Exploring Allosteric Modulators of Opioid Receptors: Structural and Functional Analysis

by

Matthew Alexander Stanczyk

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Doctoral Committee:

Professor John Traynor, Chair
Professor Henry Mosberg
Associate Professor Manojkumar Puthenveedu
Professor Alan Smrcka
Associate Professor Andrew White

“It is not the critic who counts; not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood; who strives valiantly; who errs, who comes short again and again, because there is no effort without error and shortcoming; but who does actually strive to do the deeds; who knows great enthusiasms, the great devotions; who spends himself in a worthy cause; who at the best knows in the end the triumph of high achievement, and who at the worst, if he fails, at least fails while daring greatly, so that his place shall never be with those cold and timid souls who neither know victory nor defeat.”

— Theodore Roosevelt

Matthew Alexander Stanczyk

mastan@umich.edu

ORCID iD: 0000-0001-6010-5750

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List of Abbreviations, Acronyms, and Symbols

α2AR	alpha2-adrenergic receptor
AC	adenylate cyclase
ago-PAM	positive allosteric modulator with agonist properties
β1AR	beta1-adrenergic receptor
β2AR	beta2-adrenergic receptor
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary cells
CI	confidence interval
CFP	cyan fluorescence protein
DAMGO	[D-Ala$_2$,N-Me-Phe$_4$,Gly-ol$_5$]-enkephalin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DOPr	delta opioid receptor
DPN	diprenorphine
DPDPE	[D-Phe, D-Phe]-enkephalin
EC_{20}	20% effective concentration
EC_{50}	50% effective concentration
EKAR	extracellular signal-regulated kinase activity reporter
ERK	extracellular regulated kinase
FBS	fetal bovine serum
FLAG epitope	DYKDDDDK
FRET	fluorescence resonance energy transfer
G protein	guanine nucleotide binding protein
GDP	guanosine diphosphate
GIRK	G protein-coupled inwardly rectifying potassium channel
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine-5'-triphosphate xi

GTPγS	guanosine-5'-O-(3-thio)triphosphate
GTPγ^{35}S	guanosine-5'-O-(3-[$_{35}$S]thio)triphosphate
HEK293	human embryonic kidney 293 cells
IBMX	3-isobutyl-1-methylxanthine
K_{off}	ligand dissociation rate
K_{on}	ligand association rate
Leu-Enk	leucine-enkephalin
M2R	muscarinic acetylcholine receptor M2
MD	molecular dynamics
Mg/kg	milligram/kilogram
Met-Enk	methionine-enkephalin
MOPr	mu opioid receptor
mu-PAM	positive allosteric modulator of the mu opioid receptor
mu-SAM	silent allosteric modulator of the mu opioid receptor
MWC	Monod-Wyman-Changeux
Na^+	sodium ion
NAM	negative allosteric modulator
NLX	naloxone
NTI	naltrindole
NOPr	nociceptin/orphanin FQ peptide receptor
PAM	positive allosteric modulator
PDB	protein data bank
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
SAM	silent allosteric modulator
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SNC80	4-[(*R*)-[(2*S*,5*R*)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-*N,N*-diethylbenzamide
TEV	tobacco etch virus
THC	(-)-Δ9-tetrahydrocannabinol
TM	transmembrane

Abstract

Pain is one of the most common medical ailments experienced worldwide, affecting roughly 20% of the global population. Despite its prevalence and decades of research, few options provide adequate pain relief and restore normal human function. The current gold standard class of drugs for acute and chronic pain is the opioid analgesics. Opioids act at the mu opioid receptor to provide robust analgesia and act as central nervous system depressants. However, their use is limited because opioids produce respiratory depression and have a high liability for abuse. The prevalence of these side effects has led to the current opioid epidemic and a call for more effective pain management therapeutics.

Positive allosteric modulators and biased agonism at opioid receptors are two emerging paradigms for designing safer pain therapeutics. Positive allosteric modulation refers to ligands that bind to sites topographically distinct from the orthosteric pocket on receptors to enhance orthosteric ligand efficacy and/or affinity. In theory, these compounds could enhance activity of endogenous opioid peptides released during pain states, minimizing global receptor activation in brain regions responsible for addiction and respiratory depression. Biased agonism refers to preferential activation of one intracellular signaling pathway over another, usually G protein vs β-arrestin in the case of opioid receptors. Evidence suggests β-arrestin biased ligands produce more side effects compared to G protein biased ligands. It is well established for other receptor types, such

as the muscarinic and cannabinoid systems, that allosteric modulators are capable of influencing receptor bias promoted by orthosteric ligands. However, no studies to date have examined this phenomenon at the mu opioid receptor.

The work outlined in this dissertation explores the cellular consequences of allosteric modulation of opioid receptors. The results suggest that using positive allosteric modulation is a novel approach to promote signaling bias at opioid receptors. At the delta opioid receptor, the positive allosteric modulator (PAM), BMS 986187, activates the receptor in the absence of orthosteric ligand. BMS 986187 promotes signaling bias favoring G protein activation over β-arrestin recruitment via the allosteric site. This bias is engendered by lack of receptor phosphorylation and ultimately translates into decreased receptor desensitization.

This work also demonstrates how BMS 986187 and a structurally unrelated PAM, BMS 986122, facilitate bias onto orthosteric ligands at the mu opioid receptor. While BMS 986187 elicits β-arrestin bias, BMS 986122 promotes G protein bias, despite both compounds acting at a shared receptor site.

G protein bias is seen as favorable for mu opioid analgesics, as they produce pain relief with reduced side effects. Based on this hypothesis, BMS 986122 was chosen as a scaffold for optimization efforts through medicinal chemistry. Over 40 analogs were synthesized and tested for efficacy in enhancing orthosteric ligand functional activity. Ultimately, a lead compound was identified for future drug development efforts (CCG-258188). This drug produces a 5-fold larger effect than the original lead PAM, BMS 986122. This work sets the stage for further pharmacokinetic optimization and in vivo validation with the promise of providing a safer opioid analgesic.

Chapter 1 : General Introduction

Pain and Opioids

Pain is the most common medical condition worldwide (Sauver *et al.*, 2014), costing the United States approximately 100 billion dollars a year in lost work time and healthcare expenditures (Melnikova, 2010). Meanwhile, an estimated 25% of the United States population (~75 million people) experience moderate to severe chronic pain (Reinke, 2014). Additionally, individuals who have persistent pain are four times more likely than those without it to suffer from anxiety or depression and more than twice as likely to have difficulty working (Katz, 2002; Gureje *et al.*, 2016). Pain has both physiological and psychological components, both of which play an important role in the perception of pain and overall quality of life (Lamé *et al.*, 2005). Modulating either or both of these components effectively reduces pain and improves quality of life (Katz, 2002). Despite efforts to design alternative pain treatment, the most commonly prescribed treatment for pain is opioid analgesics.

