Aminobutyric acid
GDP	Guanosine diphosphate
GIRK	G-protein-coupled inwardly-rectifying potassium channel

GNTI	5'-Guanidinonaltrindole
GPCR	G-Protein Coupled Receptor
GRK	G-protein-coupled receptor kinase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GTPγS	Guanosine-5'-O'- (γ-thio)-triphosphate
HIV	human immunodeficiency virus
IL	Intracellular loop
LH	Leutenizing hormone
mDOR	mice δ opioid receptor
mKOR	mice κ opioid receptor
mMOR	mice μ opioid receptor
MMT	methadone maintenance therapy
NAc	Nucleus accumbens
norBNI	Norbinaltorphimine
NTI	Naltrindole
NTX	Naltexone
PBS	phosphate buffer saline
SAR	structure-activity relationship
PKA	protein kinase A
TM	Transmembrane
VTA	Ventral tegmental area

Abstract

DISCOVERY OF MOR SELECTIVE, REVERSIBLE OPIOID ANTAGONIST FOR POTENTIAL USE IN TREATMENT OF DRUG DEPENDENCE

Abdulmajeed M. Jali, M.S.

A thesis submitted in partial fulfillment of the requirements of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2017

Director: Dana E. Selley, Ph. D., Department of Pharmacology and Toxicology

Opioid dependence/addiction is a major public health problem that is associated with multiple health and social costs. Pharmacotherapeutic treatment has been relatively effective, but the risk of relapse after treatment remains high. Naltrexone, an opioid antagonist, is FDA-approved for long-term maintenance therapy to reduce relapse risk. However, naltrexone is accompanied by side-effects that are due to lack of selectivity among opioid receptor types. Based on the message-address concept and molecular modeling studies, 18 novel compounds designed to bind selectively to the MOR based on interaction with a key aromatic residue, were synthesized by our collaborators. The purpose of this study was to identify MOR-selective antagonists from this series of compounds. Using radioligand

and GTPγS binding assays in transfected cells and native tissues, two compounds were discovered with the high MOR-selectivity and low efficacy required to serves as lead ligands in future discovery efforts toward next-generation opioid antagonists.

Chapter.1 Introduction

1.1 Opioids

The Sumerians are recognized to be the first people who harvested the opium poppy plant around 3400 BC. They called it *Hul Gil*, the “joy plant. The opium resin, which is derived from the poppy plant, *Papaver somniferum*, contains the active opiate alkaloids morphine and codeine (Figure 1). Its first recorded use as an analgesic was 2,500 years ago (Booth, 1999; Jaffe & Martin, 1990).

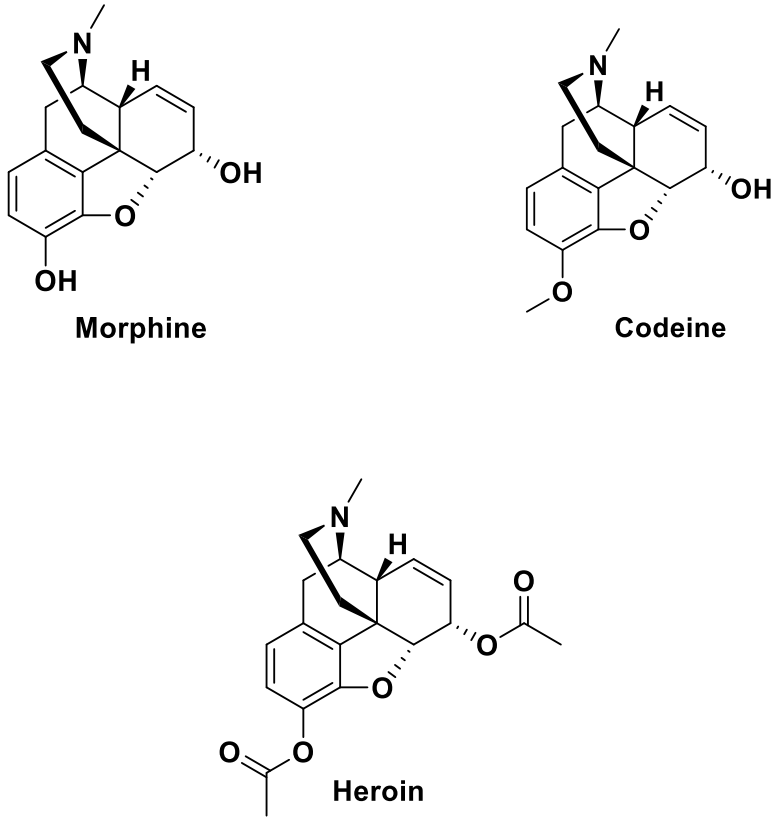


Figure 1. Chemical structures of morphine, codeine, and heroin

Following the spread of the opium poppy's cultivation and use throughout the globe, several events occurred in the 19th century that led to increased understanding of both the medicinal benefits and abuse and addiction liability of opioids, the latter of which can cause devastating consequences. In 1806 Friedrich Serturner, a German physician, was the first to isolate the active ingredients in opium and named it morphine after Morpheus, the Greek god of dreams (Brownstein, 1993). Dr. Charles Wood, a Scottish physician, invented the hypodermic needle and used it to inject morphine to relieve pain. Morphine was first used as an adjunct to general anesthetics when Claude Bernard used it for premedicating experimental animals. He found that it reduced the required amount of chloroform to induce anesthesia. Dr. Eduard Livenstein from Germany presented the first document that comprehensively describes addiction to morphine, including withdrawal syndrome and relapse, and argued that craving for morphine was a physiological response. In an attempt to obtain a safer drug than morphine the English researcher C.R. Wright synthesized 3,6-diacetylmorphine in 1898 (Figure 1), also named "heroin", which was briefly promoted as more effective and less addictive than morphine before market withdrawal (Booth, 1999; Brownstein, 1993; Musto, 1999).

The term opioid is now used to classify any compound that exhibits morphine-like responses and includes naturally occurring opiates, synthetic and semi-synthetic opioid agonists, partial agonists and antagonists, and endogenous opioid peptides along with their synthetic analogues (Jaffe & Martin, 1990).

1.2 Opioid receptors

In 1973, three laboratories almost simultaneously demonstrated the presence of specific opioid receptors by radioligand binding studies in homogenized membrane fractions of rat brain (Pert & Snyder, 1973; Simonet al., 1973; Terenius, 1973). Consistent with earlier evidence from pharmacological studies, opioid receptors were also shown to exist in the gastrointestinal system as illustrated by Terenius (Terenius, 1972) and Synder and Creese (Creese & Snyder, 1975). The concept of multiple opioid receptor types preceded their identification by two decades when nalorphine produced biphasic effects in human clinical studies. Low doses of nalorphine could block morphine analgesia, which however, was retrieved at higher doses of nalorphine. Those findings could most readily be explained if nalorphine acted on a second receptor (Snyder & Pasternak, 2003). More detailed studies with different opioid drugs in the chronic spinal dog were conducted by Martin et al. (Martin et al., 1976) and revealed the existence of three hypothesized opioid receptor types. Each receptor type was named for the prototypical drug that activated the receptor. The mu (μ) opioid receptor (MOR) was named for morphine, the kappa opioid (κ) receptor (KOR) was named for ketocyclazocine, and the sigma (σ) receptor was named for n-allylnormetazocine (SKF 10,047). Subsequent purification and cloning studies uncovered that protein corresponding to σ receptor binding sites do not possess pharmacological properties of opioid receptors (Hanner et al., 1996; Kekuda et al., 1996; Traynor & Elliott, 1993). Binding studies in rat brain and studies utilizing isolated peripheral organ bioassays suggested the presence of the delta opioid δ receptor (DOR) which was selective for the synthetic enkephalin analogue [D-Ala^2, D-Leu^5] encephalin (DADLE) (Lord, 1977). Two years later Chang and Cuatrecasas (Chang and Cuatrecasas, 1979) confirmed the existence of opioid

receptors with a purely δ binding profile in NG 108-15 cells. The synthesis of highly selective KOR ligands, such as U50,488, yielded more information on KOR binding sites (Vonvoigtlander et al, 1983).

In the 1990s, multiple cloning studies definitively identified the three distinct types of opioid receptors that were already hypothesized based on pharmacological evidence. The first opioid receptor to be cloned was the DOR using cDNA from the mouse DOR from NG108-15 (Evans et al., 1992). The cloning of MOR and KOR followed by using their homology to the DOR (Chen et al., 1993; S. Li et al, 1993; Meng et al., 1993; Minami et al., 1993; Wang et al., 1993).

1.3 Opioid peptides

The concept of endogenous opioid ligands arose after the discovery of opioid receptors. This was supported by the production of analgesia during electrical stimulation of specific brain regions (Mayer & Liebeskind, 1974). This analgesic effect was blocked by naloxone, an opioid antagonist. Three major endogenous opioid peptides have been characterized so far. Met-enkephalin and leu-enkephalin were the first endogenous peptide to be isolated and sequenced (Hughes et al., 1975). Both enkephalins are derived from one precursor polypeptide, proenkephalin, and bind with the highest affinity to the DOR (Comb et al., 1982; Hughes et al., 1975; Traynor & Elliott, 1993). The second one is β-endorphin, discovered by Li et al. in 1976, which binds to the MOR and DOR with relatively equal affinity, and derived from the polypeptide precursor, pro-opiomelanocortin (POMC) (C. H. Li et al., 1976). The third endogenous peptide is dynorphin, which has the highest affinity for KOR and derived from the polypeptide prodynorphin (Chavkin & Goldstein, 1981; Goldstein et al., 1979). All opioid peptides are cleaved from their larger polypeptide

precursors by proteolytic cleavage at dibasic residues, and expression of the gene encoding each particular precursor determines the opioid peptide(s) that is used by each particular opioidergic cell (Froehlich, 1997) (Figure 2).

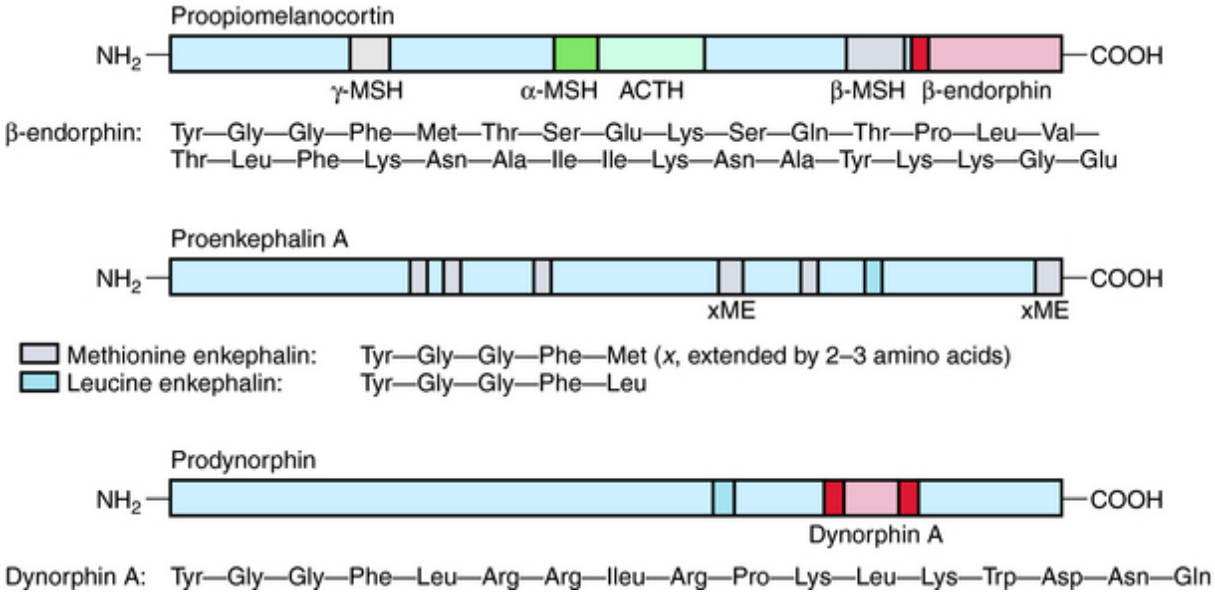


Figure 2. Three major families of opioid peptides, B-endorphin, Met-enkephalin and Leu-enkephalin, Dyndorphin A, are derived from distinct precursor molecules and are encoded by three different genes (Clinical Gate, 2015).

1.4 Physiological effects

Due in part to the widespread distribution of opioid receptors in the brain, opioids, such as morphine, are involved in multiple physiological effects. Several studies have shown that most of the clinically relevant effects of opioids are mediated through the MOR, which has been confirmed in knockout mice genetically lacking the MOR (Kieffer, 1999). The major therapeutic effect induced by opioid agonists is analgesia. Opioids not only increase the threshold of stimulus intensity required for detection of a noxious stimulus, but also alter pain perception in CNS; therefore they alleviate the negative emotional state that occurs during pain (Jaffe &

Martin, 1990). Beyond alleviation of negative emotions, euphoria has been reported among a large portion of patients, yet some patients experience dysphoria. It is thought that the MOR in the ventral tegmental area (VTA) and nucleus accumbens (NAc) is responsible for mediating reward, a common response to MOR agonists and other drugs of abuse (V David & Cazala, 2000; Fields & Margolis, 2015; Jaffe & Martin, 1990; Olmstead & Franklin, 1997; Y. Zhang et al., 2009). Opioids also display antitussive effects due to inhibition of cough reflex in the medulla (Jaffe & Martin, 1990). One of the therapeutic limitations of opioids is respiratory depression, which results from inhibition of respiratory centers in the brainstem and accounts for the potential for lethality in opioid overdose (Pattinson, 2008). Constipation is another common side effect. The mechanism by which constipation occurs is decreasing propulsive bowel contractions and increasing non-propulsion contractions of intestinal smooth muscle, along with increasing anal sphincter tone, mainly through effects on the enteric nervous system. Other opioid physiological effects include miosis, histamine release, prolactin release, ACTH stimulation, LH and oxytocin inhibition, emesis, sedation and convulsion, the latter two of which occur mainly at relatively high doses (Jaffe & Martin, 1990).

Fortunately, tolerance develops to the lethal effects of opioids, but also to analgesia. Very little tolerance develops to constipation and miosis. MORs also mediate opioid dependence, which leads to the withdrawal syndrome upon the abrupt reomval of the agonist or precipitation by administration of an antagonist. Also, cross-tolerance has been observed between different opioid agonists, further supporting the evidence that the effects of most clinically relevant opioid agonists are mediated through the same receptor: MOR (Jaffe & Martin, 1990).

Opioids have a high degree of abuse liability, and it is well known that chronic use of opioids can lead to tolerance, dependence, and in many cases, addiction. Addiction can be broadly defined as a state of continued, compulsive, relapsing drug-seeking behavior even in the face of negative emotional, social, legal and monetary consequences. A major focus in opioid research has been the discovery of novel drugs that can effectively treat opioid addiction. Therefore, a better understanding of opioid receptor signaling could provide useful information for discovery of novel drugs that are useful in the treatment of opioids dependence or addiction.

1.5 Opioid receptor signaling

Opioids produce their effects through the activation of opioid receptors which belong to G-Protein Coupled Receptor (GPCRs) Superfamily. GPCRs are the largest class of membrane proteins in the human genome. GPCRs share common features; each consists of a single polypeptide with an extracellular N-terminus, an intracellular C-terminus, and seven hydrophobic transmembrane domains (TM1-TM7) linked by three intracellular loops (IL1-IL3) and three extracellular loops (EL1-EL3). Opioid receptors belong to the opsin family, or rhodopsin-like family (Mombaerts, 2004). GPCRs produce intracellular signaling primarily by the activation of guanine nucleotide-binding regulatory proteins (G-proteins). G-proteins are heterotrimeric proteins composed of α, β, and γ subunits. Those subunits are made up of a large family and can form several combinations that reflect the complexity of transduction of signals via G-proteins. There are 18 genes for the α subunits, which are categorized into four families, α_s, $\alpha_{12,}$ α_i, and α_q. β and γ subunits have 5 and 12 known types, respectively. These different subunits have a substantial role in

signaling as they can be stimulatory or inhibitory based on the G-protein family to which they coupled (Hamm, 1998; Hildebrandt, 1997).

Opioid receptors are coupled to the inhibitory type of G-proteins, $G_{i/o}$. They can be activated by either endogenous or exogenous ligands, such as β-endorphin or morphine. The binding of an agonist to a GPCR stabilizes an active conformation of the receptor. This will facilitate the release of guanosine diphosphate (GDP) that is prebound to the Gα subunit for cytoplasmic guanosine triphosphate (GTP), a process known as guanine nucleotide exchange (Tuteja, 2009). The release of GDP from the Gα subunit is caused by an increase in GDP dissociation rate (Florio & Sternweis, 1989; Lorenzen et al., 1993), possibly culminating in decreased GDP affinity (Breivogel et al., 1998; Selley et al., 1997). After the binding of GTP, the Gα subunit dissociates from the βγ subunits, which remain associated with each other as a heterodimer, and both the α and βγ subunits become free to interact with various effectors and either increase or decrease their activity depending on the specific G-protein subunit and effector protein. The termination of G-protein activity is controlled by the intrinsic GTPase activity of the Gα subunit, which catalyzes the hydrolysis of GTP to GDP, therefore inactivating G-protein signaling and allowing the reassociation of Gα with the Gβγ complex (Tuteja, 2009) (Figure 3).

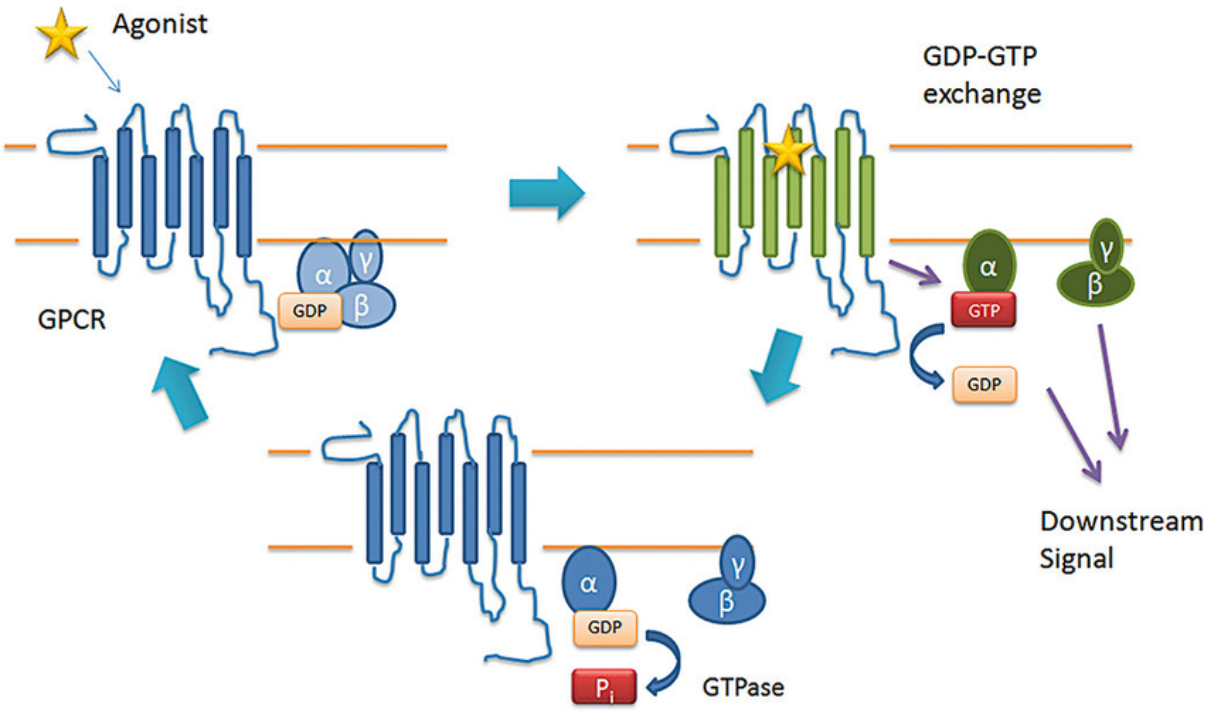


Figure 3. Schematic representation of G-protein activation/inactivation cycle (Smith et al., 2010).