Opioids have been used for the treatment of pain for thousands of years (Macht, 1915; Askitopoulou, Ramoutsaki and Konsolaki, 2002; Pasternak and Pan, 2013). They have current advantages in that they are potent, cheap and effective. However, patients taking opioids for extended periods of time experience tolerance, where the patient requires a higher dose to achieve the same amount of pain relief. Tolerance is a major limiting factor of opioid therapy, as higher doses lead to increased side effects including

respiratory depression, constipation, drowsiness. Enhanced side effects as a result of higher doses can result in coma or death (Thompson *et al.*, 1995).

The term ‘Opioid analgesic’ comprises derivatives of the active alkaloids of the *Papaver somniferum (opium)* plant. Poppy plants have been used to treat pain as far back as the Mesopotamian era (3000 BC) (Brownstein, 1993). The first active component of opium, morphine, was isolated in 1806 by Friedrich Serturner and its use became widespread following the invention of the hypodermic needle in the 1850s (Brownstein, 1993). Consequently, addiction to opioids became more prevalent. Using the original morphine compound, many semi-synthetic compounds were developed over the years to improve upon its analgesic properties. Heroin was first synthesized in 1898 and marketed as "non-addictive" morphine, a claim which turned out to be far from the truth. This was followed in the 1940’s by the synthesis of other alkaloid derivatives such as nalorphine and synthetic compounds that bear little structural relationship to morphine, such as methadone (Keats and Telford, 1956).

Despite the synthesis of numerous opioid analgesics, none were significantly more effective than morphine. Further, there was still little biological understanding surrounding their action until the 1960s and 1970s. It was at this time that the first endogenous opioid peptides and their receptors were discovered (Martin, 1967; Pert, Pasternak and Snyder, 1973; Hughes, 1975; Pasternak, Goodman and Snyder, 1975; W R Martin *et al.*, 1976; Lord *et al.*, 1977). The main endogenous opioids known today are beta-endorphin, enkephalins and dynorphins and they activate the mu, delta, and kappa opioid receptors, respectively. Where the mu receptor was named due to the function of morphine at this receptor, the kappa for the function of ketocyclazocine, and delta for studies in the mouse

vas deferens (Mansour *et al.*, 1988). At the same time a seminal study conducted by Martin et al (W.R. Martin *et al.*, 1976) in the chronic spinal dog model, determined the symptoms elicited by agonists for the mu, kappa and sigma (now known not to be a 7 transmembrane GPCR) receptors. Ultimately, it was concluded that the clinically useful opioid analgesics acted primarily at the mu opioid receptor. Shortly after, the delta OR was cloned, followed quickly by the mu opioid receptor was cloned in 1993 (Chen *et al.*, 1993; Thompson *et al.*, 1993; Wang *et al.*, 1993). This discovery was instrumental in analgesic drug discovery. Recent work has documented the crystal structure of a high-affinity antagonist and agonist bound mu opioid receptor (Manglik *et al.*, 2012; Huang *et al.*, 2015).

Despite a long history, the use of opioids to treat pain has been controversial. It was understood that opioids were highly addictive and much of this fear left pain patients suffering as they were drastically undertreated (Melzack, 1990). Two seminal papers published by Melzack and Portenoy et al. in the 1980s and '90s highlighted the lack of adequate pain management in a world gripped by the fear of an opioid addiction epidemic (Portenoy and Foley, 1986; Melzack, 1990). The resulting shift in opinions toward opioid use in managing pain persists today. Opioid prescriptions rose significantly in the United States between 1990 and 2015. Concurrently, U.S. opioid overdose deaths more than tripled over the same time period (Paulozzi, Budnitz and Ms, 2006; Okie, 2010; Bohnert *et al.*, 2011; Kolodny *et al.*, 2015; Scholl *et al.*, 2018). Despite efforts to curb the epidemic, experts estimate the number of opioid overdose deaths will reach 500,000 between 2019 and 2025 in the U.S. alone (Chen *et al.*, 2019). Recent efforts to curtail prescribing are inadequate, and broad-sweeping restrictions will likely leave legitimate pain patients

without sufficient pain management. There is a clear need to develop analgesics that harness the pain-relieving effects of opioids without the catastrophic side effects.

Opioid Receptor Activation

The mu, delta and kappa opioid receptors are members of the Class A G protein-coupled receptor superfamily. GPCRs transmit signals received from the binding of orthosteric ligands and propagate these signals internally through heterotrimeric G proteins (Fig 1.1). Opioid receptors couple to the Gi/o subfamily of G proteins, which lead to inhibition of neurotransmission. Upon receptor activation, the heterotrimeric G protein dissociates into α and βγ subunits. The α subunit inhibits the production of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase. The βγ subunit can interact with multiple protein complexes such as K_{ir} channels and Ca channels, leading to a hyperpolarization of the neuron. βγ also recruits G protein receptor kinases (GRK) to the plasma membrane and facilitates receptor phosphorylation on the C terminal tail. This process is the fundamental beginning of receptor desensitization whereby receptor activation is abrogated by cellular compensatory mechanisms (Al-Hasani and Bruchas, 2013; Williams *et al.*, 2013).

Receptor phosphorylation leads to recruitment of the β arrestin proteins. β-arrestins can scaffold to numerous proteins and abrogate G protein signaling via the incorporation of receptors into clathrin-coated pits. However, β-arrestins can also act as signaling molecules and receptors may continue signaling even when translocated to internal pools (Dewire *et al.*, 2007; Stoeber *et al.*, 2018). In addition, receptors can engage β-arrestins via tail or core engagement. Tail interaction relies on phosphorylation of residues on the c terminal tail. In contrast, the core complex with the receptor involves

coordination between the "finger loop region" of β-arrestin and the helical bundle of the receptor (Latorraca *et al.*, 2018). Differential binding of these β-arrestins is also linked to differences in signaling downstream of the receptor, with the core engagement appearing dispensable for internalization and signaling but necessary for desensitization of G protein signaling (Cahill *et al.*, 2017). Indeed, modern understanding of opioid receptor signaling is far more complex than previously appreciated.