Effectors that are modulated by $G_{i/o}$ include inhibition of adenylyl cyclase, activation of inwardly rectifying potassium channels (GIRK), inhibition of calcium channels, especially the N and P/Q types, activation of phospholipases C/A_2, Mitogen-activated protein kinases (MAPK), and Phosphoinositide 3-kinases (PI3) . It was initially thought that βγ subunit did not directly modulate effector activity, but later studies revealed that it can directly modulate effector activity either positively or negatively (Tuteja, 2009). There are also other intracellular kinases that are indirectly modulated by G-proteins. The phosphorylation of active GPCRs, including opioid receptors, by G-protein Coupled Receptor Kinases (GRK) leads to desensitization of GPCR-mediated G-protein activation and coupling of the receptor to the clathrin-mediated enodocyitc process, which leads to GPCR internalization. β-arrestins can also act as a scaffold to recruit alternative downstream signaling pathway such as MAPK or protein kinase B (Akt). The MAPK family is composed of 12-15 gene

products with the most well-described being extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Al-Hasani & Bruchas, 2011).

1.6 In vitro studies of opioids

1.6.1 Radioligand binding

The first step in initiating a cellular response is the binding interaction between a ligand and a receptor. Therefore, the relative affinity of a given ligand for the target receptor versus other related receptors will in part determine its selectivity of action. Our radioligand binding studies were designed to determine the binding affinity of novel ligands for each of the three major opioid receptor types, MOR, KOR and DOR, and to compare the relative binding affinities of each ligand between receptor types to determine the selectivity for each receptor. To accomplish this goal, we utilized indirect competition binding assays. Radiolabeled ligands that reversibly bind to the desired receptor were used to label each receptor of interest, mouse receptors that are stably expressed in Chinese hamster ovary (CHO) cells, and a range of concentrations of each novel compound was added to determine the competition binding affinity for each receptor type. This approach avoids the need to radiolabel each novel compound, and can be used to rapidly screen multiple compounds. We used mouse receptors because our collaborators will use mice for in vivo evaluation of the novel ligands. Thus, we can correlate the in vitro and in vivo data with the same species. The use of heterologously transfected cell lines allows for a high level of receptor expression, which provides a high ratio of specific to non-specific binding, and avoids the complication of co-expression of additional opioid receptor types in each cell line. A detailed explanation of the radioligand binding assay is provided in Chapter 2. One disadvantage of all ligand-receptor binding assays is that they

cannot determine the functional activity of the ligand when it binds to the receptor of interest. For this, an assay of functional receptor activity must be conducted, as introduced in the next section.

1.6.2 Functional activity of opioid ligands

As mentioned in section 1.4, the biological responses of opioids are mainly mediated by the MOR and our novel ligands of interest were designed to bind selectively to the MOR. Therefore, the efficacies of our novel ligands were tested in the same MOR-expressing CHO cells (mMOR-CHO) used in the MOR radioligand binding assays. Further comparative studies were also conducted in mouse thalamus, which expresses a high density of MOR with relatively low expression of other opioid receptor types, in order to determine the relative efficacy of our novel lead compounds compared to one of our first-generation lead compounds, 17-cyclopropylmethyl-3,14β-dihydroxy-4,5 α-epoxy-6α-[(3'-isoquinolyl)acetamido]morphinan (NAQ), in a more native biological system.

As previously described in section 1.5, the MOR is a GPCR that is coupled mainly to the $G_{i/o}$ subfamily of G-proteins. Because there are multiple spatial and temporal cellular events that occur between the binding of an agonist to a receptor and the production of a biological response, it is critical to consider whether to measure a proximal or distal signaling step in signal transduction following GPCR activation. Multiple cellular signaling events at various steps in MOR signaling pathways have been examined to assess the efficacy of agonists, and several of these are briefly discussed below.

Intracellular Ca^{+2} is a second messenger downstream in the signaling pathway for certain GPCRs. Unlike receptors that are naturally coupled to activation

of $G\alpha_{q,}$, $G\alpha_{i/o}$-coupled receptors cannot generally induce a ligand-dependent increase in intracellular Ca^{+2}. In order for $G_{i/o}$ to cause a robust increase in intracellular Ca^{+2} in the cellular model used in this study, transfection of a chimeric $G\alpha_q$ -protein ($G\alpha_{qi5}$ or $G\alpha_{qo5}$) containing the 5-residue C-terminal sequence of $G\alpha_{i/o}$ that is required for receptor coupling or a promiscuous G-protein ($G\alpha_{16}$ or $G\alpha_{15}$) is required (R. Zhang & Xie, 2012). This requires additional manipulation of the cell system. In addition, Ca^{2+} release is three steps downstream in the signaling pathway, which includes G-protein mediated activation of the effector phospholipase C, synthesis of the second messenger inositol triphosphate (IP_3), and IP_3-induced release of Ca^{2+} from the endoplasmic reticulum. Therefore, there is substantial signal amplification that occurs between G-protein activation and stimulation of intracellular Ca^{+2} release (Tuteja, 2009; Yan et al., 2016). In contrast, measurement of an event that is proximal to the receptor and therefore subjected to minimal signal amplification would provide better discrimination between full and partial agonists (Keen, 1991).

Adenylyl cyclase (AC) is a major target for the MOR so most early functional GPCR assays measured production of the G-protein mediated second messenger, cyclic adenosine monophosphate (cAMP) (R. Zhang & Xie, 2012). G-protein-mediated modulation of AC activity results in a direct measurement of the synthesis of cAMP. This is in contrast to the intracellular Ca^{2+} release assay, which measures the activity of Ca^{2+} channels that are targets of the second messenger, IP_3. Therefore, measurement of AC-generated cAMP has one less amplification step than measurement of intracellular Ca^{2+} responses. MOR-mediated inhibition of AC results in reduced production of cAMP (Traynor & Elliott, 1993). Generally, full MOR agonists produce maximal inhibition of cAMP, whereas partial agonists produce

partial inhibition of cAMP (Blake et al., 1997). However, this approach could also report misleading results, as it has been demonstrated that signal amplification occurs between GPCR-mediated activation of G-protein and inhibition of adenylyl cyclase, which would make it difficult to discriminate between full agonists and partial agonists of relatively high intrinsic efficacy (Costa et al., 1988; Tuteja, 2009; R. Zhang & Xie, 2012). Therefore, an upstream step, such as direct measurement of G-protein activity, would convey more accurate assessment of opioid ligand efficacy.

Previous studies used the activity of Gα-associated low K_m GTPase as an indicator for G-protein activation. This approach measures the increase in GTPase activity of the Gα subunit following its activation by the receptor (Koski & Klee, 1981). This method, in fact, is not ideal because it measures the hydrolysis of GTP that occurs after guanine nucleotide exchange-induced activation, which is an indirect method to measure G-protein activation. This approach provides a low signal to noise ratio because of the high level of GTP hydrolysis by non-Gα enzymatic activity, such as ATPases. It has also been shown that GTP hydrolysis can be influenced by factors that are not relevant to G-protein activation because they affect the GTPase step subsequent to G-protein activation (Selley et al., 1993). Therefore, a more direct approach is required for accurate measurement of GPCR activation.

The earliest cellular event subsequent to GPCR activation is the exchange of GTP for prebound GDP on the Gα subunit. This event is not subjected to amplification other than the initial amplification step that can occur between GPCR and G-protein. Upon the occupation of the MOR by an agonist, GTP will replace GDP on the α subunit leading to dissociation of Gα-GTP from Gβγ and subsequent modulation of downstream effectors. GTPase terminates the activation cycle by hydrolyzing GTP and thus restores the Gα-GDP subunit, which then reassociates

with the βγ dimer, terminating its interaction with effectors. The ability to measure receptor-stimulated formation of the Gα-GTP complex provides a precise evaluation of opioid efficacy. The [^{35}S]GTPγS, an analogue of GTP, membrane binding technique has been widely used to measure agonist stimulated G-protein activation by GPCRs. Indeed, this technique is experimentally more suitable for $G_{i/o}$- coupled receptors because $G_{i/o}$ is the most abundant G-protein subfamily in many cells and has the fastest GDP/GTP exchange rate among other G-proteins (C. Harrison & Traynor, 2003; R. Zhang & Xie, 2012). Thus, the [^{35}S]GTPγS membrane binding technique is the best available approach to determine the efficacy of opioid ligands on opioid receptors, including the MOR. [^{35}S]GTPγS is an analogue of GTP upon which an oxygen on the terminal phosphate is replaced with a sulphur, thus rendering the analogue resistant to hydrolysis by GTPase because of the covalent bond formation between the terminal phosphate and sulphur. [^{35}S]-labeled GTPγS competes with GTP for binding to the Gα subunit and allows the measurement of accumulation of bound [^{35}S]GTPγS due to the stability of the Gα-[^{35}S]GTPγS complex. When MOR are occupied by a ligand, the receptor will stimulate guanine nucleotide exchange on MOR-coupled G-proteins to an extent proportional to the occupancy of the receptor and the efficacy of the ligand, and the maximal magnitude of MOR-stimulated [^{35}S]GTPγS binding can be used as a measure of ligand efficacy (C. Harrison & Traynor, 2003) (Figure 4).

The radiolabeled [^{35}S]GTPγS is added to membrane homogenates in the presence of excess GDP and Na^+, which play a major role in GPCR activation. Our laboratory has published a study on the effect of GDP on various MOR ligands in mMOR-CHO cells and rat thalamus. It was found that addition of excess GDP increased the signal to noise ratio by suppressing the spontaneous $G_{i/o}$ activity in the

absence of an agonist. Also, at 10-20 μM GDP in mMOR-CHO and 30 μm in the thalamus the efficacy of full and partial agonists with high to low relative efficacy could be clearly discriminated from each other (Selley et al., 1997). Furthermore, it has been shown that Na^+ can inhibit basal [^{35}S]GTPγS binding by reducing spontaneous receptor-stimulated activity in mMOR-CHO cells and thalamus. Similarly to GDP, increasing the concentration of Na^+ magnified the relative efficacy differences among MOR agonists. 100 nM of Na^+ produced a maximal difference in relative efficacy among full and partial MOR agonists (Selley et al., 2000). Therefore, having these conditions in our assay will optimize the agonist-stimulated binding of [^{35}S]GTPγS and provide an accurate assessment of the relative efficacy of our novel ligands.

Prior to the widespread availability of the cloned opioid receptors, use of the [^{35}S]GTPγS membrane binding technique with opioids was limited to tissues endogenously expressing the receptor, such as SH-SY5Y tumor cell line (Traynor & Nahorski, 1995) or brain homogenates (Sim et al., 1995). A major disadvantage of these two systems that they frequently express two or more opioid receptors, making it difficult to determine the efficacy of nonselective opioid agonists. When opioid receptors were cloned in the 1990s, as discussed in section 1.2, not only did it help to better understand the anatomical expression pattern of opioid receptors, but also allowed the transfection of the receptors of interest into cell lines. The choice of a cell line is based on certain criteria; the main ones are lack of endogenous expression of opioid receptors, fast growth rate, easy handling, surface-adherence, and ease of transfection. The Chinese hamster ovary cell line possess these features (Pan, 2003), and was therefore chosen for use in this project.

On the other hand, it has been demonstrated previously in our laboratory that the level of receptor expression can affect the relative efficacy of MOR agonists. mMOR-CHO cells express MOR density higher than in native tissue such as the rodent thalamus. This difference in receptor expression is reflected in the efficacy of opioid agonists between mMOR-CHO cells and thalamus whereby some apparent full agonists in mMOR-CHO cells acted as partial agonists in rat thalamus, and partial agonists in mMOR-CHO cells acted as pure antagonists in rat thalamus. For example, morphine acted as a full MOR agonist in mMOR-CHO cells, but acted as a moderate efficacy partial agonist in thalamus. Another example is levallorphan, which acted as a partial agonist in mMOR-CHO cells and pure antagonist in thalamus (Selley et al, 1998; Selley et al., 1997). Hence, we tested our lead compounds in mouse thalamus to obtain results under more native conditions.

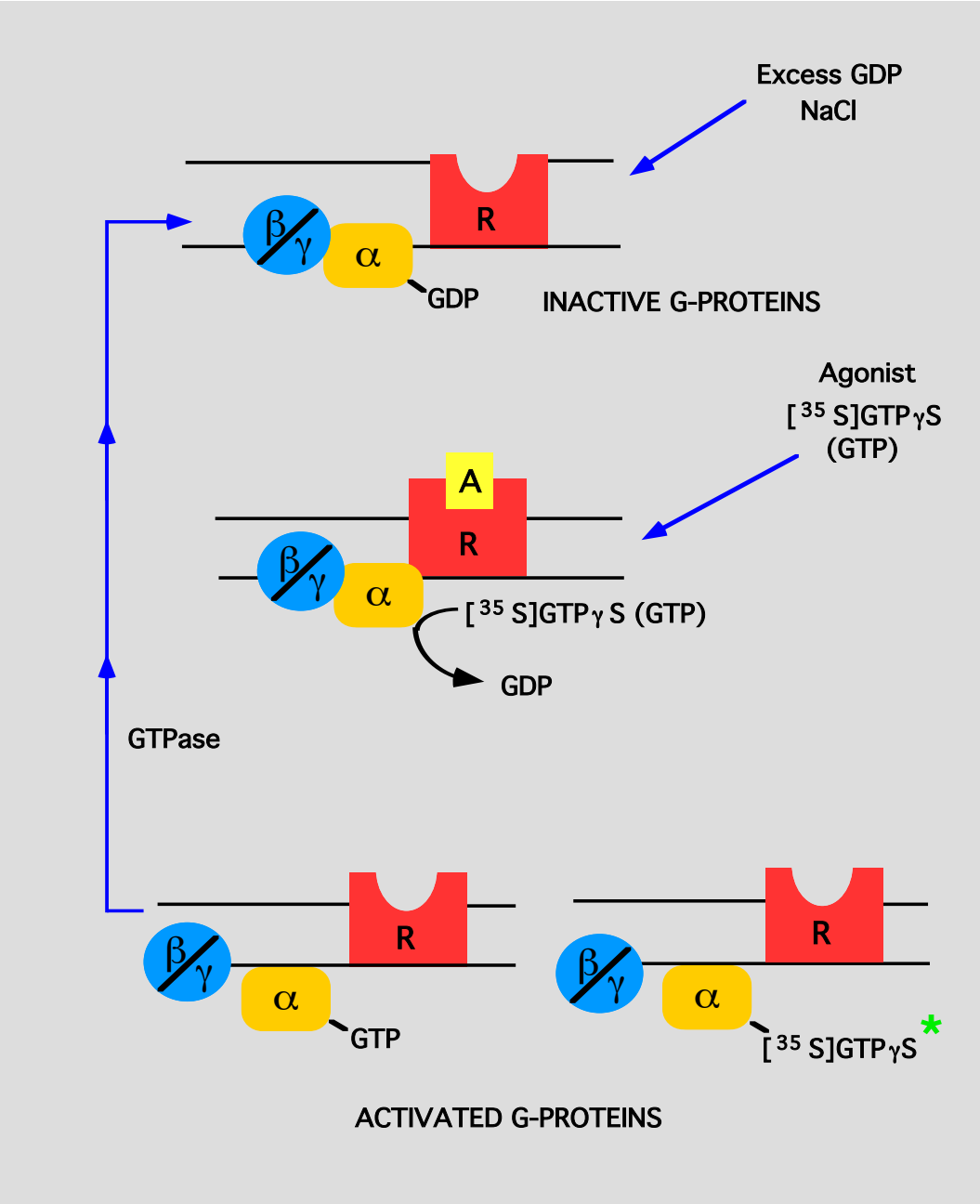


Figure 4. Schematic representation of [^{35}S]GTPγS binding assay. Relative efficacies of GPCR ligands can be determined in vitro using the [^{35}S]GTPγS binding assay.

1.7 MOR expression and role in opioid abuse and addiction

The majority of clinical effects of opioids are mediated mainly by the MOR. Evidence in favor of this comes from in vivo experiments with antisense knockdown of the MOR (Rossi et al., 1995) and in mice with genetic deletion of the MOR (Kieffer, 1999). Data from these studies revealed that both antinociceptive and abuse-related effects of opioids are abolished or greatly attenuated in MOR-knockout mice. These studies confirmed years of prior research with moderately selective opioid antagonists. For example, naloxone blocks the analgesia produced by morphine with much lower dose than blocking analgesia produced by the selective KOR agonist U-50,488 (Vonvoigtlander et al., 1983).

Opioid receptor cloning helped to map the anatomy of opioid receptor mRNAs in connection with their binding sites in the brain. The MOR is widely distributed throughout the CNS. They are found in forebrain, midbrain, hindbrain and spinal cord. The richest regions are neocortex, caudate putamen, nucleus accumbens, thalamus, hippocampus, amygdala, inferior and superior colliculi, ventral tegmental area (VTA), periaqueductal gray (PAG), locus coeruleus, nucleus tractus solitarius, spinal trigeminal nucleus and dorsal horn of the spinal cord (Mansour et al 1995; Satoh & Minami, 1995). The localization of the MOR is in agreement with the functions of the regions that mediate the clinical effects upon activation of the MOR.

Opioids have been used for the treatment of moderate to severe pain and related disorders for thousands of years. Commonly used opioids for pain treatment include morphine and codeine (natural opioids), methadone, hydrocodone, fentanyl, oxycodone, and buprenorphine. The choice of opioid is based on the conditions of particular patient and desired route of administration, but in general, higher efficacy

opioids are used to treat more severe pain than lower efficacy opioids. The advantage of opioids over other painkillers is that they not only block the perception of pain but also inhibit the pain-related negative emotional status of the patient, which improves the quality of their lives, making opioids a major drug of choice for pain treatment. The localization of the MOR in the midbrain is a major factor in mediating opioid-induced analgesia. When MORs are activated by an agonist, they induce analgesia either directly or indirectly. The direct way is mediated by inhibiting the ascending transmission of neurons from substantia gelatinosa in the dorsal horn of the spinal cord and peripheral nociceptive afferent neurons, thus decreasing nociceptive transmission from the periphery. Another mechanism of opioid-induced analgesia is indirectly stimulating the descending inhibitory pathway by acting on PAG and nucleus reticularis paragigantocellularis (NRPG). This results in higher neuronal activity through the nucleus raphe magnus, thus increasing the activation of 5-hydroxytryptamine and enkephalin-containing neurons which connect directly with the substantial gelatinosa of the dorsal horn. The net effect of this pathway is an inhibition of nociceptive transmission from the periphery to thalamus (Pathan & Williams, 2012).

Reward is another common response that is mediated by MORs that are located in VTA and NAc. The mesolimbic dopaminergic system (Figure 5) is considered a major player in mediating the positive reinforcing effects of opioids (Le Merrer et al., 2009). Several studies have shown that opioids induce positive reinforcement by increasing the level of dopamine in the mesolimbic system (Bardo, 1998; Koob, 1992; Shippenberg & Elmer, 1998; van Ree et al., 1999), and more recent studies have confirmed it is the MOR that, upon activation by opioids, produces the positive reward (Vincent David et al., 2008; Terashvili et al., 2004;

Zangen et al., 2002). Dopamine neurons of VTA project to other brain regions that are involved in reward-relevant function, including NAc, amygdala, hippocampus and prefrontal cortex. In addition to dopamine neurons, VTA contains GABA and glutamate neurons, which project to many of the same mesolimbic targets as the dopamine neurons. The mechanism by which the MOR excites VTA dopamine neurons is through inhibiting GABA interneurons that synapse on dopaminergic neurons in VTA. The elimination of tonic GABAergic inhibition by the MOR results from the activation of GIRK channels or inhibition of N and P/Q calcium channels on GABA neurons, which are major effectors of $G\alpha_{i/o}$ (Fields & Margolis, 2015). Consequently, synaptic dopamine levels will increase in terminal field regions due to a lack of suppression by GABA. Reward, or euphoria, is argued to be an emotional side effect of pain treatment by opioids, although it undoubtedly plays a role in inhibiting the negative emotional effects of pain (Miller et al., 2015).

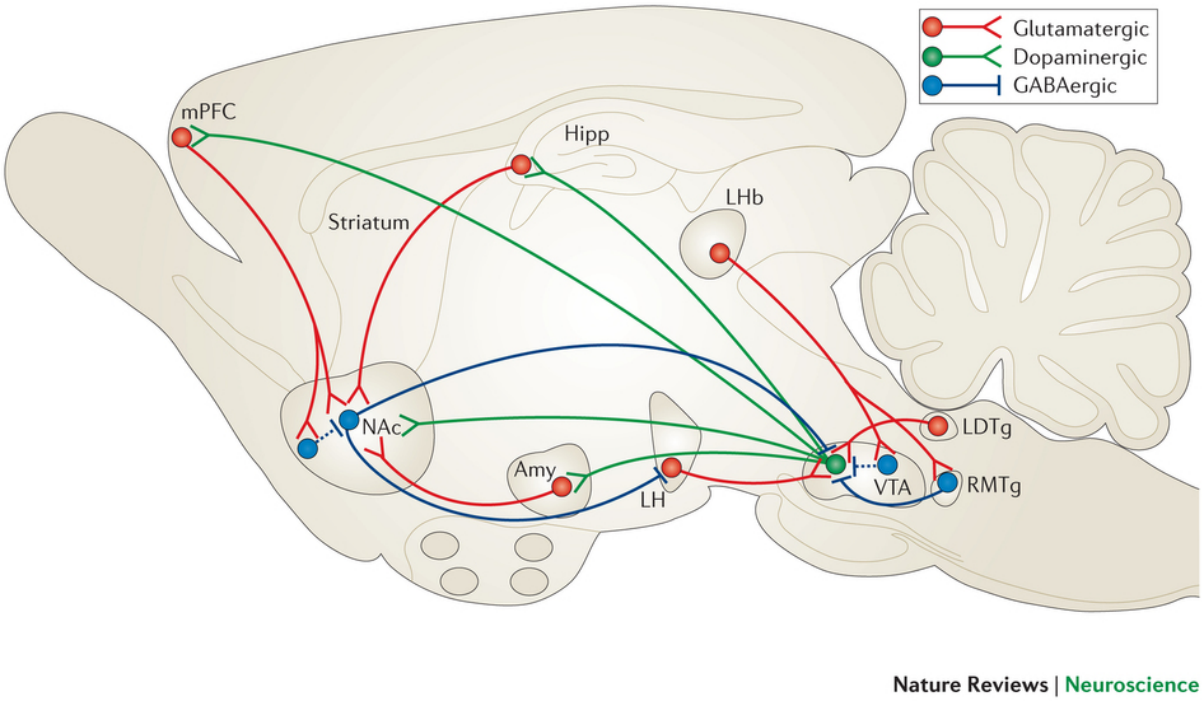


Figure 5. VTA-NAc reward circuit in the mesolimbic system (Russo & Nestler, 2013).

Despite the clinical benefits, MORs also account for multiple side effects associated with opioid use. These side effects put restrictions on opioid therapeutic use. The side effects can be divided into peripheral side effects, including constipation, urinary retention, and bronchospasm, or central side effects, including nausea, sedation, respiratory depression, and hypotension, all of which can severely affect the patients' quality of life and opioid clinical utility (Al-Hasani & Bruchas, 2011). One of the most troublesome side effects of opioids is tolerance, which is defined as the need to increase the dose of a drug to obtain the same response. Tolerance results from chronic opioid agonist use, especially at relatively high doses such as those often needed to treat severe pain. Even though tolerance develops to all actions of the MOR, the rate of tolerance development differs between different responses. For instance, tolerance to analgesia develops at a somewhat faster pace than to respiratory depression and constipation, leading to narrowing therapeutic index with chronic opioids administration (Pasternak & Pan, 2013). Repetitive use of opioids can also lead to physical dependence and/or addiction (Al-Hasani & Bruchas, 2011; Feng et al., 2012).

Opioid tolerance encompasses a diverse range of molecular and cellular mechanisms making it difficult to be incorporated into a unified theory. Certain studies suggested that NMDA receptors, enkephalin systems, and P-glycoprotein play a role in opioid tolerance (Pasternak & Pan, 2013). Also, some in vitro studies reported that chronic opioid exposure can lead to opioid receptor desensitization, internalization and downregulation, producing cellular tolerance (Dang & Christie, 2012; R. S. Harrison et al., 2010). Several studies revealed that following chronic in vivo treatment with morphine or heroin, MORs could be desensitized (uncoupled from the G-protein signaling pathway) as indicated by a reduction in agonist-

stimulated [^{35}S]GTPγS binding in the absence of downregulation of MOR binding sites (Sim-Selley et al., 2000, 2007; Sim et al., 1996). These studies further showed that MORs expressed in different brain regions were differentially sensitive to agonist-induced desensitization, such that desensitization occurred more readily in brainstem regions and spinal cord than in forebrain regions. Furthermore, it has been reported that different MOR agonists can differentially internalize the MOR, and that MOR internalization can enhance MOR re-sensitization and therefore plays a role in opposing tolerance development (Waldhoer et al., 2004).

One widely observed cellular adaptation associated with opioid dependence/addiction is the elevation of cAMP levels subsequent to chronic MOR agonist exposure. This elevation reflects cellular adaptation, rather than receptor desensitization or downregulation, to the presence of MOR agonists. This response includes upregulation of adenylyl cyclase types I and VIII, and increases in the activity of protein kinase A (PKA) and cAMP response element binding (CREB) protein (Nestler 1996). Consequently, the cAMP/PKA pathway returns to a normal level in chronic opioid exposure states. Upon the removal of the agonist or administration of an antagonist, cAMP overshoot takes place and is recognized as a potential mechanism for withdrawal symptoms (Nestler, 1996; Zachariou et al., 2008). Elevation of cAMP levels has been seen in brain regions where MORs are expressed, such as locus coeruleus, NAc, and striatal neurons (Fan et al., 2009; Zachariou et al., 2008). In addition to CREB, ΔFosB is another transcriptional factor that has a role in opioid dependence. ΔFosB is a truncated splice variant of FosB that is stable and gradually accumulates with repeated administration of drugs of abuse in NAc, hippocampus, and dorsal striatum. Unlike CREB, which is related to withdrawal symptoms by the activation of dynorphin/KOR pathway resulting in

dysphoria (Knoll et al., 2011), ΔFosB is related to pro-reward effects. The stability of and inhibition of dynorphin by ΔFosB are partially associated with the sensitization to the rewarding effects of opioids, and might contribute to relapse (Nestler, 2013; Zachariou et al., 2006).

1.8 Pharmacotherapy options

The issue of opioid tolerance and dependence/addiction has been a growing problem in the US over the past two decades. The addiction liability of opioids is very high among people who use heroin or prescription opioids (Bart, 2012). Whether opioid addiction arises from chronic use during pain treatment (Ballantyne & LaForge, 2007) or access to illicit opioids on the streets, the negative social, health and economic consequences of opioid addiction point to the need for an effective drug treatment. In 2015, opioids were in the top of overdose deaths caused by drugs of abuse with 33,000 out of 52,000 deaths (Rudd et al., 2016). Moreover, the number of overdose deaths from opioids, both prescription and heroin, went up by 2.5-fold from 2002 to 2015 (NIDA, 2014). Also, substance abuse disorders cost the nation approximately $600 billion yearly. Effective pharmacotherapeutic treatments have shown a reduction in the costs associated with opioid addiction. For example, one year of methadone maintenance treatment costs an average of $4,700 per patient, whereas one year of imprisonment costs approximately $24,000 per person (Volkow, 2012).

The currently approved treatments by FDA for opioid dependence are based on the long-term maintenance therapy with either an agonist, partial or full, or an antagonist. The long-term agonist maintenance therapies include the MOR full agonist, methadone, and the MOR partial agonist, buprenorphine. Both drugs are

synthetics and have a long half-life that allows one daily dose, lasting up to 28 hours for methadone and 37 hours for buprenorphine (Bart, 2012). Methadone and buprenorphine are typically used as replacement therapy for opioid dependence. Opioid-withdrawal syndromes occur when there is a rapid decline in blood drug level following the termination of repeated exposure, thus using a MOR agonist will diminish withdrawal symptoms by maintaining a steady-state level of drug that makes patients feel essentially normal, rather than euphoric or dysphoric. After stabilizing patients on a dose that alleviates withdrawal symptoms, patients can be tapered off the drug until detoxification is achieved (Schuckit, 2016). Yet, a large portion (40-60%) of patients relapse to abusing either heroin or other opioids (Volkow, 2012). Like most opioid agonists, methadone and buprenorphine have the potential to cause respiratory depression when overdosed (especially with the higher efficacy agonist, methadone), and to be abused. Also, cardiac side effects have been associated with methadone during induction period or upon discontinuation (Bart, 2012).

Another disadvantage of long-term agonist maintenance therapy is the potential for immune system impairment. Functional, binding, and molecular studies indicate the expression of opioid receptors on cells from the immune system. Opioids may act like cytokines to modulate the immune system through interaction with opioid receptors, either centrally or peripherally, yet the mechanism is complicated and not well understood (Feng et al., 2012). Nonetheless, evidence suggests an immunosuppressive role of opioids. One study showed a significant reduction in natural killer cell activity when rats were injected systemically with morphine, and naltrexone fully reversed this effect (Vallejo et al., 2004). A similar effect was seen on T-lymphocyte proliferation in rats following morphine injection (Flores et al.,

1994). This immunosuppressive action of opioids is mediated by the MOR, as confirmed by a study in which morphine did not affect immune system function in mice lacking the MOR (Vallejo et al., 2004). Moreover, the prevalence of human immunodeficiency virus (HIV), hepatitis C virus (HCV) and other infectious diseases is high among opioids abusers, both in injecting or non-injecting individuals. These diseases are transferred by sharing contaminated injection equipment and by involving in risky sexual behavior sometimes associated with drug use (Volkow, 2012). In fact, one study by Mahajan et al (Mahajan et al., 2002) explored the significant role of MOR agonists in the pathogenesis of HIV. Using an astrocytoma cell line and normal human astrocytes, this study showed that morphine inhibited local production of HIV-1 protective chemokines leading to encephalopathy. Morphine, also, enhanced the expression of an HIV-1 entry co-receptor gene within the CNS. These effects were reversed by the selective MOR antagonist, β-fenaprexamine, indicating a primary role of the MOR in the susceptibility of the CNS to HIV infection. Therefore, the immune system of individuals who are on agonist maintenance therapy or in a relapse period will be affected by the persistent activation of the MOR.

Opioid agonists have also shown a paradoxical effect, in which they can activate pronociceptive systems leading to pain hypersensitivity and short-term tolerance. Instead of analgesia, repeated use of opioid agonists can induce hyperalgesia, which is defined as a reduction in pain threshold. Evidence suggests that opioids can stimulate certain effectors in opposition to their inhibitory role, at ultralow doses or after chronic agonist exposure (Ghelardini et al., 2015). In a study with cultured dorsal root ganglion neurons, micromolar concentrations of opioid agonists decreased the action potential duration, while nanomolar concentrations

increased it (Shen & Crain, 1989). In a clinical study using a cold pressor test for detecting pain threshold in patients on methadone maintenance therapy (MMT) and normal subjects, patients on MMT detected pain earlier and were less tolerant to pain than normal individuals (Doverty et al., 2001), implying that prolonged activation of the MOR was related to opioid-induced hyperalgesia. This was supported by Clark et al (Li et al., 2001) using the mouse strain, CXBK, with reduced MOR expression in the CNS, which showed that the when the CXBK mice were injected with morphine and there was no signs of hyperalgesia.

To avoid the aforementioned disadvantages and to prevent relapse, patients can be switched to an antagonist maintenance therapy. The only opioid antagonist approved by the FDA for the treatment of opioid addiction/dependence is naltrexone (NTX). It is a semi-synthetic antagonist that was synthesized as a congener of oxymorphone in the 1960s. Compared to methadone or buprenorphine, NTX possesses essentially zero intrinsic efficacy at the MOR; thus it will not induce any side effects that results from MOR activation (Bart, 2012). Patients do not develop tolerance to nor dependence on NTX. Also, NTX is not only approved for relapse prevention of opioid dependence but is also used in the treatment of alcohol dependence (Stotts et al.,2009). Moreover, NTX lacks addiction liability, is not reinforcing, and has no potential for abuse or diversion for unprescribed use. It can also block the effect of parenterally administered heroin, hydromorphone or morphine for 24, 48, and 72 hours, respectively (Tai and Blaine, 1997). The main lethal effect of opioid agonist overdose is respiratory depression, which is not caused by NTX when overdosed. The hypothesis behind using NTX maintenance therapy is to prevent drug-induced relapse and thereby minimize the effects of factors that cause negative reinforcement, such as drug-associated cues and social stressors.

With NTX occupying the MOR, patients will not be able to relieve the resulting negative emotional state through opioid use, hence the behavior of relapsing will ultimately cease (Bart, 2012). Unlike methadone and buprenorphine, NTX is relatively safe for patients with HCV, HIV, and who consume large amounts of alcohol (Schuckit, 2016). To avoid precipitating withdrawal symptoms, individuals need to be tapered off with an agonist first and then switched to NTX to prevent relapse. The antagonist maintenance therapy requires a shorter period of time required for patients to be on drug. A clinical study showed that 6-month retention rates in treatment following extended release NTX are similar to one-year retention in methadone maintenance (Stotts et al., 2009).

One of the long-term effects of NTX is the upregulation of MOR. It has been found that after chronic exposure to NTX, the density of MOR increased in brain and a 7315c cells model. This was demonstrated by the increased number of maximum binding sites for MOR agonists, but there was no change in the affinity of MOR for MOR agonists. The potency of the MOR agonists was not influenced in an AC inhibition assay, but the efficacy increased. This could be due to the upregulated MOR that can activate more G-proteins subunits which can cause greater maximal inhibition of AC (Côté et al., 1993). Although it is unclear whether MOR upregulation by chronic NTX treatment occurs equally among CNS regions, one implication of these findings is that sensitization to a subset of MOR agonist effects could occur following such treatment. While this could pose some potential risks depending on which opioid actions become sensitized, one could also speculate that it might lead to improvement in MOR-mediated functions of the endogenous opioid system.

The most common reported side effects of NTX are nausea, headache, depression and dysphoria (Stotts et al., 2009). These side effects are thought to be

caused by the non-selectivity of NTX for opioid receptors. The selectivity of NTX for MOR over DOR is fairly high, with 435-fold selectivity (K_i= 0.33 and 143.5 nM, respectively), but the selectivity over KOR is modest, with only a 4.4-fold selectivity ratio (KOR K_i= 1.44 nM). Because a large dose of NTX is required to outcompete self-administered agonists and prevent relapse, there can be off-target effects. NTX can bind to DOR and KOR at sufficiently high doses. The intrinsic efficacy of NTX at KOR is higher than at MOR and DOR; NTX is a partial agonist at KOR (Bidlack, 2014). The activation of KOR in VTA is linked with dysphoria due to inhibition of dopamine release (Lalanne et al., 2014). Human and animal studies reported dysphoric effects upon activation of KOR by either endogenous or exogenous agonists (Lalanne et al., 2014). While NTX is a partial agonist at KOR, it is an antagonist at DOR, which has a positive role in modulating mood. Mice with genetic knockout or antagonism of the DOR displayed depressive-like behavior and anxiety, whereas activation of the enkephalin/DOR system enhanced mood activity (Lutz & Kieffer, 2013). Therefore, it is possible that relatively high doses of NTX could produce pro-depressive, dysphoric effects via partial agonism at KOR or antagonism of DOR, although thus far there is little direct evidence of these hypothetical mechanisms. However, it should also be noted that blocking peripheral KOR has been associated with cardiac side effects, as KORs are expressed in heart tissue, but their physiological role is not well understood (Peng et al., 2012). A human study with the selective KOR antagonist JDTic, a 4-phenylpiperidine derivative ((3*R*)-7-hydroxy-N-[(2*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide), showed that bradycardia and ventricular tachycardia were produced in the JDTic group (Chavkin

& Martinez, 2015). Consequently, with a drug that can selectively antagonize MOR, potentially serious cardiac side effects could be avoided.

Several drug discovery attempts have been conducted to avoid these side effects of NTX by synthesizing a compound with better MOR selectivity. However, the current MOR antagonists do not have the desired characteristics for clinical use. For example, cyprodime has a lower affinity for MOR than NTX or naloxone. Also, cyprdime has only moderate selectivity for MOR over DOR and KOR (K_i value ratios are approximately 45 over KOR and 40 over DOR). Other MOR-selective antagonists act as irreversible antagonists, such as β-funaltrexamine and colcinamox, which are not favored because of covalent bond formation with the receptor that will lead to a long-term reduction of available receptors. Even though CTAP and CTOP are MOR-selective antagonists, the fact that they are peptides limits their medical application due to poor bioavailability when given orally or parenterally. Peptides are vulnerable to metabolic inactivation and elimination, and most cannot penetrate blood-brain barrier (Li et al., 2009). Like NTX, naloxone is a relatively non-selective opioid antagonist, but is short-acting and useful for reversing opioid-induced respiratory depression by IV injection (Dorp et al., 2007). However, it has very poor oral bioavailability, making it impractical for use in long-term maintenance therapy.