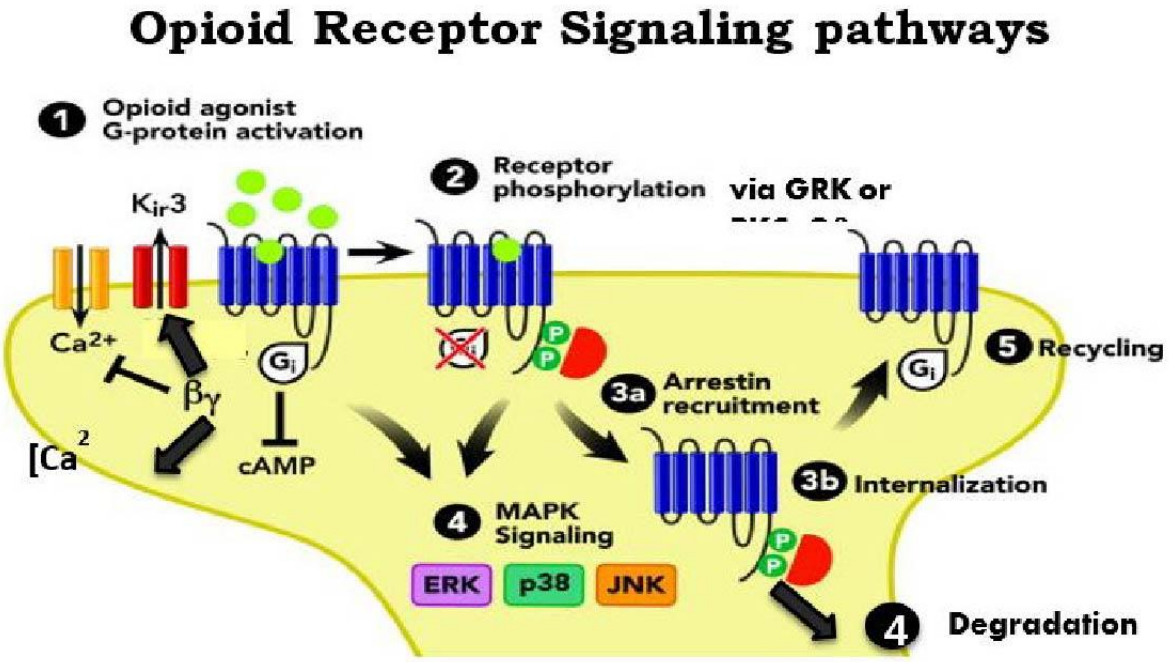


Figure 1.1 Opioid Receptor Signaling Pathways. Reprinted with permission from Anesthesiology (Al-Hasani and Bruchas, 2013)

Biased Agonism in Opioid Drug Discovery

Ligands can couple differentially to the downstream effectors outlined above (i.e., G protein and β-arrestin), a phenomenon referred to as biased agonism. Biased agonism

has been described for many years across many receptor types, although its quantification has been recently aided by the development of more advanced functional assays. Seminal papers highlighted biased agonism at opioid receptors. In particular, morphine analgesia in β-arrestin 2 knockout (KO) mice was observed to be enhanced on the hot plate test (Bohn *et al.*, 1999); in wild type mice (WT) morphine led to a maximum effect of 50% MPE that was reduced to 0% MPE by 90 minutes. Strikingly, the authors noted a near maximal effect (90% MPE) of morphine in β-arrestin 2 KO mice at 150 minutes post-injection. In addition, GTPγ^{35}S binding assays, a measure of G protein activation, performed in membranes from the periaqueductal gray region of β-arrestin 2 KO mice demonstrated an increase in morphine potency and efficacy compared to similar membranes from WT animals, when stimulated with DAMGO, a full agonist.

Two additional studies from the same group examined opioid-related side effects in WT and the β-arrestin 2 KO mice. The first study found that there was a lack of tolerance to the antinociceptive effects of morphine in the KO mice, although physical dependence did develop. (Bohn *et al.*, 2000). The second study demonstrated a significant reduction of opioid-induced constipation and respiratory depression even though analgesia was enhanced in the KO mice (Raehal, Walker and Bohn, 2005).

Collectively, this set of studies set the stage for vigorous development efforts of agonists at the mu opioid receptor with the prevailing theory that G protein biased ligands lead to reduced respiratory depression, constipation, and tolerance. A recent *tour de force* in pharmacology and medicinal chemistry characterized a series of biased mu opioid ligands and evaluated their preclinical therapeutic windows (Schmid *et al.*, 2017; reviewed in Stanczyk and Kandasamy, 2018). In this study, the therapeutic window

represented the separation between the doses at which respiratory depression and analgesia were 50% of maximum effect (ED_{50}) The investigators found a significant correlation between *in vitro* bias and *in vivo* therapeutic window, controlling for complex parameters in pharmacokinetics, providing significant support for the development of G protein mu opioid biased ligands. One compound in particular, SR17018, had a G protein bias factor ranging from 30-85 depending on which cell line and receptor species was used for the assays. SR 17018 had a similar ED_{50} as morphine (6.9 vs 5.9 mg/kg) and produced a maximum effect in the hot-plate analgesia assay. However, when measuring breathing rate (a measure of respiratory depression), morphine suppressed breathing rate to 25% of baseline levels with and ED_{50} of 33mg/kg whereas SR 17018 only suppressed breathing rate to 70% of baseline levels up to a tested dose of 48 mg/kg. Using a ratio of these ED_{50} values as a "therapeutic window" estimate, Morphine had a window of 5 vs 29 for SR17018 (Schmid *et al.*, 2017).

Table 1.1 ED50 Values and Therapeutic Windows for SR Compounds. Adapted from *Schmid et al 2017* (Schmid *et al.*, 2017)

Table 3. ED_{50} Values and Therapeutic Windows for SR Compounds

Agonist	ED_{50} (95% CI)				Therapeutic Window			
	Hot Plate	Tail Flick	$\%O_2$ Saturation	Breath Rate	O_2/HP	O_2/TF	BR/HP	BR/TF
Fentanyl	0.24 (0.16 to 0.32)	0.14 (0.07 to 0.20)	0.71 (0.60 to 0.85)	0.52 (0.37 to 0.67)	3	5	2	4
SR-11501	4.6 (2.9 to 6.3)	11 (5.6 to 16)	18 (13 to 27)	7.6 (4.6 to 11)	4	2	2	1
Morphine	5.9 (4.4 to 7.5)	3.8 (2.5 to 5.2)	79 (62 to 104)	33 (23 to 42)	13	21	5	9
SR-14969	1.7 (0.88 to 2.5)	2.3 (1.4 to 3.2)	21 (16 to 29)	20 (9.8 to 30)	13	9	12	9
SR-14968	0.44 (0.31to 0.57)	0.61 (0.32 to 0.89)	14 (11 to 18)	11 (5.6 to 17)	31	23	28	20
SR-15098	12 (9.5 to 15)	13 (8.9 to 18)	538 (276 to 3359)	174 (70 to 277)	44	40	14	13
SR-15099	8.4 (6.2 to 11)	7.4 (4.9 to 9.9)	560 (260 to > 5000)	206 (25 to 386)	67	75	28	28
SR-17018	6.9 (4.7 to 9.1)	7.7 (4.1 to 11.3)	719 (278 to > 5000)	197 (24 to 370)	105	93	29	26

Bias at delta and kappa opioid receptor is less well understood. At the delta opioid receptor, few rigorous studies determine the bias between ligands mathematically.