Up until very recently, no non-peptidyl, reversible, highly selective MOR antagonists have been discovered. Based on molecular modeling, use of the message-address concept, and in vitro pharmacological screening studies, the objective of this study is to identify a compound has the mentioned features.

1.9 MOR mutational studies

Since MOR mediates most of the physiological responses of clinically relevant opioids, it was important to investigate the MOR structure and compare to other opioid receptors to understand the structural basis of the function and regulation of the MOR. All opioid receptors share around 60-70% similarity at the level of amino acids, particularly in the transmembrane and intracellular regions, which correlates highly with the common signaling mechanism among opioid receptor types. On the other hand, the N-terminus, C-terminus, and second and third extracellular loops exhibit most of the dissimilarity, which is consistent with the different affinities for different opioid ligands, as well as some differences in receptor regulation by intracellular proteins that interact with the C-terminus (Pasternak & Pan, 2013). Site-directed mutagenesis studies revealed several amino acid residues required for ligand binding and selectivity. Results from these studies illustrate the complexity of ligand-receptor interaction due to the high degree of reliance on several conserved amino acids. While more than 20 amino acids can affect ligand binding affinity, two seem to play an important role in ligand binding affinity, His^{297} and Asp^{114}. A D114N receptor mutation decreased the binding affinity for DAMGO and morphine, while the affinities of partial agonists, nalbuphine and buprenorphine, and antagonists, naloxone and diprenorphine, were not influenced (Bot et al., 1998; Xu et al., 1999). In comparison, the mutation H297N decreased partial agonists' affinities for MOR, but not DAMGO and morphine (Bot et al., 1998).

As for MOR selectivity, three amino acids have been identified as key residues, Asp^{147}, Asn^{150}, Trp^{318}. The significant role of Trp^{318}, located in EL3, has been illustrated by two studies. W318K/L mutation produced a reduction of binding affinity for MOR ligands, like DAMGO, fentanyl, and naltrexone. Also, Trp^{318} serves

as a key residue for binding MOR-selective ligands and excluding DOR-selective ligands. This was depicted when the W318L mutant produced a notable increase in the affinity of DPDPE, a DOR selective ligand. The W318L mutant was intentionally designed to mimck the DOR, as leucine occupies the analogous position of tryptophan in DOR (Bonner et al., 2000). Additionally, the other study confirmed the role of Trp^{318} by constructing a W318A mutant that resulted in undetectable affinity levels for MOR ligands (Xu et al., 1999).

Asn^{150} is a conserved residue that is located in TM7 in all opioid receptors. Asn^{150} mutation increased the affinity for MOR agonists, but not antagonists, when substituted with alanine (N150A) (A Mansour et al., 1997). Also, the conserved aspartate residue located in TM3 (Asp^{147}) was shown to play a role in binding of MOR agonists only. However, another study excluded the role of Asp^{147} as a requirement for high affinity binding (Surratt et al., 1994).

Since Trp^{318} is only found in the MOR and its mutation affected the binding affinity of MOR antagonists, manipulation of Trp^{318} and NTX docking studies were conducted to explore the interaction of substituted antagonists with MOR to assist in the design of novel MOR-selective antagonist compounds.

NTX is considered a universal template for designing opioid receptor antagonists. Naltrindole (NTI), norbinaltrophimine (norBNI), and guanidinenaltrindole (GNTI) are successfully modified derivatives of NTX. A molecular modeling study of the opioid receptors using NTX as the probe showed an aromatic binding pocket was formed between C(6) of NTX and Trp^{318} of MOR, but not in DOR or KOR, thus suggesting a critical role of Trp^{318} in MOR selectivity. This could serve as an alternative address binding domain. Based on this hypothesis, 16 compounds were synthesized by introducing a heteroaromatic moiety onto the 6-position of NTX,

either in α or β configuration. An amide group was used to link the side chain of each moiety to the morphinan skeleton; thus, these compounds are naltrexamine derivatives. Out of the 16 compounds, two were identified as promising leads, NAQ and 17-Cyclopropylmethyl-3,14**β**-dihydroxy-4,5**α**-epoxy-6**β**-(4′pyridylcarboxamido)morphinan (NAP). Both had high affinity for the MOR (K_i= 0.55 and 0.37 nM, respectively). The K_i ratio of NAQ for DOR/MOR was 241 and for KOR/MOR was 48, while the NAP K_i ratio for DOR/MOR was 747 and for KOR/MOR was 163. Further molecular modeling study verified that NAP and NAQ recognized the alternative address domain in the MOR (Li et al., 2009). Recently, this Trp^{318} residue was mutated to alanine (W318A) and expressed in CHO cells to test the binding affinity of NTX, NAP, and NAQ. This study revealed that the affinity for NTX was not affected by the mutation, whereas the affinities for NAQ and NAP were dramatically reduced to undetectable values (Table 1) (Zaidi et al., 2013).

Table 1. Binding of ligands to site directed mutated MORs (Zaidi et al., 2013).

Compounds	Wild type MOR (nM) ± SEM		W318A MOR (nM) ± SEM	
	IC_{50}	K_i	IC_{50}	K_i
NTX	3.90 ± 2.96	1.85 ± 1.41	10.35 ± 1.64	4.91 ± 0.78
NAP	2.29 ± 0.15	1.09 ± 0.07	>1000	ND[a]
NAQ	5.42 ± 0.70	2.57 ± 0.33	>1000	ND[a]

[a,] not detectable.

1.10 Hypothesis

Based on the previously discussed premise regarding the potential role of W318 in MOR selectivity and through application of the message-address concept for designing compounds, we hypothesize that introducing an indole ring at position 6 of naltrexamine will lead to enhanced MOR affinity relative to the DOR or KOR possibly through the formation of Pi-Pi stacking with the W318 residue in the MOR. Therefore, 18 compounds were synthesized using naltrexone as the parent compound and naltrexamine as an intermediate parent. These compounds are substituted either in the α or β configuration. The indole rings are attached to the morphinan skeleton at the 5-member ring, 6-member ring, or a substituted mutiple carbon linker at position 3 on indole rings (Figure 6).

This study will determine the affinity of the synthesized compounds by radioligand competition binding at mMOR, mDOR, and mKOR that are heterologously and stably expressed in CHO cells, hence the potential confound of the existence of more than one opioid receptor will be avoided in this system. Next, the efficacy for MOR-mediated G-protein activation will be evaluated using [^{35}S]GTPγS binding in mMOR-CHO cells. Furthermore, the efficacy of the most MOR-selective lead compounds will be compared to the efficacy of known opioid compounds with low efficacy at the MOR in both mMOR-CHO cells and mouse thalamus, the brain region expressing the highest MOR density relative to other opioid receptor types. We hypothesize that 6-indole-substituted naltrexamine analogs that are highly MOR-selective will retain low efficacy for MOR activation.

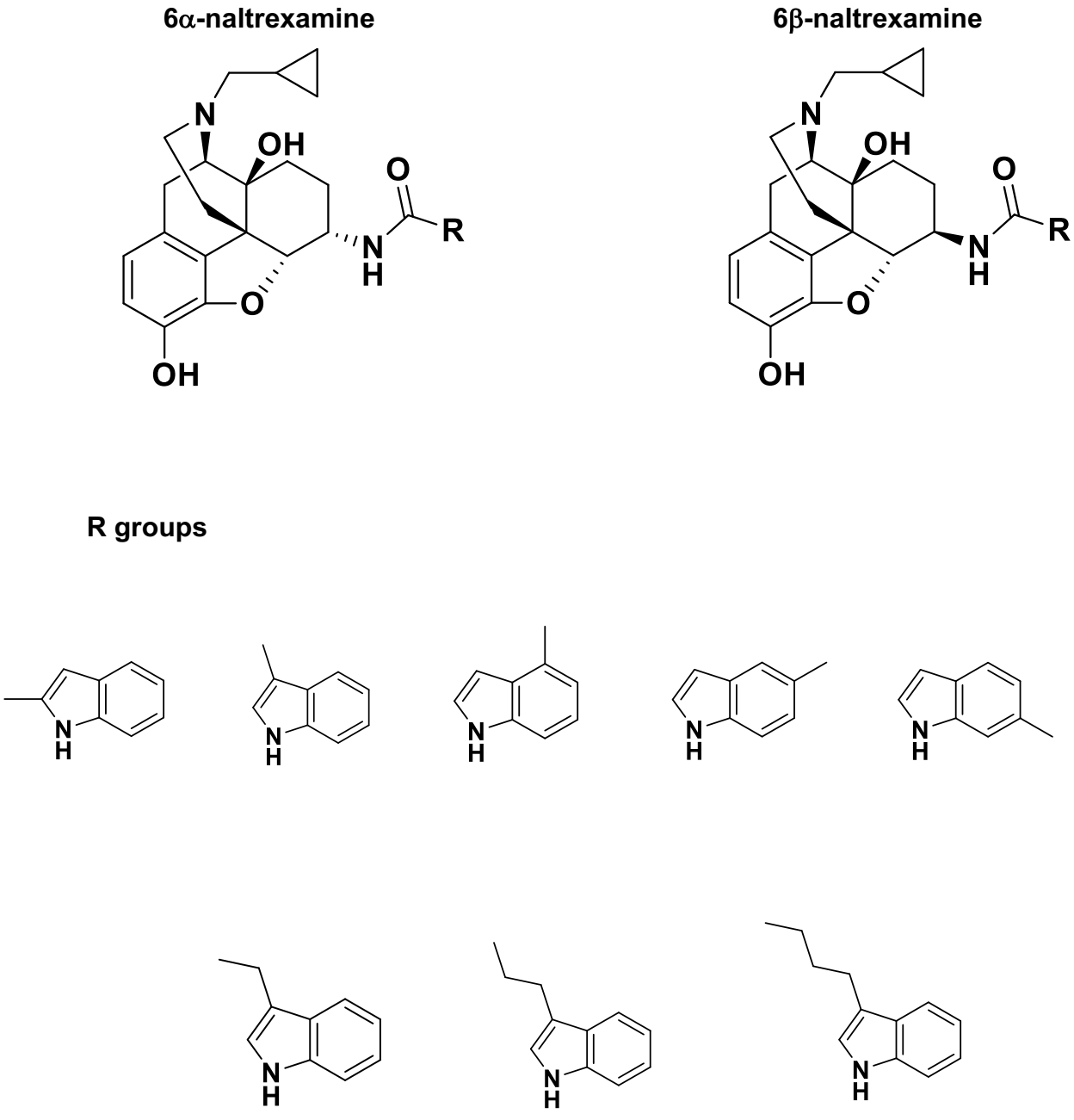


Figure 6. The indole analogues of 6α-naltrexamine (dashed line) and 6β-naltexamine (solid line) were synthesized by Samuel Obeng in Dr. Zhang's lab.

Chapter 2. Experimental Procedures

2.1. Chemical synthesis

The novel compounds were synthesized by Samuel Obeng in Dr. Zhang's lab.

2.2 Materials

2.2.1 Chemicals

[^{3}H]naloxone ([^{3}H]NLX) (70 Ci/mmol), ([^{3}H]dieprnorphine ([^{3}H]DIP) (25.1 Ci/mmol), NTX, SNC80, U50,488, [D-Ala^2 ,N-MePhe^4, Gly^5-ol] enkephalin (DAMGO), Guanosine-5'-O'- (γ-thio)-triphosphate (GTPγS), [^{35}S]GTPγS, GDP, Trizma base (Tris), Adenine deaminase (ADase), hydrochloric acid (HCl), magnesium chloride, sodium chloride (NaCl), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and Econo-1 scintillation fluid.

2.2.2 Cell culture

Stable CHO cell lines with heterologous expression of mMOR, mDOR, mKOR were used. Cells obtained from liquid nitrogen storage were thawed. Before transferring the cells into a culture dish (55 cm^2) containing 9 ml of pre-warmed complete growth medium (1:1 mixture of 500 ml of DMEM/F12 media including Fetal Bovine Serum (FBS), penicillin/streptomycin, and G418), 1 ml of the same growth medium was slowly added into the cryovial to accelerate warming. Next, resuspended cells were incubated at 37 ^{o}C with CO_2 and 95 % humidity for 24 hours to allow the formation of a cell monolayer, after which the media was replaced with fresh culture media. mMOR-CHO cells were cultured in 1:1 mixture of 500 ml of

DMEM/F12 media including 10% FBS, 1% penicillin/streptomycin, and 0.5% G418. The same culture media was used for mDOR and mKOR except that 5% FBS was used. The cells were split after a period of time in which they were allowed to grow until 85-95% confluency was attained. As for splitting cells, first the old media was aspirated and the cells were rinsed with 5 ml phosphate buffered saline (PBS), then 3 ml trypsin was added and the cells were placed in the incubator for 5 min or until all cells are completely detached. After aspirating trypsin, cells were suspended in 10 ml culture media and portion of the cells was transferred to another culture dish containing 10 ml media. The cells were placed back in the incubator. The media would be replaced with a fresh one periodically. For the next splitting, the surface area of culture dish was changed from 55 cm^2 to 152 cm^2 to increase the number of growing cells. The cells then would be split from one dish to eight or ten dishes. When the cells were confluent, they were harvested. First, the media was removed and each culture dish was rinsed with 5 ml PBS and another 5 ml PBS was added to each dish. Cells were then scrapped off the dishes using a Teflon cell scraper and then transferred into a 50 ml centrifuge tube, which was then centrifuged at 1,000 x g for 10 minutes. The supernatant was decanted and the cells were suspended in membrane buffer (50 mM Tris, 3 mM $MgCl_2$, and 1 mM EGTA, PH 7.4). The cells were then homogenized using a Polytron and centrifuged at 5,0000 x g for 10 minutes at 4°C. The supernatant was then decanted and the cell pellet was homogenized to re-suspend in membrane buffer. Membrane protein levels were determined by the method of Bradford (Hammond & Kruger, 1988). The membranes were then aliquoted at 3 mg membrane protein per cryovial and stored at -80 ^oC until use.

2.3 Membrane preparation

Membranes prepared from MOR-, DOR-, and KOR-CHO cells were recovered in the same manner, as follows. Cells were obtained from -80^{o}C storage, thawed and then transferred into a centrifuge tube containing 4 mL membrane buffer. Membranes were homogenized and the suspension was then centrifuged at 50,000 x g for 10 minutes at 4°C. The supernatant was decanted and the pellet was re-suspended in 4 mL binding assay buffer (50mM Tris-HCl, 3 mM $MgCl_2$, and 0.2 mM EGTA; pH 7.7) and homogenized, The Bradford assay was used to measure the protein concentration in the membrane preparations to be used in the assay. For [^{35}S]GTPγS binding assays, MOR-CHO membranes were prepared as described above; however, GTPγS assay buffer was used instead (50 mM Tris-HCl, 3mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl; pH 7.7). Thalamus from adult male ICR mouse (obtained pre-dissected from -80°C storage and provided by Dr. Laura Sim-Selley) was also prepared in the same way to be used in [^{35}S]GTPγS binding assays.

2.4 Radioligand binding assay

For the determination of affinity and selectivity of the compounds under investigation, radioligand binding assays were used. [^{3}H]NLX was used to label MOR and [^{3}H]DIP was used to label both DOR and KOR. Prior to performance of competition binding assays with the novel compounds, radioligand saturation binding assays were conducted to determine K_D and B_{max} values of the radiolabeled ligands in each cell line. Varying concentrations of [^{3}H]NLX or [^{3}H]DIP were incubated in binding assay buffer with 30 μg of MOR-, DOR-, or KOR-CHO cell membrane protein in the absence and presence of 5 μM of naltrexone, SNC80 or U50,488, respectively, to determine specific binding at each receptor.

To determine the affinity of the novel compounds at MOR, 25-30 μg mMOR-CHO membranes were incubated with 1-2 nM [^{3}H]NLX and varying concentrations of each test compound in a 500 μL total volume for 90 minutes at 30^{o}C. Naltrexone (5 μM) was used to determine non-specific binding. Total specific binding was measured in the absence of competitor compounds. The incubation was terminated by rapid filtration through Whatman GF/B glass fiber filters, followed by three washes with 1 mL per wash of cold 50 mM Tris-HCl buffer (pH 7.4). The filters containing bound radioligand were transferred to scintillation vials, which then were filled with 4 mL Econo-1 scintillation fluid. Bound radioactivity was measured by liquid scintillation spectrophotometry at 45% efficiency for ^{3}H.

The affinity of the novel compounds for DOR and KOR was determined according to the same procedure described above, with the following exceptions. Membranes (25-30 μg protein) were obtained from DOR- or KOR-CHO cells, respectively. The radioligand was [^{3}H]DIP, 1.26 nM for DOR and 0.95 nM for KOR. Non-specific binding was determined in the presence of 5 μM SNC80 or U50,488 for DOR and KOR, respectively. All KOR binding assays were conducted by Samuel Obeng.

All competition binding assays were conducted in triplicate and replicated at least three times. All saturation binding assays were conducted in duplicate and replicated at least twice. All binding assay reagents were kept on ice during sample preparation until transfer to sample tubes for incubation at 30°C.

2.5 [^{35}S] GTPγS functional assay

To determine the functional activity of the novel compounds at the primary target receptor, MOR, the ligand-stimulated [^{35}S]GTPγS binding assay was

performed in membranes from the same MOR-CHO cell line in which MOR binding affinity was determined. The efficacy of the novel compounds was measured at MOR relative to a maximally effective concentration (3 μM) of the full agonist DAMGO. Membranes (10 μg protein) were incubated in GTPγS assay buffer with 0.1 nM [^{35}S] GTPγS, 20 μM GDP, and varying concentrations of each novel compound in a 500 mL total volume for 90 minutes at 30 ^{o}C. Unlabeled GTPγS (20 μM) was used to detect non-specific binding. Basal binding was measured in the absence of any MOR ligand. The incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three rinses with 1 mL per rinse of cold 50 mM Tris-HCl buffer (pH 7.4). The filters containing bound radioligand were transferred to scintillation vials, which then were filled with 4 mL Econo-1 scintillation fluid. Bound radioactivity was measured by liquid scintillation spectrophotometry at 90% efficiency for ^{35}S.

[^{35}S]GTPγS binding assays in mouse thalamus were conducted following the same procedure except that 8 μg membrane protein, 10 μM DAMGO, and 30 μM GDP were used, and the incubation period was 2 hours.

All first-tier screen [^{35}S]GTPγS binding assays were conducted in duplicate and repeated at least three times. Second-tier screens were also conducted in MOR-CHO cells and mouse thalamus to compare the lead novel ligands to known opioid ligands, and these assays were conducted in duplicate and replicated at least four times. All assay reagents were kept on ice during sample preparation until transfer to sample tubes for incubation at 30°C.

2.6 Data analysis

Results were reported as mean ± SEM. Radioligand binding curves and concentration-effect curves were fit by nonlinear regression analysis with Prism 6.0 software (GraphPad Software, San Diego, CA) to obtain K_D, B_{max}, IC_{50}, Hill slope, E_{max}, and EC_{50} values. The raw data were transformed to the appropriate format for analysis using Microsoft Excel software and then applied to Prism. For competition analysis, four-parameter fit was used with the total specific binding (top) constrained to 100% and complete competition (bottom) constrained to 0% to determine the IC_{50} and Hill slope values. For [^{35}S]GTPγS binding concentration-effect curves, four-parameter fit was used with the minimum (bottom) constrained to 0% to determine the E_{max}, EC_{50} and Hill slope values. For saturation analysis of specific binding, three-parameter fit was used with the minimum constrained to 0 to determine the radioligand K_D, B_{max} and Hill slope values.

The Cheng-Prusoff equation was used to calculate K_i values [$K_i = IC_{50} / (1+(L^*/K_D^*))$] where L^* is the concentration of the radioligand and K_D^* is the equilibrium dissociation constant of the radioligand. The efficacy of the tested compounds was calculated as relative to net DAMGO-stimulated [^{35}S]GTPγS binding, which is defined as [E_{max} as % Maximal Stimulation = (net stimulation by ligand/net stimulation by 3 μM DAMGO) x 100%] in MOR-CHO cells or [E_{max} as % Maximal Stimulation = (net stimulation by ligand/net stimulation by 10 μM DAMGO) x 100%] in mouse thalamus.

One-way ANOVA with the post-hoc Newman-Keuls test was conducted to determine significant differences in E_{max} values between the lead compounds and known opioid ligands in MOR-CHO cells and mouse thalamus. Student's t-tests were conducted to determine whether the Hill slope values of the novel compounds were

significantly different from 1. The significance cut-off (α) was set at 0.05 in all statistical tests.

Chapter. 3 Results

3.1 Affinity of the indole derivatives of 6α and 6β-naltrexamine for MOR

Radioligand binding assays were performed to determine the binding affinity of the indole derivatives of naltrexamine for the mouse MOR-CHO cells using mMOR-CHO cell membranes. Methodological details for these assays are provided in Chapter.2.

3.1.1 Affinity of the indole derivatives of 6α-naltrexamine for MOR

Compared to known compounds, like NTX and NAQ, the MOR binding affinity of the novel 6-substituted indole analogs did not differ substantially. For the purpose of deciphering structure-activity relationships among the novel ligands, the compounds were divided into three groups according to the position of attachment of the indole ring substituent to the morphinan skeleton. From the results obrained, it was noticed that the position where the indole rings are attached to the morphinan skeleton did not affect the binding affinity greatly. Altough there was variation in the affinity within each group among the α configuration analogues, the magnitude of the difference was relatively small. For example, compound 106 (with substitution at position 7 of the indole ring) had the highest affinity among the α analogues, which was only 3.3-fold higher than compound 092 (with substitution at position 5 of the indole ring) (Figure 7 and Table 2). Moreover, it was observed that the MOR binding affinity increased with increasing length of the alkyl chain at position 3 of the indole

ring. Interestingly, all compounds had Hill slope values greater than 1. Nevertheless, it should be noted that compound 099 (with no linker) in which the indole ring is directly attached to the carbonyl group had almost the same K_i value as compound 102 with three a carbon-length spacer, indicating a biphasic relationship of spacer length and binding affinity.

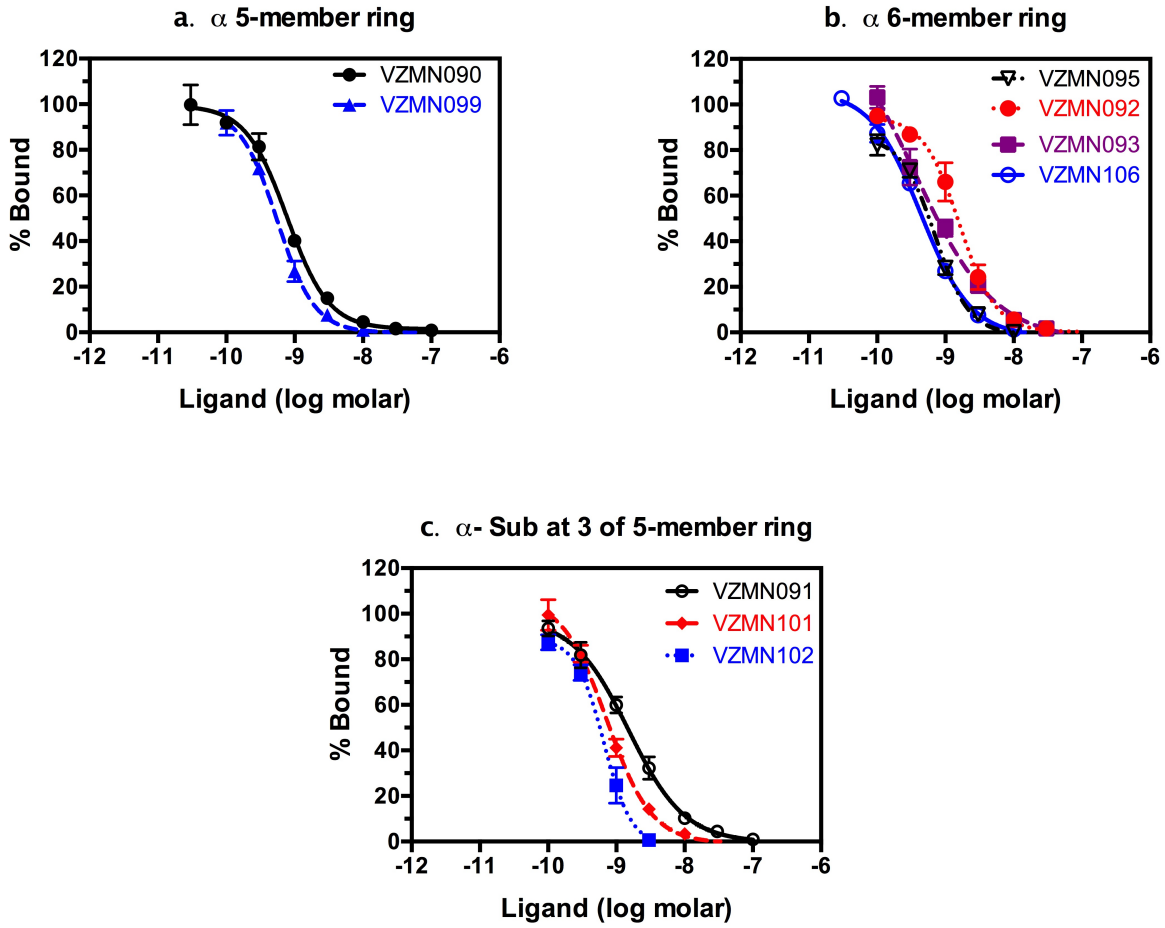


Figure 7. Inhibition of [^{3}H]naloxone binding to MOR by the indole derivatives of 6α-naltrexamine. A) compounds 090 and 099 (with substitutions at positions 2 and 3 of the indole ring). B) compounds 095, 092, 093, and 106 (with substitutions at positions 4, 5, 6, and 7 of the indole ring). C) compounds 091, 101, and 102 (with increasing the length of the alky chain with one, two, and three carbon atoms, respectively, at position 3 of the indole ring). Data are presented as mean values ± SEM from at least three experiments. The concentration of [^{3}H]naloxone in MOR binding assays was 1.51 ± 0.06 nM and the total binding in the absence of the competitor was 1.72 ± 0.07 pmol/mg.

Table 2. MOR K_i(nM) and Hill slope values for the indole derivatives of 6α-naltrexamine.

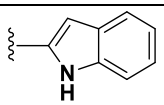
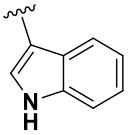
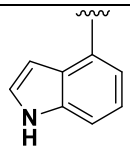
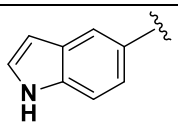
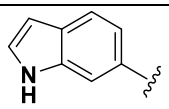
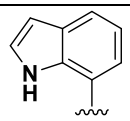
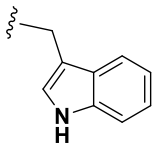
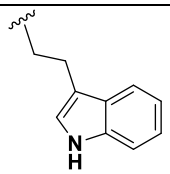
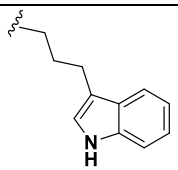
substitution position	Code	R	K_i (nM) ± SEM	Hill slope± SEM
Substitutions at α 5-member ring	VZMN090		0.36 ± 0.03	-1.42 ± 0.18
	VZMN099		0.28 ± 0.04	-1.54 ± 0.08*
Substitutions at α 6-member ring	VZMN095		0.26 ± 0.04	-1.29 ± 0.07*
	VZMN092		0.76 ± 0.11	-1.61 ± 0.1*
	VZMN093		0.43 ± 0.05	-1.24 ± 0.12
	VZMN106		0.23 ± 0.01	-1.37 ± 0.08*
Alkyl chain linker at position 3 of α 5-member ring	VZMN091		0.74 ± 0.12	-1.06 ± 0.05
	VZMN101		0.43 ± 0.04	-1.59 ± 0.16*
	VZMN102		0.29 ± 0.03	-1.85 ± 0.12*

Table 2. MOR K_i(nM) and Hill slope values for the indole derivatives of 6α-naltrexamine. The novel compounds are divided into three groups according to the position of substitution in the indole ring. Data are presented as mean values ± SEM from at least three experiments. *, Hill coefficient was $p < 0.05$ different from one as determined by Student's *t*-test.

3.1.2 Affinity of the indole derivatives of 6β-naltrexamine for MOR

Unlike α analogues, β analogues had less variation in their binding affinities with 2.6-fold difference between the compound with the highest (compound 104) and lowest affinity (compound 094). The K_i values of the β analogues were smaller than those of α analogues. Most K_i values were either in 0.2 nM range or less (Table 3 and Figure 8). However, the binding affinity was close to the affinity of NTX (0.33 nM), indicating that the β configuration did not affect the affinity substantially. As with their respective α analogues, several β analogues also had Hill slope values of greater than 1. Overall, the binding affinity of 6α and 6β-naltrexamine indole derivatives showed considerably high affinity for MOR.

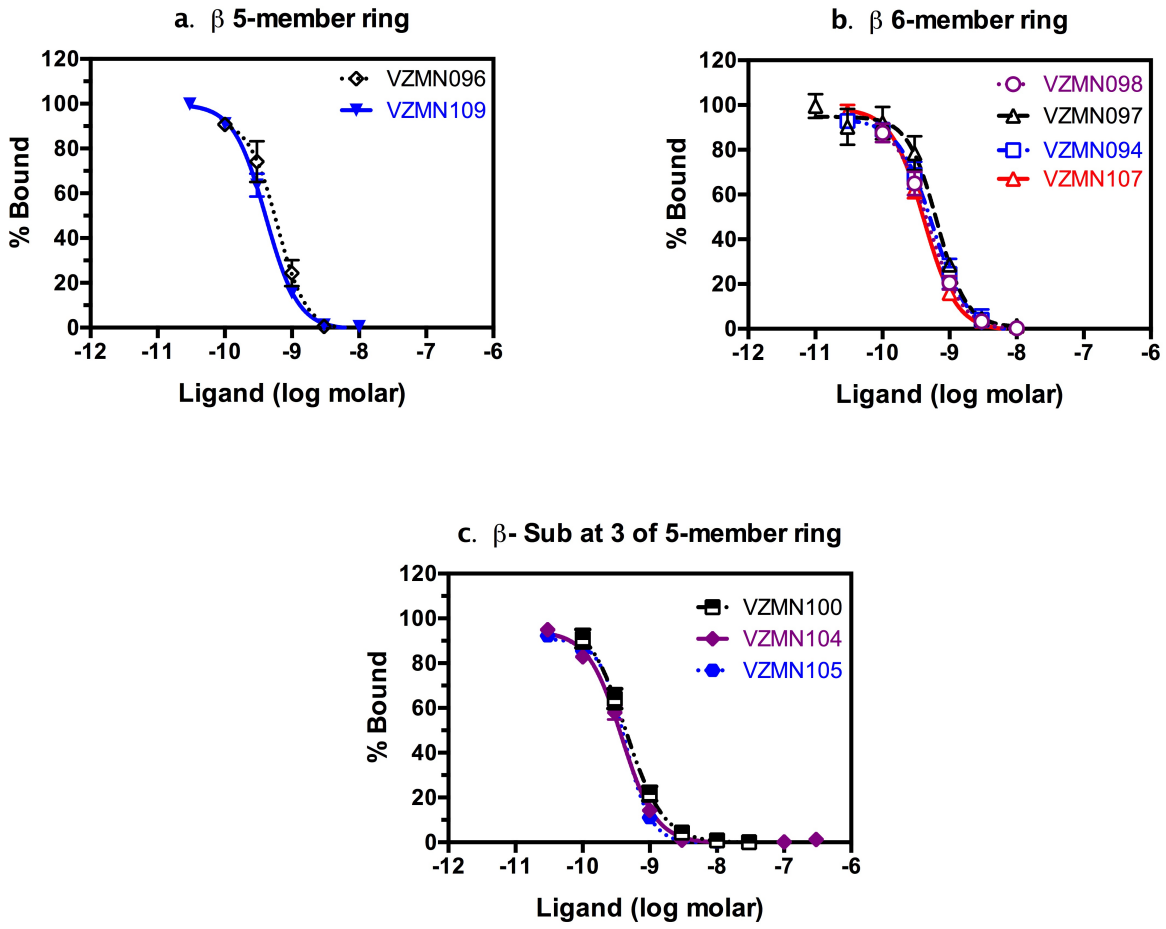


Figure 8. Inhibition of [^{3}H] naloxone binding to MOR by the indole derivatives of 6β-naltrexamine. A) compounds 096 and 109 (with substitutions at positions 2 and 3 of the indole ring). B) compounds 098, 097, 094, and 107 (with substitutions at positions 4, 5, 6, and 7 of the indole ring). C) compounds 100, 104, and 105 (with increasing the length of the alky chain with one, two, and three carbon atoms, respectively, at position 3 of the indole ring). Data are presented as mean values ± SEM from at least three experiments.

Table 3. MOR K_i(nM) and Hill slope values for the indole derivatives of 6β-naltrexamine.

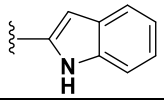
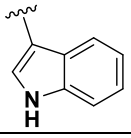
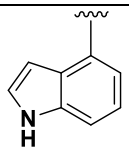
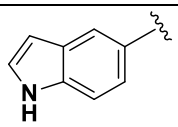
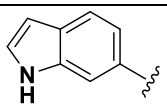
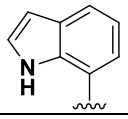
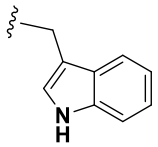
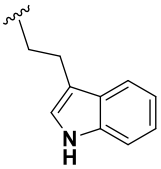
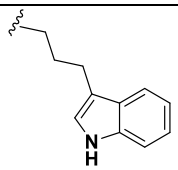
substitution position	Code	R	K_i (nM) ± SEM	Hill slope± SEM
Substitutions at β 5-member ring	VZMN096		0.28 ± 0.03	-2.2 ± 0.31#
	VZMN109		0.19 ± 0.01	-1.93 ± 0.07#
Substitutions at β 6-member ring	VZMN098		0.23 ± 0.03	-1.58 ± 0.09#
	VZMN097		0.28 ± 0.03	-2.17 ± 0.64
	VZMN094		0.42 ± 0.03	-1.59 ± 0.07#
	VZMN107		0.18 ± 0.01	-1.8 ± 0.05#
Alkyl chain linker at position 3 of β 5-member ring	VZMN100		0.24 ± 0.03	-1.58 ± 0.08#
	VZMN104		0.16 ± 0.01	-1.65 ± 0.13#
	VZMN105		0.20 ± 0.02	-1.93 ± 0.2#

Table 3. MOR K_i(nM) and Hill slope values for the indole derivatives of 6β-naltrexamine. The novel compounds are divided into three groups according to the position of substitution in the indole ring. Data are presented as mean values ± SEM from at least three experiments. #, Hill coefficient was $p < 0.05$ different from one as determined by Student's *t*-test.

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3.2 Affinity of the indole derivatives of 6α and 6β-naltrexamine for KOR

3.2.1 Affinity of the indole derivatives of 6α-naltrexamine for KOR

From the results obtained, it can be seen that the position of the substitution on the indole ring did not affect the binding affinity for KOR substantially. Among the indole analogues of 6α-naltrexamine, compound 092 had the least affinity for KOR with K_i value of 3.44 nM whilst compound 102 had the highest affinity for KOR with subnanomolar K_i value. The other α indoles had K_i values ranged from 1 to around 1.5 nM (Table 4). Increasing the length of the linker between the indole ring and morphinan skeleton led to higher affinity (compounds 091, 101, and 102). Compared to NTX, the affinity of α analogues for KOR were either slightly lower or higher. However, none of the indole analogues of 6α-naltrexamine had lower affinity for KOR than NAQ.

3.2.2 Affinity of the indole derivatives of 6β-naltrexamine for KOR

Interestingly, all the indole analogues of 6β-naltrexamine produced very high affinity for KOR. Unlike the indole analogues of 6α-naltrexamine, the range of K_i values of the indole analogues of 6β-naltrexamine did not exceed 1 nM except for compound 098, which had ~ 2nM (Table 5). Elongating the length of the linker did not have a pattern of either increasing, as in α analogues, or decreasing the binding affinity.

3.3 Affinity of the indole derivatives of 6α and 6β-naltrexamine for DOR

3.3.1 Affinity of the indole derivatives of 6α-naltrexamine for DOR

Generally, the affinity of the indole derivatives of 6α-naltrexamine for DOR is lower than for KOR. From the results obtained, it could be assumed that the position of the substitution had a greater impact on the affinity of the indole derivatives of 6α-naltrexamine for DOR than for KOR. Compounds 102 and 092 had the highest and lowest affinity for DOR, K_i = 6.73 nM and 26.7 nM, respectively. The range of K_i values for other compounds was between 10-25 nM (Table 4). The alkyl chain length that links the indole ring to the morphinan skeleton did not have an effect on the affinity when it is elongated by one more carbon atom.

3.3.2 Affinity of the indole derivatives of 6β-naltrexamine for DOR

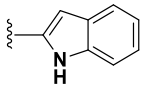
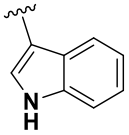
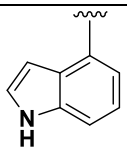
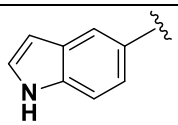
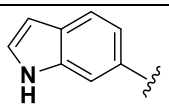
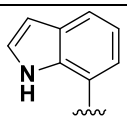
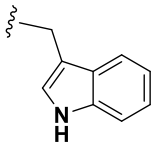
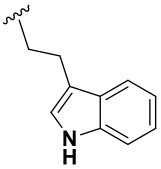
The position of the substitution on the indole ring on the β configuration produced greater variation on the affinity within the indole derivatives of 6β-naltrexamine. Two compounds had the lowest affinity with K_i of 50 nM or above, 098 and 104. Compound 096 had the highest affinity, K_i = 1.54 nM. The indole derivatives of 6β-naltrexamine had a wider range of K_i values than the α indoles, which was from 1.54 to 77.28 nM (Table 5). Increasing the length of the linker between the indole ring and morphinan skeleton did not have a pattern in either enhancing or decreasing the affinity.