However, the delta receptor is a promising target for biased agonist since such as approach could lead to separation of the beneficial effects (anti-depressant; relief of inflammatory pain) with less propensity to tolerance and the proconvulsive side-effects (Pradhan *et al.*, 2009, 2010, 2012). At the kappa opioid receptor, reports suggest that G protein biased ligands avoid the dysphoria associated with receptor activation, although the correlations lack the strong pharmacokinetic validation displayed for the mu opioid receptor (Brust *et al.*, 2016; Bohn and Aubé, 2017).

As the number of biased ligands increases, skepticism surrounding their purported efficacy has mounted due to poor clinical translation. There are currently no clinically available biased agonists for any receptor, although this is most likely due to the nascent rigorous quantification methods. At the mu opioid receptor, TRV130, found by screening, is the only biased ligand to date to make it through clinical trials. Preclinical as well as phase 1 and 2 data was extremely promising: *In vitro,* it was shown that TRV130 has a significantly reduced efficacy at recruiting β-arrestin 2 relative to morphine, while maintaining a maximum effect in cAMP inhibition. In addition, this compound was shown to have reduced receptor internalization and C-terminal tail phosphorylation, two characteristics of low β arrestin recruiting ligands. These *in vitro* data were paired with *in vivo* data, where TRV130 showed rapid-onset analgesia at doses lower than morphine, whereas respiratory depression and constipation were minimal compared to morphine. These data were followed up with successful phase 1 and phase 2 trials which promised effective analgesia with reduced side effects (Singla *et al.*, 2017). However, TRV130 failed to show statistically significant reductions in side effects at doses that were equianalgesic to morphine in phase 3 testing (Cook, Burt and Singla, 2019). In defense

of this failure, the TRV130 compound was very limited in bias towards G protein (~3-fold) (DeWire *et al.*, 2013), however, this failure has cast significant doubt on biased opioid drug discovery efforts.

Allosteric Modulation of Opioid Receptors

Another potential therapy for pain is through the use of positive allosteric modulators of opioid receptors. Allosteric modulators bind to sites topographically distinct from the orthosteric pocket. Allosteric modulators can be envisioned to create a novel 'receptor conformational state' to modulate the affinity and/or efficacy of co-bound orthosteric ligands. Allosteric modulators can show positive, negative, or silent cooperativity with orthosteric ligands and are deemed PAM's, NAM's and SAM's respectively. In addition, some PAMs can activate receptors in the absence of orthosteric ligands, also called Ago-PAMs (Conn, Christopoulos and Lindsley, 2010; Keov, Sexton and Christopoulos, 2011). Recent discoveries from crystal structures have identified a number of different binding sites on GPCRs (Lu and Zhang, 2018).

Positive allosteric modulators possess multiple theoretically advantages over traditional orthosteric ligands (Burford, Traynor and Alt, 2015). First, despite general structural conservation across GPCR families, allosteric sites are believed to be less conserved compared orthosteric sites, this can allow for subtype selectivity within receptor families. Secondly, positive allosteric modulators given in the absence of any exogenous orthosteric agonist will maintain the temporal and spatial fidelity of endogenous signaling. As shown in figure 1.2 (top), following pain or stress, endogenous opioid release occurs in a pulsatile manner. This is in stark contrast to the prolonged exposure to exogenous compounds. With positive allosteric modulators, the endogenous

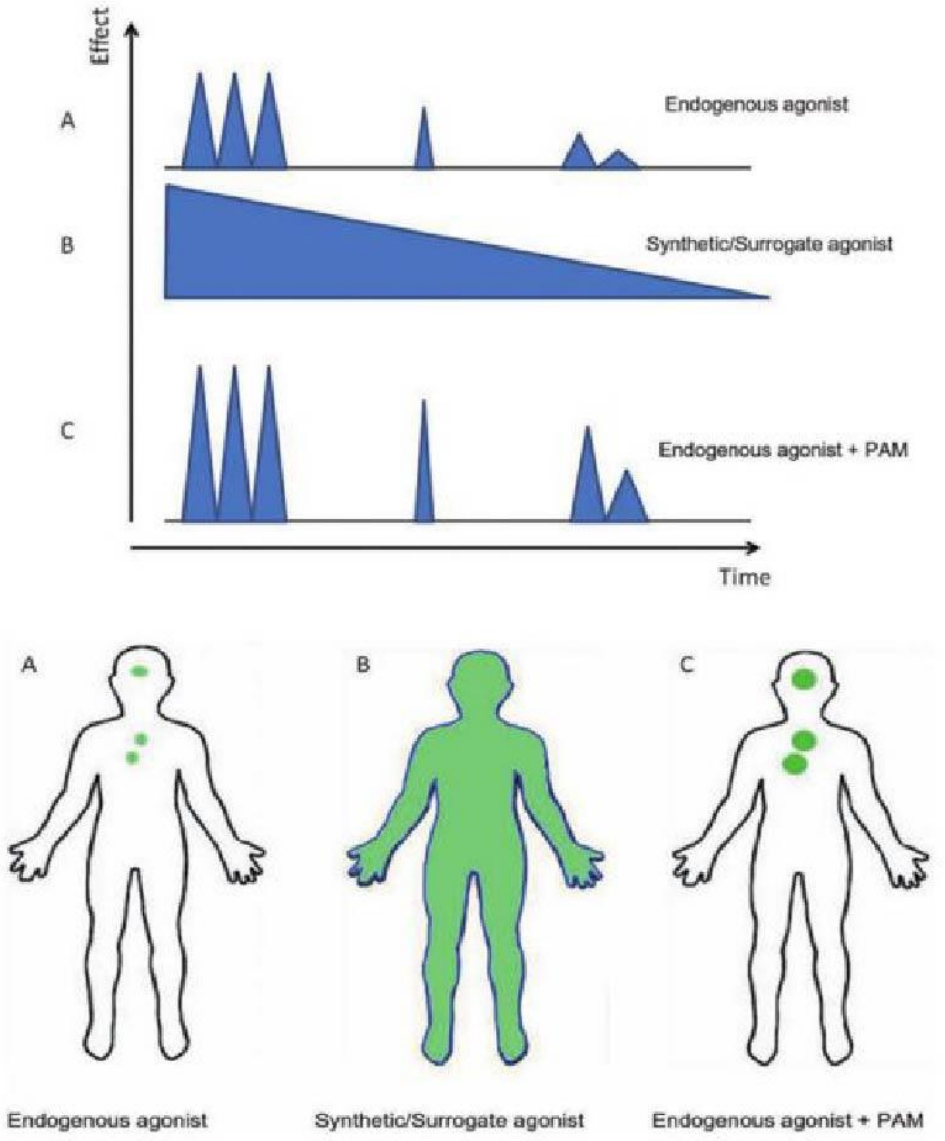


Figure 1.2 Advantages of Allostery. By enhancing endogenous response, allosteric modulators can maintain the temporal (top) and spatial (bottom) fidelity of analgesia elicited in a pain state. This presents the theoretical advantage of targeting only receptors responsible for analgesia in tightly controlled time frames. Figure reprinted with permission from *British Journal of Pharmacology* (Burford, Traynor and Alt, 2015).