3.4 Selectivity for MOR over KOR and DOR of indole derivatives 6α and 6β-naltrexamine

Neither the substitutions nor the configuration greatly enhanced the selectivity for MOR over KOR (Table 4 and 5). Compound 106 in the α configuration and compound 098 in the β configuration had the highest selectivities with 7.4 and 8.7, respectively, affinity ratio for MOR over KOR. It is worth mentioning that both of these compounds were linked to the 6-position carbon of the morphinan skeleton via the 6-member ring of the indole group. However, the position of the substitution in the indole ring was different, with compound 106 linked at the 7 position and compound 098 at the 4 position, which are opposite (para) to each other on the 6-member ring. In general, the α indole analogues had better selectivity for MOR over KOR than the β indole analogues.

In contrast to selectivity over KOR, the selectivity for MOR over DOR was significantly enhanced by the substitution and the configuration (Table 4 and 5). Among α analogues, compound 106 had the best selectivity, 60-fold selective for MOR over DOR. It was noticed that the β analogues generally had higher selectivity for MOR over DOR than α analogues. Compound 104 was 461-fold selective for MOR over DOR. Compound 098 had also good selectivity for MOR over DOR, at 212-fold selective. It can be seen from the selectivity ratios of these novel compounds that compound 106, in the α configuration, and compound 098, in the β configuration, had good selectivity for MOR over both KOR and DOR.

Table 4. K_i values of indole derivatives of 6α-naltexamine at KOR and DOR and selectivity summary.

		K_i (nM) ± SEM			**Selectivity Ratio**	
compound	R	μ	κ	δ	κ/μ	δ/μ
NTX[a]		0.33 ± 0.02	1.44 ± 0.11	143.5 ± 13.7	4.4	435
NAQ[a]		1.11 ± 0.07	13.3 ± 1.1	169.9 ± 15.0	12	146
VZMN090		0.36 ± 0.03	0.93 ± 0.13	14.2 ± 2.8	2.5	39.2
VZMN099		0.28 ± 0.04	0.98 ± 0.13	10.5 ± 2.9	3.4	37.0
VZMN095		0.26 ± 0.04	1.49 ± 0.35	9.3 ± 2.8	5.6	35.1
VZMN092		0.76 ± 0.11	3.44 ± 0.99	26.7 ± 7.7	4.5	35.0
VZMN093		0.43 ± 0.05	1.63 ± 0.28	12.8 ± 3.4	3.8	29.4
VZMN106		0.23 ± 0.01	1.69 ± 0.35	13.7 ± 1.4	7.4	60
VZMN091		0.74 ± 0.12	3.13 ± 0.45	9.2 ± 2.7	4.2	12.2
VZMN101		0.43 ± 0.04	1.36 ± 0.18	25.1 ± 6.8	3.1	57.3

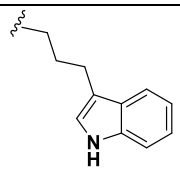
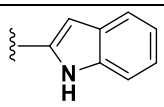
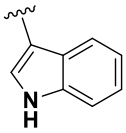
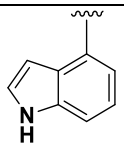
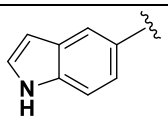
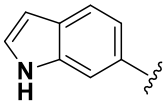
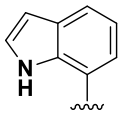
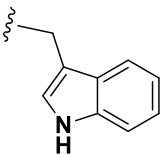
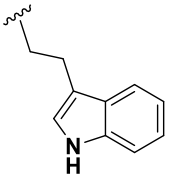
VZMN102		0.29 ± 0.03	0.15 ± 0.03	6.73 ± 0.40	0.5	23.0

Table 4. K_i values of indole derivatives of 6α-naltexamine at KOR and DOR and selectivity summary for MOR over both KOR and DOR. Data are presented as mean values $\pm$ SEM from at least three experiments. The concentration of [^{3}H] dipernorphine in KOR binding assays was 0.95 $\pm$ 0.07 nM and the total binding in the absence of the competitor was 0.43 $\pm$ 0.01 Pmol/mg. The concentration of [^{3}H] dipernorphine in DOR binding assays was 1.26 $\pm$ 0.17 nM and the total binding in the absence of the competitor was 1.0 $\pm$ 0.06Pmol/mg. [a]NTX and NAQ K_i values (Yuan et al., 2015)

Table 5. K_i values of indole derivatives of 6β-naltexamine at KOR and DOR and selectivity summary

		K_i (nM) ± SEM			**Selectivity Ratio**	
compounds	R	μ	κ	δ	κ/μ	δ/μ
VZMN096		0.28 ± 0.03	0.18 ± 0.002	1.54 ± 0.47	0.6	5.4
VZMN109		0.19 ± 0.01	0.16 ± 0.01	7.17 ± 1.2	0.83	37.3
VZMN098		0.23 ± 0.03	1.94 ± 0.30	49.8 ± 12.7	8.2	212
VZMN097		0.28 ± 0.03	0.51 ± 0.04	17.7 ± 4.8	1.8	63
VZMN094		0.42 ± 0.03	0.17 ± 0.01	6.2 ± 0.8	0.7	26
VZMN107		0.18 ± 0.01	0.52 ± 0.09	30.4 ± 9.5	2.8	162
VZMN100		0.24 ± 0.03	0.95 ± 0.11	37.0 ± 1.2	3.9	153.5
VZMN104		0.16 ± 0.01	0.39 ± 0.05	77.1 ± 28.3	2.3	461.6

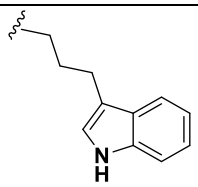
VZMN105		0.20 ± 0.02	0.18 ± 0.04	25.3 ± 4.3	0.83	124.5

Table 5. K_i values of indole derivatives of 6β-naltexamine at KOR and DOR and selectivity summary for MOR over both KOR and DOR. Data are presented as mean values ± SEM from at least three experiments

3.3 Efficacy of the indole derivatives of 6α and 6β-naltrexamine at mMOR-CHO

To determine the efficacy of the novel indole 6-substituted naltrexamine analogs for MOR-mediated G-protein activation, concentration-effect curves for ligand-stimulated [^{35}S]GTPγS binding were examined in mMOR-CHO cell membranes. The stimulation by each compound was normalized to that of a maximally effective concentration (3 μM) of the full MOR agonist DAMGO, so that the E_{max} values derived from the curve fits represent the relative efficacy of each compound (E_{max} of DAMGO = 100%).

3.3.1 Efficacy of the 6α-naltrexamine indole derivatives at mMOR-CHO

The results show that the position of substitutions on the indole rings did not substantially affect potency or efficacy. All α analogues had low to moderate efficacy to activate the MOR (Figure 9 and Table 6). Compound 106 (substitution at position 7) had the lowest efficacy, with a relative E_{max} of 19% (of DAMGO), while compound 099 was the most potent, an EC_{50} value of 0.1 nM. Increasing the length of the alkyl chain at position 3 led to enhancement of both potency and efficacy, and compound 102 with a three carbon linker was the most efficacious. However, compound 099 with no spacer between the indole and the morphinan skeleton was more potent than the compounds that had additional carbon spacers. All the α analogues produced Hill slope values that were not significantly different from 1.

3.3.2 Efficacy of the 6β-naltrexamine indole derivatives at mMOR-CHO

In contrast to their respective α analogues, the β analogues had greater variation in efficacy and potency as a function of position of substitution. E_{max} values ranged from as low as 17% of DAMGO to as high as 92% of DAMGO (Figure 10 and Table 7). EC_{50} values also varied from sub-nanomolar to 14nM. Compounds 097 and 098 were lowest in efficacy with E_{max} values of 17% and 22% of DAMGO, respectively. However, compounds 096, 109, and 094 were the most efficacious, with E_{max} values of 92%, 79%, and 72% of DAMGO, respectively. The other compounds had low to moderate efficacy, ranging from approximately 26% to 55%. Most compounds had Hill slope that were not different from 1.

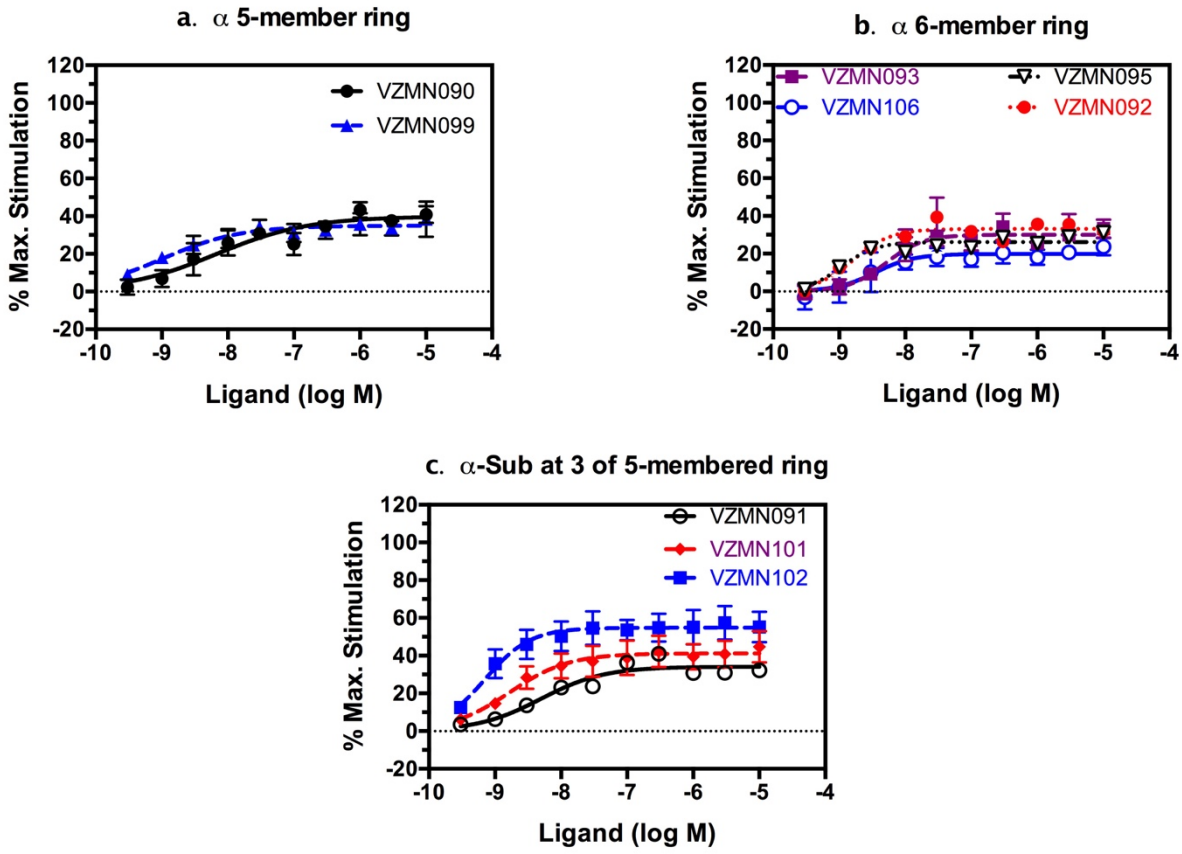


Figure 9. Concentration-effect curves of indole derivatives of 6α-naltrexamine for the stimulation of [^{35}S]GTPγS binding in mMOR-CHO cells. A) compounds 090 and 099 (with substitutions at positions 2 and 3 of the indole ring). B) compounds 095, 092, 093, and 106 (with substitutions at positions 4, 5, 6, and 7 of the indole ring). C) compounds 091, 101, and 102 (with increasing the length of the alky chain with one, two, and three carbon atoms, respectively, at position 3 of the indole ring). Data are presented as mean values ± SEM from at least three experiments. Net agonist stimulated [^{35}S]GTPγS binding was 173.37 ± 15.63 fmol/mg and the basal binding in the absence of an agonist was 48.63 ± 2.95 fmol/mg.

Table 6. E_{max} (%DAMGO), EC_{50}, and Hill slope values for indole derivatives of 6α-naltrexmine.

Compound	E_{max} (% DAMGO)	Log EC_{50}	Hill slope
VZMN090	36.91 ± 2.40	-8.40 ± 0.24 (6.75 nM)	1.63 ± 0.49
VZMN099	33.33 ± 3.51	-9.02 ± 0.06 (0.10 nM)	0.89 ± 0.11
VZMN095	28.90 ± 0.95	-8.52 ± 0.29 (4.99 nM)	1.28 ± 0.50
VZMN092	32.24 ± 1.25	-8.81 ± 0.02 (1.54 nM)	1.50 ± 0.38
VZMN093	26.62 ± 0.66	-8.26 ± 0.09 (6.05 nM)	1.69 ± 0.53
VZMN106	19.11 ± 3.31	-8.67 ± 0.27 (3.85 nM)	1.92 ± 0.30
VZMN091	34.13 ± 0.97	-8.31 ± 0.02 (4.75 nM)	0.90 ± 0.04
VZMN101	41.55 ± 6.14	-8.68 ± 0.04 (2.09 nM)	0.87 ± 0.10
VZMN102	54.89 ± 6.34	-9.14 ± 0.09 (1.89 nM)	1.57 ± 0.40

Table 6. E_{max} (%DAMGO), EC_{50}, and Hill slope values for indole derivatives of 6α-naltrexmine. E_{max} (%DAMGO), EC_{50}, and Hill slope values for indole derivatives of 6α-naltrexmaine derived from Concentration-effect curves for the stimulation of [^{35}S]GTPγS binding in mMOR-CHO cells. Data are presented as mean values ± SEM from at least three experiments.

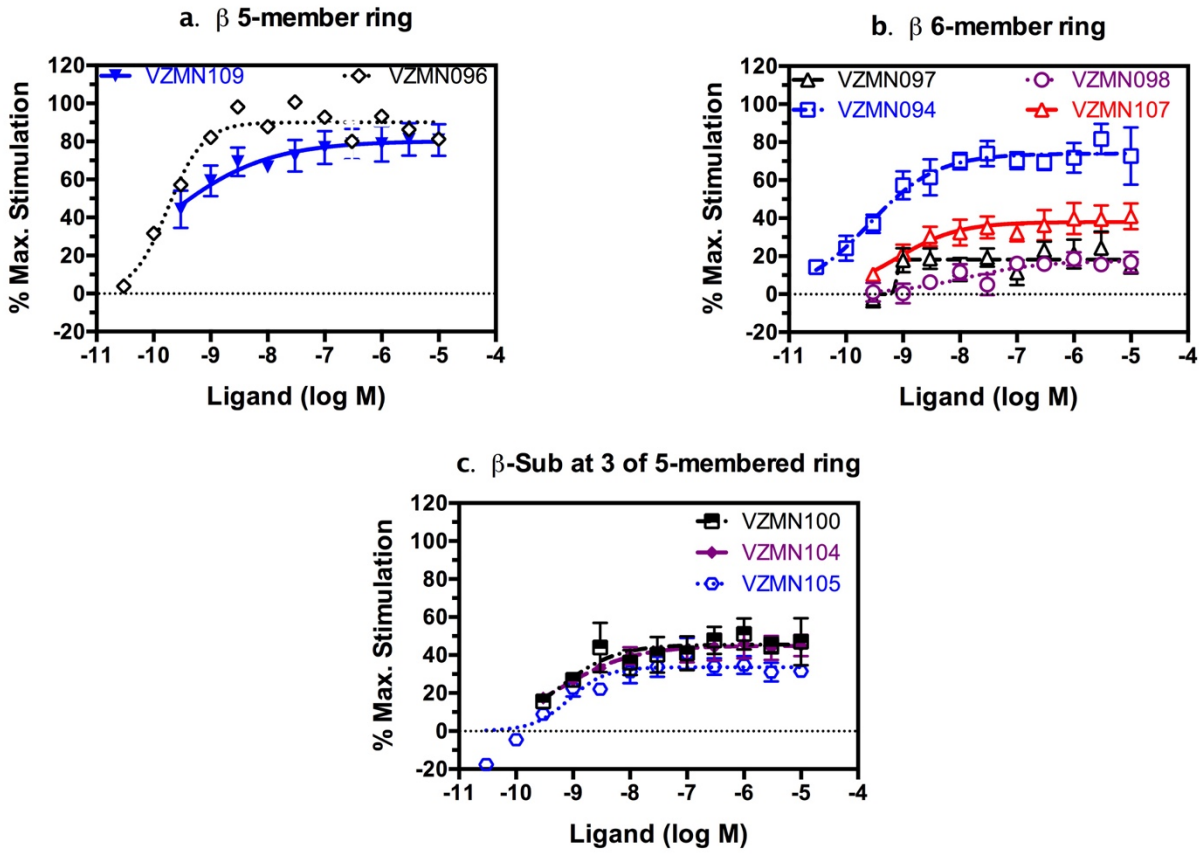


Figure 10. Concentration-effect curves of indole derivatives of 6β-naltrexamine for the stimulation of [^{35}S]GTPγS binding in mMOR-CHO cells. A) compounds 096 and 109 (with substitutions at positions 2 and 3 of the indole ring). B) compounds 098, 097, 094, and 107 (with substitutions at positions 4, 5, 6, and 7 of the indole ring). C) compounds 100, 104, and 105 (with increasing the length of the alky chain with one, two, and three carbon atoms, respectively, at position 3 of the indole ring). Data are presented as mean values ± SEM from at least three experiments.

Table 7. E_{max} (%DAMGO), EC_{50}, and Hill slope values for indole derivatives of 6β-naltrexamine

Compound	E_{max} (% DAMGO)	Log EC_{50}	Hill slope
VZMN096	92.42 ± 2.81	-9.69 ± 0.02 (0.21 nM)	1.54 ± 0.22
VZMN109	79.48 ± 7.40	-9.75 ± 0.18 (0.22 nM)	0.65 ± 0.09
VZMN098	16.22 ± 1.40	-8.22 ± 0.33 (8.15 nM)	1.62 ± 0.62
VZMN097	22.32 ± 3.50	-8.64 ± 0.19 (2.97 nM)	6.01 ± 4.40
VZMN094	71.79 ± 4.37	-9.72 ± 0.04 (0.19 nM)	0.93 ± 0.17
VZMN107	37.94 ± 5.85	-9.10 ± 0.11 (1.92 nM)	0.82 ± 0.07
VZMN100	48.27 ± 4.45	-9.04 ± 0.24 (1.44 nM)	0.91 ± 0.34
VZMN104	45.08 ± 5.18	-9.29 ± 0.16 (1.73 nM)	0.68 ± 0.03°
VZMN105	36.29 ± 4.11	-8.99 ± 0.07 (1.04 nM)	0.85 ± 0.12

E_{max} (%DAMGO), EC_{50}, and Hill slope values for indole derivatives of 6β-naltrexmaine derived from Concentration-effect curves for the stimulation of [^{35}S]GTPγS binding in mMOR-CHO cells. Data are presented as mean values ± SEM from at least three experiments. °, Hill coefficient was $p < 0.05$ different from one as determined by Student's *t*-test.

3.4 Correlation of K_i values with EC_{50} values at MOR

Analysis was conducted in order to determine the correlation of the affinity with the potency at MOR of the novel ligands. Results showed that the K_i values did not correlate with EC_{50} values ($r^2 = 0.034$, $p = 0.465$; Figure 11). Further analysis of the α or β analogs, each as a separate group, also showed no correlation between K_i values and EC_{50} values ($r^2 = 0.004$ and 0.008, $p = 0.815$ and 877, respectively). Although this finding was somewhat surprising, multiple factors could potentially account for such results. For example, different assay conditions in receptor binding versus functional assays might play a role in these results. This will be discussed further in Chapter 4.