signal will be enhanced only when the native neurotransmitter is released. This is important for opioid receptors as prolonged receptor activation is more likely to lead to the development of tolerance. In addition, as shown in figure 1.2 (bottom), endogenous opioids released in painful conditions may be confined to regions of the body responsible for pain modulation. Allosteric modulators can enhance this signal at these specific sites, contrasting the whole-body activation elicited by exogenous orthosteric ligands. For opioid receptors, this presents the benefit of enhancing analgesia produced by endogenous opioids while potentially sparing receptor activation in brain regions responsible for respiration, reward and GI transit.

Finally, allosteric compounds can bias receptor signaling, either alone or in conjunction with orthosteric ligands (Keov, Sexton and Christopoulos, 2011; Davey *et al.*, 2012; Khajehali *et al.*, 2015). These compounds present a novel strategy to engender G protein bias in orthosteric ligands and widen the corresponding therapeutic window of opioids (Fig 1.3).

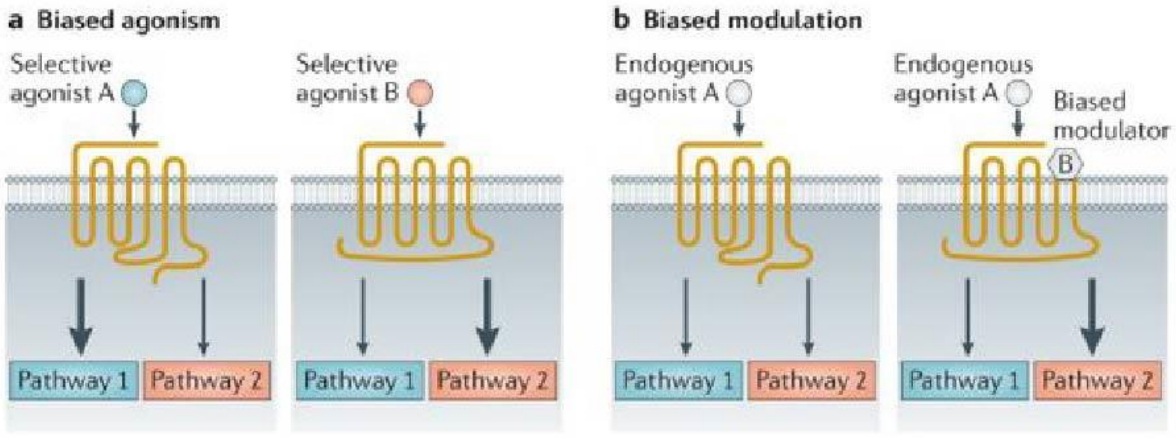
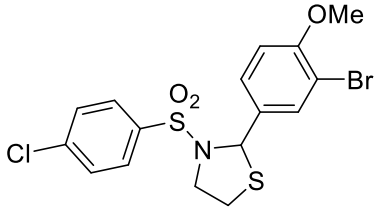


Figure 1.3 Biased Signaling at GPCRs. (a) Biased agonism via orthosteric site allows distinct receptor signaling cascades. (b) Orthosteric ligands with no bias can be engendered with bias via allosteric modulation. Reprinted with permission from *Nature Reviews Drug Discovery* (Kenakin and Christopoulos, 2013)

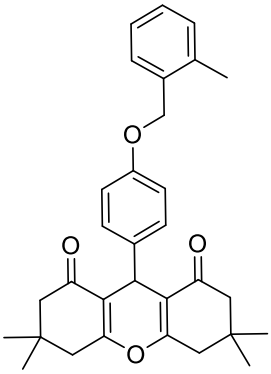
Two novel positive allosteric modulators, BMS 986122 and BMS 986187, were recently discovered via high-throughput screening to act via the mu and delta receptors, respectively (Burford *et al.*, 2013, 2015). BMS 986122 was identified as a PAM for the mu-opioid receptor (mu-PAM) and increases the potency of the endogenous opioid peptide Met-Enkephalin from 169 nM ±16 to 49 nM ± 7, a 3.5-fold increase. Conversely, BMS 986187 was characterized as a delta opioid receptor positive allosteric modulator (delta-PAM) that shifted Leu-enkephalin affinity from 221 nM (119-324 95% C.I) to 7 nM (3-12 95% C.I.), representing a 32-fold shift. Additional studies have shown that the mechanism by which these allosteric modulators enhance ligand efficacy/affinity is through negative cooperativity with sodium ions (Livingston and Traynor, 2014). It is known that sodium ions act a conserved site on opioid receptors (and many class A GPCR's) to maintain quiescent receptor states, acting as negative allosteric modulators (NAM's) (Shang *et al.*, 2014). The evidence presented in Livingston and Traynor indicated positive allosteric modulators may function through disruption of this site. Furthermore, recent evidence suggests that BMS 986187 and BMS 986122 are preferentially active at the delta and mu receptors respectively but are not selective such that they act via a putative conserved site on mu, delta, and kappa opioid receptors (Livingston *et al.*, 2018). However, much about the function of these allosteric modulators at opioid receptors remains unknown.

Collectively, the discovery of allosteric compounds for opioid receptors represents a significant shift from traditional opioid drug discovery efforts. In addition, these compounds serve as useful tools to investigate the structure and function of PAMs at

opioid receptors with respect to their ability to engender bias alone or in combination with a traditional opioid.



BMS 986122



BMS 986187

Figure 1.4 Structures of BMS 986122 and BMS 986187

Aims

The purpose of the work described in this thesis is to further explore the consequences of allostery at opioid receptors. Studies to date have characterized how positive allosteric modulators of the mu-opioid receptor can alter the efficacy and binding affinity of orthosteric ligands. However, the consequences of receptor activation extend far beyond proximal receptor interactions. Future allosteric drug development requires a deeper understanding of the impact on signaling bias and structure-activity relationships at opioid receptors, two elements that are highlighted in subsequent sections.