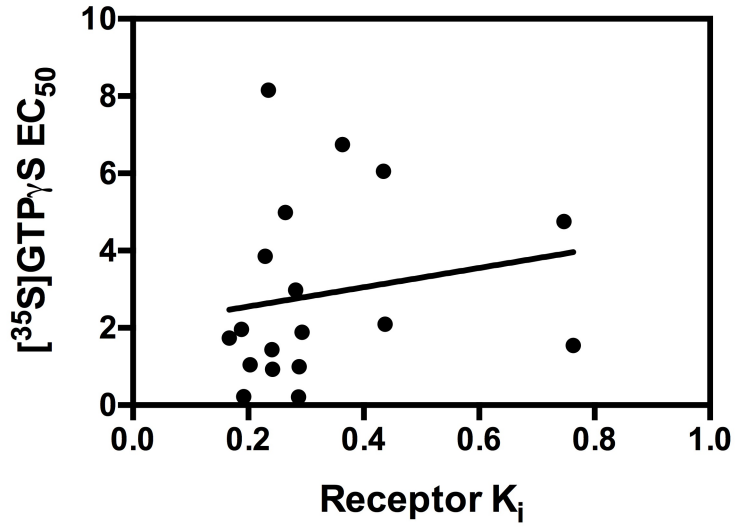


Figure 11. Correlation of K_i values with EC_{50} values in mMOR-CHO cells.

3.5 Efficacy comparison to known compounds (NAQ and nalbuphine) in mMOR-CHO

Based on the initial screen for efficacy of the indole analogues, compounds 098 and 106 had the lowest efficacy. To determine how these compounds compared

with known opioid compounds with low efficacy for MOR activation, a direct comparison was conducted in mMOR-CHO cells using the [35S]GTPγS binding assay. As in the initial functional screen, all compounds were compared with the full MOR agonist DAMGO. However, one parameter was changed from the initial screen. Three concentration points per log unit were used in this study, as opposed to two concentrations per log unit in the initial screen, in order to obtain a more accurate assessment of curve-fit parameters with these low efficacy compounds. The results (Figure 12 and Table 8) showed that compounds 098 and 106 appeared to have slightly lower efficacy than the low efficacy partial agonist nalbuphine, which is available clinically. They also had slightly lower apparent efficacy than the MOR-selective low efficacy partial agonist NAQ. As expected, NTX was the least efficacious with less than 10% of maximum DAMGO stimulation, which is consistent with its accepted action as a MOR antagonist. Statistical analysis, however, did not find any significant differences in E_{max} values between compounds 98, 106, nalbuphine and NAQ, although all four of these ligands had significantly higher efficacy than NTX and lower efficacy than DAMGO. The full MOR agonist DAMGO produced maximum activation of MOR. In this particular assay, both compounds 106 and 098 had Hill slopes were not significantly different from 1. Under these experimental conditions, both compounds 098 and 106 had relative efficacy values that were essentially the same as nalbuphine and NAQ (Figure 13).

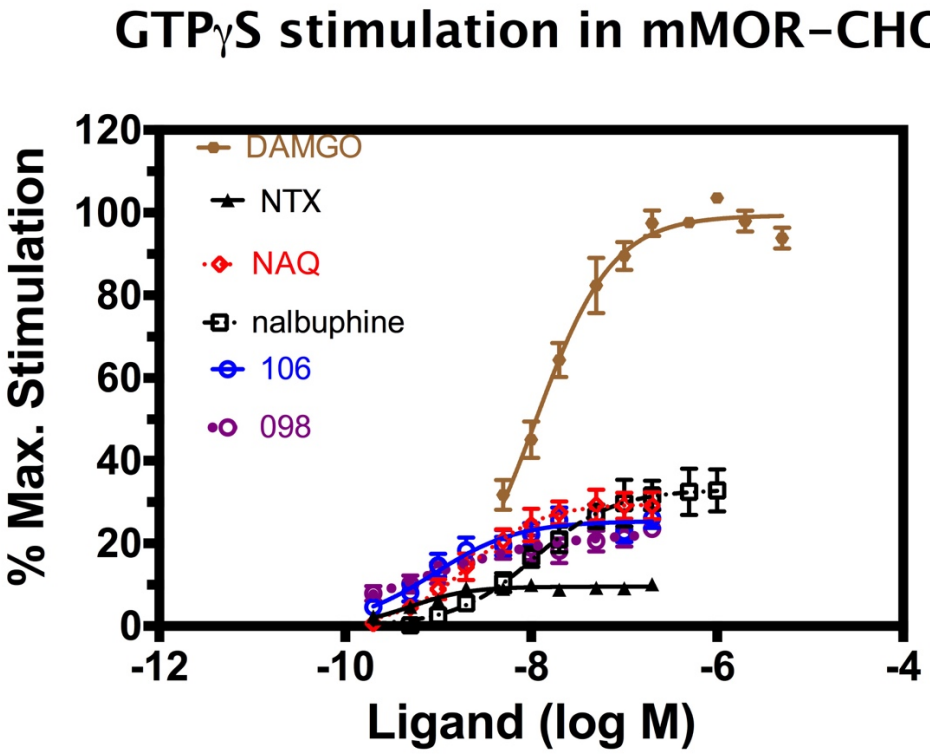


Figure 12. Concentration-effect curves of compounds 106, 098, NAQ, nalbuphine, NTX, and DAMGO for the stimulation of [^{35}S]GTPγS binding in mMOR-CHO cells from side-by-side comparison experiments. Data are presented as mean values ± SEM from at least three experiments. Net agonist stimulated [^{35}S]GTPγS binding was 167.58 ± 9.01 fmol/mg and the basal binding in the absence of an agonist was 32.54 ± 1.77 fmol/mg.

Table 8. E_{max} (%DAMGO), EC_{50}, and Hill slope values for compounds VZMN098, VZMN106, nalbuphine, NAQ, NX, and DAMGO from side-by-side

Compound	E_{max} (% DAMGO)	EC_{50} (Log M)	Hill slope
VZMN098	23.18 ± 2.05	-9.15 ± 0.24 (0.84 nM)	0.76 ± 0.10
VZMN106	25.46 ± 1.58	-9.01 ± 0.11 (1.12 nM)	1.05 ± 0.11
nalbuphine	33.83 ± 3.54	-7.92 ± 0.04 (12.1 nM)	0.96 ± 0.07
NAQ	29.53 ± 2.85	-8.64 ± 0.1 (2.47 nM)	1.18 ± 0.01
NTX	9.73 ± 0.72	-9.29 ± 0.11 (0.60 nM)	1.38 ± 0.45
DAMGO	100.00 ± 1.45	-7.94 ± 0.08 (12.42 nM)	1.07 ± 0.08

E_{max} (%DAMGO), EC_{50}, and Hill slope values for derived from Concentration-effect curves of compounds VZMN098, VZMN106, nalbuphine, NAQ, NX, and DAMGO for the stimulation of [^{35}S]GTPγS binding in mMOR-CHO cells. Data are presented as mean values $\pm$ SEM from at least three experiments.

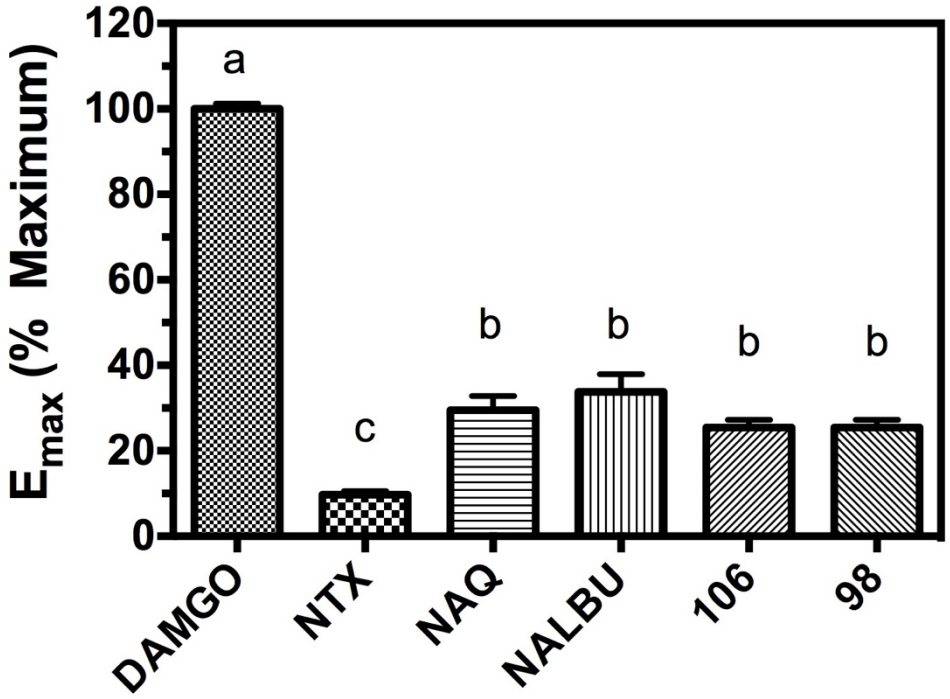


Figure 13. Comparison of ligand E_{max} values in mMOR-CHO cells. Values are mean E_{max} ± SEM derived from the curve fits shown in Figure 11. Values that do not contain any of the same letter designations are $p < 0.05$ different from each other as determined by ANOVA with post-hoc Newman-Keuls test.

3.6 Efficacy comparison to NAQ in mouse thalamus

To compare the relative efficacy of these novel lead 6-indole substituted compounds with NAQ under more native conditions, [^{35}S]GTPγS binding was conducted in membranes from mouse thalamus. Thalamus was chosen because it is the highest MOR-expressing region of the brain, and contains only very low levels of KOR and DOR. Although DAMGO produced robust stimulation of [^{35}S]GTPγS binding in the thalamus, compounds 098 and 106 produced very low stimulation similar to NAQ. It was difficult to obtain an accurate E_{max} or EC_{50} values due to the very low level of stimulation produced by 098, 106, and NAQ. The E_{max} value for compound 106 was only 11% of DAMGO, compared to 16% for NAQ, and the concentration-effects curves for compound 098 could not be unambiguously fit (Table 9 and Figure 14). Under these experimental conditions, compound 106 had a relative efficacy value that was essentially the same as NAQ (Figure 15) and a Hill slope that was not significantly different from 1.

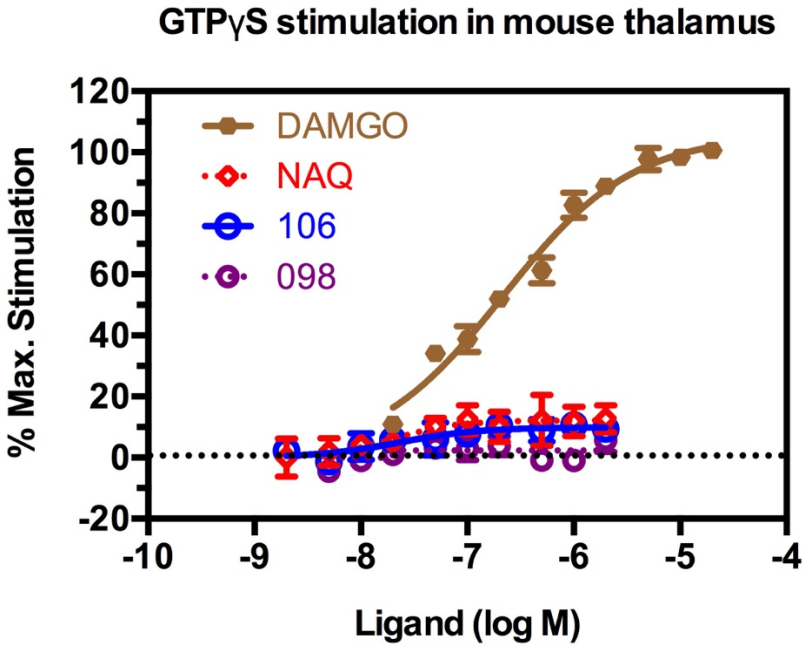


Figure 14. Concentration-effect curves of compounds 106, 098, NAQ, and DAMGO for the stimulation of [^{35}S]GTPγS binding in membranes that were prepared from mouse thalamus from side-by-side comparison experiments. Data are presented as mean values ± SEM from four experiments in which four mice thalami were used. Net agonist stimulated [^{35}S]GTPγS binding was 221.13 ± 5.73 fmol/mg and the basal binding in the absence of an agonist was 125.19 ± 4.13 fmol/mg.

Table 9. E_{max} (%DAMGO), EC_{50}, and Hill slope values for compounds VZMN098, VZMN106, NAQ, and DAMGO in mouse thalamus.

Compound	E_{max} (% DAMGO)	EC_{50} (Log M)	Hill slope
VZMN098	ND[a]	ND[a]	ND[a]
VZMN106	11.21 ± 0.96	-7.62 ± 0.23 (37 nM)	1.36 ± 0.20
NAQ	16.35 ± 4.36	-7.47 ± 0.13 (42 nM)	3.07 ± 1.38
DAMGO	106.0 ± 1.83	-6.67 ± 0.06 (214 nM)	0.75 ± 0.06

E_{max} (%DAMGO), EC_{50}, and Hill slope values for derived from Concentration-effect curves of compounds VZMN098, VZMN106, and DAMGO for the stimulation of [^{35}S]GTPγS binding in mouse thalamus. "a" denotes that those values were not detectable. Data are presented as mean values ± SEM four experiments in which four mice thalami were used.

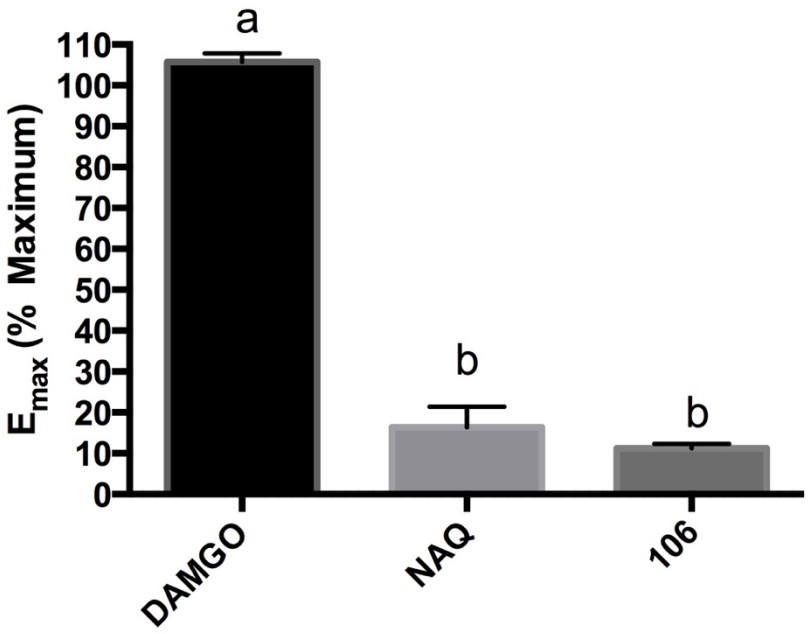


Figure 15. Comparison of ligand E_{max} values in mouse thalamus. Values are mean E_{max} ± SEM derived from the curve fits shown in Figure 13. Values that do not contain any of the same letter designations are $p < 0.05$ different from each other as determined by ANOVA with post-hoc Newman-Keuls test.

Chapter 4. Discussion

The identification of nonpeptidyl, highly selective, reversible MOR selective antagonists is essential for the treatment of opioid dependence/addiction. The reason that blocking MOR could help in treating opioid addiction/dependence is that MOR mediates not only the analgesic effects of clinically prescribed opioids, but also the abuse-related effects of prescribed opioids and heroin (Kieffer, 1999). Also, a MOR selective antagonist could serve as a tool in MOR structural characterization and opioid agonists functional studies. As previously mentioned in the introduction, so far, the current MOR antagonists have disadvantages that limit either their clinical or research applications. Previous molecular modeling and docking studies of NAP and NAQ in all opioid receptor types revealed an alternative address domain in the MOR. The W318 residue, which is located at the border of TM7 and EL3 of the MOR, is recognized by NAP and NAQ through the formation of a Pi-Pi stacking interaction (Zaidi et al., 2013). Based on this modeling result, 18 indole analogues of 6α and 6β-nlatrexamine were designed and synthesized based on the massage-address concept. This study hypothesized that introducing the indole ring at position 6 of the morphinan skeleton would yield compounds that prefer a Pi- Pi-stacking interaction with the W318 residue in MOR. These novel compounds, should they provide the required high selectivity and low efficacy at the MOR, could be developed and formulated for use in the treatment of opioid dependence/addiction.

First, receptor binding assays were conducted to determine the affinity of the novel compounds for MOR and the selectivity for MOR over KOR and DOR. In this assay, the novel compounds compete with labeled naloxone for binding to MOR, and

labeled diprenorphine for binding to KOR and DOR. CHO cells that stably and heterologously express MOR, KOR, or DOR were used to determine the relative affinities and therefore selectivity for MOR compared to the other two opioid receptors. Such cell systems eliminate the chance of binding of the radioligand to another opioid receptor and provide more precise results because it allows measurement of competition of the novel ligands to a single receptor type.

The MOR binding data revealed that all of the indole analogues of 6α and 6β-naltrexamine had very high affinity with mostly subnanomolar K_i values (Tables 2 and 4). This means that the position of the substitution on the indole ring did not have a substantial effect on the affinity for MOR. Compound 106 (the 7-substituted indole analogue of 6α-naltrexamine) had the highest affinity among α analogues, with a K_i value of 0.23 nM, while compound 092 (the 5-substituted indole analogue of 6α-naltrexamine) had the least affinity, with a K_i value of 0.76 nM (Figure 7 and Table 2). It was noticed that increasing the length of the linker at position 3 on the indole ring by one or two additional carbons enhanced the binding affinity (compounds 091, 101, and 102 in the α configuration). Nonetheless, it should be noted that there is some sort of biphasic relationship regarding the distance between the indole ring and the morphinan skeleton. Compound 099 is directly attached to the carbonyl group and had almost the same K_i value as compound 102 with the three-carbon linker. This suggests that there is no single distance between the substituent and the morphinan skeleton that is optimal for MOR affinity. Further docking study could demonstrate how directly attaching the substituent or varying the length of the linker affects the mode of interaction of the novel compounds with MOR. For example, it might facilitate interaction with the W318 residue of MOR by the indole ring. As for the indole analogues of 6β-naltrexamine, compound 104 (with

the two-carbon linker at position 3 of the indole ring) had a K_i value of 0.16 nM which was the best affinity and 2.6-fold greater than the worst affinity, which was obtained with compound 094 (the 6 substituted indole analogue) (Figure 8 and Table 3). Although the α and β configurations showed high binding affinity for MOR, the extent of the affinity variation within each configuration differed. It was observed that the indole analogues of 6α-naltrexamine had somewhat greater variation in their K_i values than did the indole analogues of 6β-naltrexamine. The K_i values for the indole analogues of 6α-naltrexamine ranged from 0.23 to 0.76 nM, while the K_i values of the indole analogues of 6β-naltrexamine ranged from 0.16 to 0.42 nM. Generally, the indole analogues of 6β-naltrexamine had better affinity for MOR than their respective α analogs. On the other hand, when compared to the affinity of NAQ and NAP for the MOR, the introduction of the indole ring to the morphinan skeleton did not substantially alter the binding affinity. The K_i values of NAQ, NAP, and NTX lie within the obtained K_i range for the novel compounds (1.11 nM, 0.37 nM, and 0.33 nM, respectively), when taking experimental variance into account. Interestingly, most compounds had Hill slope values of more than one. Only three compounds in the α indole analogues had Hill slopes that were not significantly different from 1 (090, 093, and 091). For the β indole analogues, all compounds exhibited Hill slopes that were significantly different from 1 except for compound 097. This could indicate positive cooperativity in binding to the MOR. One explanation for the high Hill slope could be that the indole ring of the compound recognizes W318 in MOR first and that facilitates the docking of the whole compound into the binding pocket. However, further docking study of compounds with high Hill slope will be needed to illustrate the most likely mode of binding of these indole analogues.