Chapter 2: BMS 986187 is a G protein biased Ago-PAM at the Delta Opioid Receptor

Chapter two investigates the cellular consequences of delta opioid receptor activation by BMS 986187 in the absence of orthosteric ligand. There have been numerous reports in the literature of allosteric ligands driving biased signaling absent

orthosteric ligand (Gregory, Sexton and Christopoulos, 2007; Valant *et al.*, 2012). The initial discovery paper highlighted BMS 986187 as a positive allosteric modulator; however, no studies were performed on its function in the absence of orthosteric ligand (Burford *et al.*, 2015). The data presented in this chapter highlight BMS 986187 as an Ago-PAM of the delta opioid receptor. In addition, despite showing potent G protein activation, BMS 986187 shows low levels of receptor internalization and recruitment of β-arrestin 2 (Stanczyk *et al.*, 2019). The signaling bias is further explored to identifying a low level of receptor phosphorylation as a leading hypothesis for the observed signaling profile.

Chapter 3: BMS 986187 and BMS 986122 engender divergent bias at the mu opioid receptor

Evidence from Chapter 2 suggested that BMS 986187 action extended beyond the delta opioid receptor. This was followed by a study showing BMS 986187 acts as a positive allosteric modulator at the mu and kappa opioid receptor. Moreover, BMS 986187 and BMS 986122 appear to modulate receptor function at the two receptors via a conserved or possibly overlapping binding site(s). Considering the overlapping binding site, and the impact allosteric ligands can have on receptor bias (Chapter 2), we investigated how these structurally distinct modulators impacted signaling bias of four orthosteric ligands (DAMGO, methadone, fentanyl, morphine). This mechanism of this observed bias was then studied with a focus on related signaling partners.

Chapter 4: Structure-activity relationship of BMS 986122

Chapter 3 identified BMS 986122 as promoting G protein bias at the mu opioid receptor. This is a highly favorable profile according to reported literature. This work

describes efforts to optimize the efficacy of BMS 986122 for future drug development. Insights are provided into functional groups required for PAM function. Additionally, a more complex understanding of allosteric/orthosteric interaction is provided.

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Chapter 2 : The delta opioid receptor positive allosteric modulator BMS 986187 is a G protein biased allosteric agonist

Summary

The delta opioid receptor (DOPr) is an emerging target for the management of chronic pain and depression. Studies have highlighted the potential of biased signaling, the preferential activation of one signaling pathway over another downstream of DOPr, to generate a better therapeutic profile. BMS 986187 is a recently discovered positive allosteric modulator (PAM) of the DOPr. Here we ask if BMS 986187 can directly activate the receptor from an allosteric site in the absence of orthosteric ligand and if a signaling bias is generated. Using various clonal cell lines expressing DOPr we investigated the effects of BMS 986187 on events downstream of DOPr by measuring G protein activation, β-arrestin 2 recruitment, receptor phosphorylation, loss of surface receptor expression, ERK 1/2 phosphorylation, and receptor desensitization. We show that BMS 986187 is a G protein biased allosteric agonist relative to β-arrestin 2 recruitment. Despite showing direct and potent G protein activation, BMS 986187 has a low potency to recruit β-arrestin 2. Data suggests this is the result of limited receptor phosphorylation and ultimately leads to low receptor internalization and a slower onset of desensitization. This is the first evidence of biased agonism mediated through direct binding to an allosteric site on an opioid receptor in the absence of occupancy of the orthosteric site. Our data suggests

that agonists targeting DOPr, or indeed any GPCR, through an allosteric site may be a novel way to promote signaling bias and thereby potentially produce a more specific pharmacology than can be observed by activation *via* the orthosteric site.

Introduction

Chronic pain and depression are two of the most common medical ailments experienced worldwide and are often comorbid. For example, an estimated 25% of the United States population (75 million people) experience moderate to severe chronic pain (Reinke, 2014), while an estimated 15-20% experience depression (Kessler and Bromet, 2013). Opioid analgesics that target the mu opioid receptor (MOPr) are the most widely prescribed drugs for both chronic and acute pain, but suffer from serious side effects including respiratory depression and abuse liability (McNicol et al., 2017; Przewłocki & Przewłocka, 2001). Treatments for depression are varied, but under the best circumstances only an estimated 50% of patients show full remission (Rush *et al.*, 2006). Mounting evidence suggests that agonists targeting the delta opioid receptor (DOPr), a G protein coupled receptor (GPCR), are effective in preclinical models of chronic pain and depression and could provide for new therapies (Jutkiewicz *et al.*, 2005; Bie and Pan, 2007; Cahill, Holdridge and Morinville, 2007; Kabli and Cahill, 2007; Saitoh and Yamada, 2012).

The development of DOPr agonists, such as BW373U86, SNC80 and related compounds, as medications has been limited due to on-target side effects, namely a propensity to cause convulsions and the rapid development of tolerance both in rodent and non-human primate models (Jutkiewicz *et al.*, 2005; Danielsson *et al.*, 2006; Pradhan *et al.*, 2012; Lutz, Pierre-Eric and Brigitte, 2014). Until recently, all compounds developed

as DOPr agonists targeted the orthosteric site on the receptor. However, the discovery of allosteric modulators that act at DOPr, in particular BMS 986187 (Burford et al., 2015), presents an opportunity to interrogate this receptor in a novel way. An allosteric modulator is a compound that binds to a site on a GPCR other than the endogenous ligand or orthosteric site, and by doing so modulates the affinity and/or efficacy of an orthosteric ligand. Allosteric modulators can either be positive (PAM), negative (NAM), or silent (SAM) with regards to their effect on orthosteric ligands (Conn, Christopoulos and Lindsley, 2010; Keov, Sexton and Christopoulos, 2011). Modulators may also possess direct intrinsic pharmacological activity themselves. Such compounds are commonly referred to as “ago-PAM’s” or “ago-NAM’s” depending on the nature of this activity (Langmead and Christopoulos, 2006; Kenakin, 2007).

One potential benefit of allosteric modulators is to engender biased agonism, or functional selectivity (Kenakin and Christopoulos, 2013). Biased agonism is the preferential activation, or inhibition, of certain downstream signaling cascades over others, classically G protein activation over β-arrestin recruitment (Whalen, Rajagopal and Lefkowitz, 2011; Kenakin *et al.*, 2012; Kenakin and Christopoulos, 2013; Schmid *et al.*, 2017). Thus, in theory, a drug could promote downstream effectors associated with beneficial actions while bypassing the effectors associated with the unwanted effects. Multiple studies have suggested biased agonism stemming from orthosteric activation of the DOPr, although pertinent rigorous bias calculations are rarely performed (Audet *et al.*, 2008), see for review (Pradhan *et al.*, 2012). Evidence suggests that β-arrestin 2 mediated internalization of the DOPr might be associated with some of the negative effects of DOPr agonists. Indeed, a number of ligands that fail to internalize the DOPr

such as ARM390, despite potent G protein activation, have shown reduced tolerance (Pradhan *et al.*, 2009, 2010, 2012) and reduced propensity to cause convulsions (Pradhan *et al.*, 2011) in animal models. This suggests an agonist that preferentially activates G protein over β-arrestin 2 recruitment may have reduced on-target side effects that have limited the utility of other DOPr ligands, such as SNC80. However, this does conflict with recent experiments in β-arrestin knockout mice suggesting that β-arrestin 2 recruitment does not contribute significantly to the onset of convulsions (Dripps *et al.*, 2017).

To date, no studies have examined the role allosteric modulation plays in functional selectivity at the DOPr. BMS 986187 shows probe dependence at the DOPr and our prior work suggests it may be a directly acting allosteric agonist as evidenced by its ability to inhibit adenylyl cyclase (AC) in the absence of orthosteric agonist (Burford et al., 2015). To this end, we set out to elucidate the nature of this ago-PAM activity. We found that BMS 986197 is an allosteric agonist with biased signaling towards G protein pathways over the recruitment of β-arrestin 2.

Results

BMS 986187 stimulates GTPγ^{35}S Binding via DOPr

BMS 986187 has been shown to produce inhibition of forskolin-stimulated cAMP production in the absence of orthosteric ligand, demonstrating that it has direct agonist action *via* an allosteric site (Burford et al., 2015). However, inhibition of adenylate cyclase (AC) is a highly amplified signaling output and requires low efficacy in a compound, although it does require prior stimulation of heterotrimeric Gαi/o proteins. To demonstrate that BMS 986187 can directly stimulate DOPr to activate Gαi/o, we performed GTPγ^{35}S

binding assays as previously described (Traynor and Nahorski, 1995) in HEK 293 cells expressing human DOPr (Figure 2.1A). BMS 986187 stimulated GTPγ^{35}S binding in a concentration dependent manner giving a potency value (EC_{50}) of 301 ± 85 nM. In brain homogenates from C57/BL6 mice the DOPr full agonist SNC80 produced GTPγ^{35}S binding with an EC_{50} of 203 ± 31 nM (Figure 2.1B). BMS 986187 also stimulated GTPγ^{35}S binding with a weaker potency (EC_{50} of 1681 ± 244 nM), but the maximal GTPγ^{35}S response to BMS 986187 was 38% greater than that produced by SNC80. Differences in maximum and potency were significantly different as determined by non-overlapping 95% confidence intervals. To confirm the response to BMS 986187 was due to DOPr receptor activation we repeated the experiments in brain tissue from DOPr knockout mice. In brain homogenates from these mice a small degree of BMS 986187 stimulated GTPγ^{35}S binding remained, representing 20% of the BMS 986187 response observed in tissue from wild type mice, with an EC_{50} of 600 ± 397 nM. In contrast, SNC80 produced no appreciable binding over baseline in DOPr knockout mice. These findings suggest that BMS 986187 activates G protein through the DOPr at physiological receptor expression levels while also eliciting a very low level of G protein activation through a non-DOPr mediated pathway.

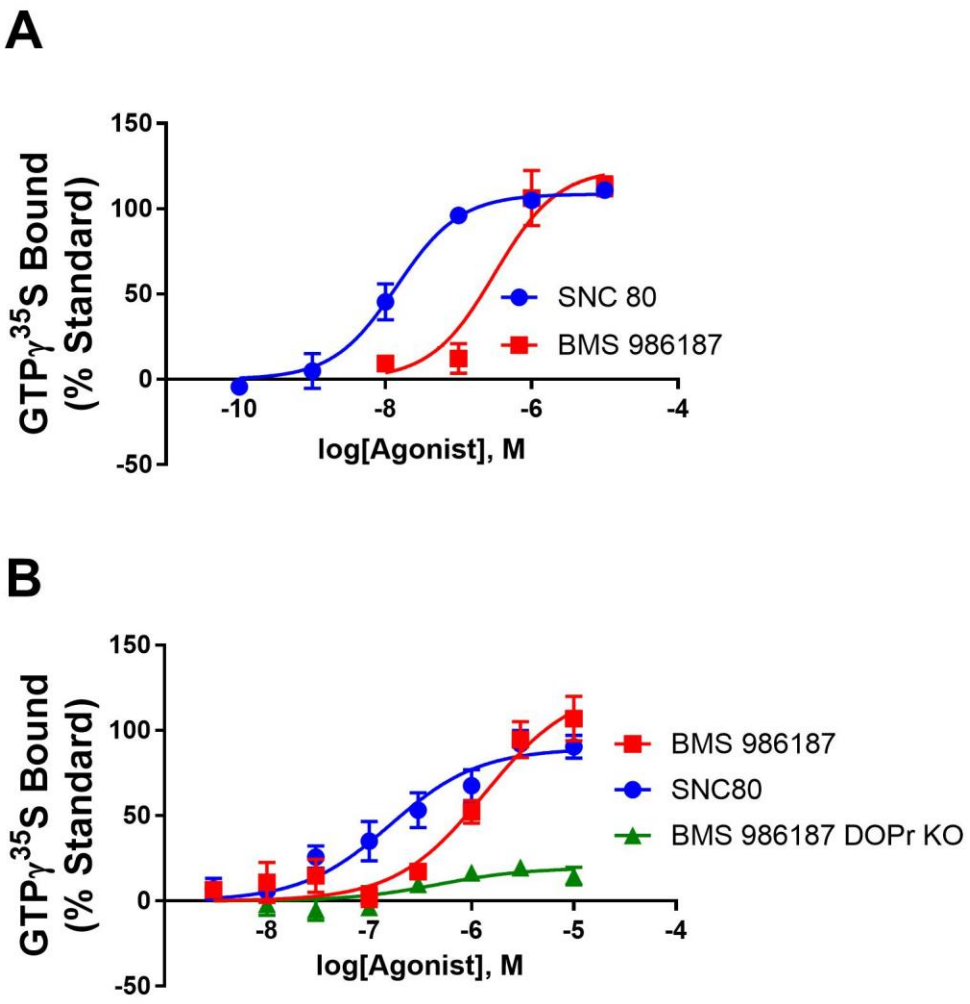

Figure 2.1: BMS 986187 elicits G protein activation. The capacity for increasing concentrations of BMS 9861897 and SNC80 to increase GTPγ35S binding was measured in membranes from FLAG-tagged HEK hDOPr cells (A) or in brain homogenates from wild type or DOPr knockout (KO) mice (B). Data are presented as percentage of the response to a maximal concentration (10 µM) of SNC80. All plotted points are the means ± SEM of five independent experiments, each run in duplicate.