KOR binding data revealed that indole analogues of 6α and 6β naltrexamine showed high binding affinity for the KOR. Among the indole analogues of 6α-naltrexamine, compounds 102 and 092 had the lowest and highest affinity (with K_i= 0.15 and 3.44 nM, respectively) (Table 4). Intriguingly, all of the indole analogues of 6β naltrexamine exhibited subnanomolar K_i values except compound 098, which was had a K_i value of ~ 2 nM (Table 5). Considering the K_i ratio of KOR/MOR, two compounds were identified with the greatest selectivity ratio: compound 106 (the 7 substituted of 6α-naltrexamine) with 7.4-fold selectivity for MOR over KOR and compound 098 (the 4 substituted of 6β-naltrexamine) with 8.2-fold selectivity for MOR over KOR (Table 4 and 5). Interestingly, increasing the length of the linker at position 3 of the indole ring in the α configuration, but not the β configuration, led to enhancement of the affinity for both MOR and KOR. Thus, the relatively low selectivity ratio of compounds in this series could be attributed to the similarity of effect of linker length on the interaction of these novel compounds with MOR and KOR. A previous docking study of NTX showed that the carbon 6 on the carbonyl group was pointing toward an aromatic binding pocket in MOR and there was also an aromatic binding pocket that was formed while interacting with KOR (Li et al., 2009). A more recent docking study of NAP and NAQ in MOR and KOR revealed that both compounds formed aromatic interaction, Pi-Pi stacking, and possibly hydrogen bond (Zaidi et al., 2013). In order to confirm this speculation with the indole analogues of 6α and 6β-naltrexamine, further docking study is required. When comparing NTX or NAQ, the selectivity ratios of the novel compounds for MOR over KOR was not enhanced substantially. The most selective compounds, 106 and 098, had a higher selectivity ratio than NTX, 7.4 and 8.2 versus 4.4, but had lower selectivity ratios than NAQ, with a selectivity ratio of 12. Generally, the introduction of

the indole substitution at position 6 of naltrexamine in either the α or β configuration did not enhance the selectivity for MOR over KOR from the previously known 6-substituted naltrexamine analogs that have already been published by the Zhang and Selley laboratories (Yuan et al., 2011, 2015).

In contrast to KOR binding data, DOR binding data showed a greater variation in the binding affinity among the indole analogues of either 6α or 6β-naltrexamine. Among the α analogues, compounds 102 and 092 produced the least and highest K_i values, 6.73 and 26.7 nM (Table 4). In contrast to the respective α analogues, the indole analogues of 6β-naltrexamine displayed a larger variation in DOR binding affinities. The K_i values ranged from 1.54 to 77.1 nM, which were exhibited by compounds 096 and 104 (Table 5). Given the low affinity of compound 104 for DOR, it showed the highest selectivity ratio for MOR over DOR among the indole analogues of 6α and 6β-naltrexamine, at approximately 460-fold selectivity for MOR over DOR. Among the indole analogues of 6α-naltrexamine, only compound 106 had a selectivity ratio larger than 50-fold for MOR over DOR, while compound 091 had the lowest, at 60- and 12-fold selectivity for MOR over DOR, respectively (Table 4). Unlike the binding affinity for MOR and KOR, increasing the length of the linker at position 3 of the indole ring in the α configuration did not produce the same effect for DOR. Intriguingly, most indole derivatives in the β configuration of 6-naltrexamine demonstrated high selectivity ratios for MOR over DOR. Six out of 9 compounds had greater than 50-fold selectivity, and 5 of these had more than 100-fold-selectivity for MOR over DOR (Table 5). Compared to NTX and NAQ, some compounds had better selectivity for MOR over DOR while others had lower selectivity ratios. In general, the indole analogues of 6α and 6β-naltrexamine displayed better selectivity for MOR over DOR than for MOR over KOR. A potential explanation for the difference in the

interaction of these ligands with DOR versus KOR was reveled by molecular modeling. A docking study of NTX in DOR showed no formation of an aromatic binding locus to which the C6 carbonyl group pointed in DOR (Li et al., 2009). Docking study of NAQ and NAP in DOR showed no formation of hydrogen bond as was the case in MOR or KOR (Zaidi et al., 2013). Considering the selectivity for MOR over KOR and DOR, compound 106 (the 7-substituted indole analogue of 6α-naltrexamine) and compound 098 (the 4-substituted indole analogue of 6β-naltrexamine) had the best overall selectivity ratios.

From our binding results, it was determined that introducing the indole ring in the hypothesized address domain produced higher affinity for MOR relative to KOR in 13 out of 18 compounds; 8 of these compounds had the indole substituent in the α configuration and 5 in the β. However, only 2 of the 18 compounds had better selectivity than NTX for MOR over KOR. Our prediction that the indole ring might form more stable aromatic interaction (e.g., Pi-Pi stacking) with the W318 residue in MOR than the corresponding Tyr residue (Y312) in KOR was based on the reduced affinity of NAQ and NAP for MOR with mutated W318 (Table 1). However, the Y312 residue in KOR might form a sufficiently stable aromatic interaction with the indole ring. It was shown that the pyridine ring of NAP and isoquinoline rings of NAQ can form aromatic interaction with Y312 in KOR, but to lesser extent than W318 in MOR (Zaidi et al., 2013). Another reason that why our novel ligands have low selectivity for MOR over KOR might be due to the formation of hydrogen bonding with different amino acid residues in both MOR and KOR. This was demonstrated with both NAQ and NAP docking studies (Zaidi et al., 2013). On the other hand, our results showed that the selectivity of our novel ligands, particularly the β analogues, for MOR over DOR was much better than KOR. In fact, all 18 of the novel indole-containing ligands

were more selective for MOR than DOR. One reason could be that the indole analogs might not form strong aromatic interaction in DOR or lack the hydrogen bond formation as in the case of NAP and NAQ (Zaidi et al., 2013). Nonetheless, only one compound of the indole series (compound 104) had greater selectivity for MOR over DOR than naltrexone. Therefore, we can conclude that although these novel ligands generally possessed reasonable selectivity for MOR over KOR or DOR, consistent with the hypothesized role of the address domain, the improvement in selectivity over NTX was modest. Nonetheless, because MOR selectivity over KOR is challenging in morphinan ligands, the fact that two ligands showed somewhat improved selectivity for MOR versus KOR compared to naltrexone is encouraging. However, our results also indicate that the indole substitution is unlikely to yield greater selectivity than substitution with a pyridine or isoquinoline ring, as in NAP and NAQ, respectively.

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Another important feature of a desired compound is to have no or low efficacy at MOR, because this is the main receptor that mediates biological responses of clinically relevant and abused opioids, and was the primary target for which these series of ligands were designed to be selective. The indole analogues of 6α and 6β-naltrexamine were therefore tested in the MOR-CHO cells using the [^{35}S]GTPγS binding functional assay to evaluate the pharmacodynamic efficacy of the novel compounds. The results were analyzed in such a way as to normalize the stimulation produced by each compound to that obtained by the full MOR agonist DAMGO, which provided a measurement of relative efficacy. The results obtained for the indole analogues of 6α-naltrexamine showed that all compounds produced low to moderate efficacy. Compound 106 had the lowest efficacy, with an E_{max} value $\approx$

19%, of DAMGO, while compound 102 had the highest E_{max} value with 54% (Figure 9 and Table 6). As for the potency, the EC_{50} values ranged from approximately 1.5 to 6.5 nM, expect for compound 099, which was the most potent with an EC_{50} value of 0.1 nM. There was a corresponding increase in the E_{max} and potency of compounds 091, 101, and 102 with the increasing the length of the alkyl chain. However, as observed previously from MOR binding results, there could be a biphasic relationship regarding the distance between the indole ring and the morphinan skeleton that affects the potency. Compound 099 was more potent than those with the alkyl chain spacers. This is consistent with the binding affinity of these compounds at MOR, which could be further explored in the future using iterative SAR and modeling study. None of the α analogues had a Hill slope significantly different from 1 in these functional assays.

Unlike the α indole analogues, the indole analogues of 6β-naltrexamine displayed a greater variation in the E_{max} values, revealing compounds of both very high efficacy and very low efficacy. Compound 096 was the most efficacious with an E_{max} value of 92% of DAMGO, while compounds 097 and 098 were the least efficacious with E_{max} values of 22% and 17%, respectively (Figure 10 and Table 7). However, the potency range was tighter in the indole analogues of 6β-naltrexamine than the range of their respective α analogs. With the exception of 098, which was the least potent with an EC_{50} value of ~14 nM, the EC_{50} values ranged from 0.2 nM to 3 nM. It was also observed that elongating the length of the alkyl chain of the carbon spacer at position 3 did not produce the same pattern of potency increase as in the α analogues. Only compound 104 had a Hill slope that was significantly different from 1.

Surprisingly, compounds 109, and 094 demonstrated high efficacy, with E_{max} values that were very close to that of DAMGO, and compound 096 was already known to have high efficacy. The chemical modifications that were applied to the novel compounds were expected to alter only the binding affinity based on previous SAR studies and the message-address concept, in which only the address domain was modified by introducing the indole ring without changing the components that are related to the efficacy. However, it is clear from the data that these modifications also affected the efficacy, producing compounds with a range of efficacies for G-protein activation ranging from low efficacy partial agonists to nearly full agonists despite the fact that these novel indole-containing ligands were based on parent compounds that are MOR antagonists. In fact, three of the 18 novel compounds in the β configuration and one in the α had relative efficacies for G-protein activation that were >50% of the full agonist, DAMGO. On the other hand, only one compound in the α configuration and two in the β had relative efficacies <25% of DAMGO, and all other compounds were in the moderate efficacy partial agonist range (~25-50% of DAMGO). Therefore, it can be concluded that modifying this hypothetical address domain also affected the functional activity of these ligands, although at present we have no accurate means to predict this structure-function relationship. Apparently, ligand interactions with GPCRs are complex, and it is difficult to predict the functional outcomes for the applied modification by the message address concept. The introduction of the indole ring to the hypothetical address domain might change the mode of ligand-receptor interaction in such way that leads to activation of the receptor. One speculation is that maybe the W318 residue in the MOR plays a role in both affinity and efficacy. Of note, both of the most optimally MOR-selective of these indole-containing ligands, compounds 98 and 102, also retained low efficacy similar

to NAQ. Therefore, further molecular modeling studies are required to interpret the SAR related to the role of 6-position substituent interactions with specific amino acid residues in the determination of ligand efficacy at the MOR.

As shown in Figure 11, the novel ligands produced K_i values that do not correlate with EC_{50} values as expected. One explanation could be the different assay conditions in the receptor binding assay versus the functional assay. The presence of GDP and NaCl in the [^{35}S]GTPγS assay can affect the affinity of opioid agonists. As previously discussed in Chapter 1.6.2, both GDP and NaCl facilitate a receptor conformation that does not have high affinity for agonists (Selley et al., 2000; Selley et al., 1997). This is manifested in compound 098, which had a K_i value less than 0.5 nM and EC_{50} value of approximately 8 nM. Another procedural factor that could have differentially affected the K_i and EC_{50} measurements is the selection of ligand concentration range for each assay. For the K_i determination, multiple ligand concentrations were selected that produced between 5 and 95% competition, with the maximum and minimum radioligand binding constrained to 100 and 0%, respectively. This was done to optimize the affinity determination. In contrast, the most important value obtained from the [^{35}S]GTPγS binding assays was the relative efficacy. Therefore, a broad concentration range was selected such that several data points would fall on the maximum plateau of the curve so the maximum effect could be determined experimentally, with only the minimum effect constrained (to 0%). Therefore, the K_i value determinations were likely to be more accurate than the EC_{50} values. Indeed, a subset of ligands (compounds 92, 93, 97, 98, 102 and 106) were re-assayed for stimulation of [^{35}S]GTPγS binding in mMOR-CHO cells using a narrower ligand concentration range, with 3 concentrations per log unit (data not shown). A correlation analysis between the EC_{50} values determined by this method

with the previously determined K_i values was then conducted for these compounds, and the correlation was highly significant ($r^2 = 0.937$, $p = 0.0015$). Therefore, future experiments comparing the K_i and EC_{50} values of all the novel 6-indole analogs under identical assay conditions, including ligand concentrations, would likely show the expected correlation between binding affinity and functional potency.

So far, when considering the binding affinity for MOR, selectivity for MOR over KOR and DOR, and efficacy at MOR, the introduction of indole ring at postiton 6 of α and β-naltrexamine revealed that 2 out of 18 novel compounds had the desired pharmacodynamic profile. Compounds 106 (the 7 substituted indole analogue of 6α-naltrexamine) and 098 (the 4 substituted of 6β-naltrexamine) were the most selective for MOR over KOR and DOR, and demonstrated the least efficacy at MOR. To further characterize the identified compounds, a side by side comparison was conducted with compounds known to have low efficacy at MOR.

The comparison was conducted in mMOR-CHO cells against NAQ, nalbuphine, NTX, and DAMGO. It can be seen from the results (Table 8 and Figure 12) that compounds 106 and 098 produced slightly lower apparent E_{max} values than NAQ and nalbuphine, but were not significantly different from NAQ (Figure 13). As expected, NTX showed the lowest E_{max} that was statistically different from the low efficacy partial agonists and DAMGO (Figure 13). Comparing all low efficacy compounds to DAMGO, there was a significant difference in E_{max} values. Compounds 098 and 106 had Hill slope values that were not different from 1.

To avoid sole reliance on data from transfected cell lines, the lead compounds were tested in membranes prepared from mouse thalamus, which expresses mostly the MOR with very low densities of other opioid receptor types. [^{35}S]GTPγS binding assays were conducted to evaluate the abilities of compounds 106 and 098 to

stimulate MOR-mediated G-protein activation in comparison with NAQ and DAMGO. It was difficult to obtain accurate E_{max} and EC_{50} values for 098 because of the very low level of stimulation produced. Compound 098 acted essentially as an antagonist in thalamus. Compound 106 and NAQ showed approximately 11% and 16% E_{max} values, respectively, indicating that both ligands acted as partial agonists of very low relative efficacy (there was no significant E_{max} difference between 106 and NAQ). Consistent with the Hill slope values obtained from mMOR-CHO cells, compound 106 produced a Hill slope that was not significantly different from 1. These results are consistent with previous studies that demonstrated the concept that a low efficacy partial agonist in a system expressing high receptor density can act as a pure antagonist in a system with a low receptor density. For instance, levallorphan acted as a low partial agonist in mMOR-CHO cells, but as a pure antagonist in rat thalamus (Selley et al., 1997). Also, NAQ acted as pure antagonist when it inhibited DAMGO-stimulated [^{35}S]GTPγS binding in MOR-CHO cells engineered to express MOR at low density (~0.4 pmol/mg), which further supported the concept that a low efficacy partial agonist can act as an antagonist in a system with low receptor density (Yuan et al., 2011). So far, compounds 106 and 098 showed promising data to serve as new lead compounds in continuing investigations with the purpose of developing novel MOR-selective antagonists as potential treatment drugs for opioid dependence/addiction.

To determine whether these newly identified leads are superior to the previously synthesized compounds, NAQ and NAP, further pharmacological characterizations are required. First, the intrinsic efficacy of the lead compounds at KOR and DOR would play a major role in considering their potential superiority over other compounds. As mentioned in Chapter 1.8 one of the side effects of NTX that

causes dysphoria is, possibly, partial agonism at KOR and antagonism at DOR. It has been illustrated that NAQ and NAP have low and moderate efficacy at KOR, respectively (Yuan et al., 2011). The antagonism, or very low efficacy, property at KOR is a desirable feature because activation of the KOR causes dysphoria (Lalanne et al., 2014). Therefore, further [^{35}S]GTPγS functional assays will be required to test the efficacy of our leads at KOR and DOR.

Moreover, the competitive property of an antagonist is essential. An irreversible (noncompetitive) antagonist is not as clinically safe because it does not dissociate from the receptor, leading to a reduction in the available number of receptors until synthesis of new receptors occurs. Also, in pharmacological studies an irreversible antagonist is not favored because it cannot be washed out from the binding locus to revive the receptors. Yet, in some studies an irreversible antagonist can be a valuable tool. NAP and NAQ are known to be competitive antagonists because they produced a right-shift in the concentration-effect curve of DAMGO (Yuan et al., 2011). For that reason, compounds 106 and 098 will need to be tested for competitive antagonism of MOR in the future.

Furthermore, the pharmacokinetic properties will need to be evaluated. In order for the lead compounds to be used for opioid dependence/addiction, they need to have the ability to penetrate into the CNS. NAQ is known to cross the blood-brain barrier (BBB), whereas NAP does not (Yuan et al., 2011). Whether similar results will be obtained with the lead indole analogues will determine their potential for application in targeting the MOR either peripherally or centrally.

Interestingly, NAQ did not exhibit precipitation of withdrawal symptoms like the known opioid antagonists, NTX and naloxone (Yuan et al., 2011). One of the main side effects of NTX is precipitating withdrawal symptoms if it is co-administered

during long-term administration of an opioid agonist. Hence, it might be worthwhile to test the withdrawal-precipitating activity of these novel leads because the benefit of not precipitating withdrawal symptoms could strengthen the potential for use of these antagonists after minimal weaning of addicts from the opioid agonist.

Eventually, following more extensive in vitro tests, such as inhibition of adenylyl cyclase and β-arrestin recruitment, of the lead compounds, in vivo tests will ultimately provide measures of effectiveness in the intact animal. In vivo evaluation of NAQ revealed that it acted as a potent antagonist and did not produce any significant agonist activity in acute antinociceptive agonistic and antagonistic effects in the tail immersion test in mice even at high doses (Yuan et al., 2011). Thus, in vivo tests would determine whether the indole leads would behave like NAQ or not.

To summarize, the introduction of an indole ring at position 6 of α and β-naltrexamine revealed 2 out of 18 novel compounds that showed promising affinity, selectivity, and efficacy data to continue investigation. However, further pharmacological characterizations are required to determine whether these indole analogues possess any advantages over the previously synthesized compounds, NAQ and NAP. Furthermore, feeding molecular and docking studies with the data obtained in this study could produce interesting results from which to interpret the SAR in terms of precise molecular interactions with the opioid receptors.

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