BMS 986187 stimulates GTPγ^{35}S binding through an Allosteric Site on DOPr

Previous work has shown that BMS 986187 does not displace the antagonist ^{3}H-diprenorphine binding from the orthosteric site (Burford et al., 2015). To verify that the agonist action of BMS 986187 is not due to interaction at the orthosteric site, GTPγ^{35}S binding was performed in membranes from HEK hDOPr cells in the presence or absence of various concentrations of orthosteric antagonists. The DOPr antagonist naltrindole (NTI) (100 nM) reduced the maximal GTPγ^{35}S binding evoked by 10 μM BMS 986187 from 99 ± 6% to 51 ± 17 % (Figure 2.2A), a statistically significant difference. Increasing the concentration of NTI by 100-fold (to 10 μM) caused no additional inhibition of BMS 986187-stimulated GTPγ^{35}S binding (50 ± 4%; Figure 2.2A). NTI (10 μM) alone failed to produce any appreciable stimulation of GTPγ^{35}S binding, consistent with its classification as a neutral antagonist (Tryoen-Toth *et al.*, 2005). The partial loss of BMS 986187-stimulated GTPγ^{35}S binding was also observed in the presence of 10 μM of the non-specific opioid antagonist naloxone. Using CHO hDOPr cells as an alternative cell line, NTI showed a concentration-dependent, but saturable, inhibition of BMS 986187 stimulation of GTPγ^{35}S binding with the lack of parallel shifts confirming the agonist action of the modulator is not due competition at the orthosteric site but rather due to negative cooperativity between the orthosteric and allosteric sites. To further verify this, we evaluated the effect of increasing concentrations of NTI on the maximal stimulation elicited by BMS 986187 and SNC80 using membranes from CHO hDOPr cells (Figure 2.2C). NTI showed a saturable inhibition of BMS 986187 stimulation of GTPγ^{35}S binding. In contrast SNC80- mediated stimulation of GTPγ^{35}S binding was fully inhibited by NTI, consistent with a competitive mechanism.

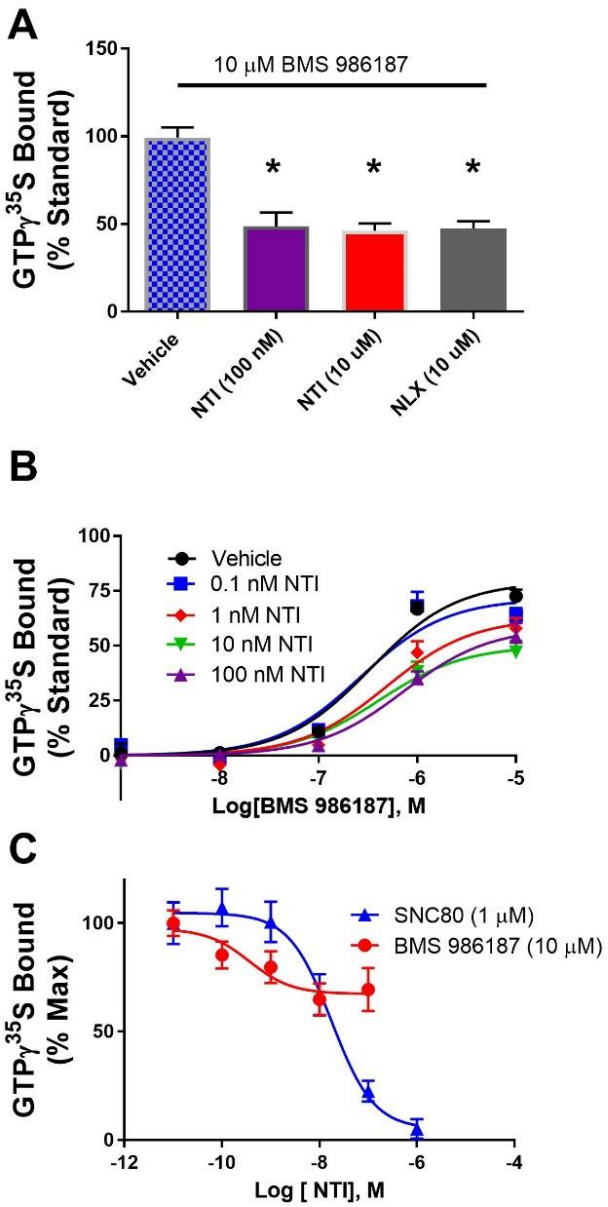

Figure 2.2 Antagonists show non-competitive interaction with BMS 986187. (A) Antagonists naltrindole (NTI) and naloxone (NLX) reduce maximal GTPγ^{35}S binding caused by BMS 986187 in FLAG-tagged HEK hDOPr cells, normalized to percent of effect of 10 μM SNC80 to control for variation between the different preparations. (B) GTPγ^{35}S concentration response curves for BMS 986187 with increasing NTI concentrations and (C) GTPγ^{35}S binding concentration response of NTI with fixed concentration BMS 986187 (10 μM) or SNC80 (1 μM), in CHO cells expressing hDOPr. All plotted points are the mean ± SEM of five (B, C) or ten (A) individual experiments, each performed in duplicate.

BMS 986187 causes a low level of DOPr internalization

Previous studies of the DOPr suggests that ligands with high efficacy at activating G protein while maintaining low efficacy at promoting receptor internalization, show reduced tolerance in animal models (Pradhan et al., 2009). To this end, we next sought to determine whether BMS 986187 would cause DOPr internalization relative to the orthosteric partial agonists TAN-67 and DPDPE and the full agonist SNC80. Preliminary studies indicated that 10 µM would be a maximal effect for all ligands and from initial time course studies (Figure 2.3A) we chose 1 h to evaluate and compare the ligands. Due to the small effect window data were pooled for analysis to provide maximum and EC_{50} values with 95% Confidence Intervals. BMS 986187 treatment resulted in low levels of internalization (7 [3.9-10.0] %) relative to TAN-67 (11 [5.2-16.2] %) < DPDPE (31 [17.7-44.1] %) and SNC80 (33 [24.7- 40.9] %) with a potency order TAN-67 (1.3 [0.15 – 11.0] nM) = SNC80 (3.7 [1.5-9.2] nM) > BMS968187 (94 [59 – 1007] nM) = DPDPE (212 [72-623] nM).

To contrast the propensity of BMS 986187 to cause internalization with its ability to activate G protein at DOPr we measured the maximal GTPγ^{35}S binding by BMS 986187 and compared this with saturating concentrations (10 µM) of the partial agonist peptide DPDPE, the partial agonist TAN-67 and the full agonist SNC80, which was used as the standard. As shown in Figure 2.4B, BMS 986187 elicited 99% ± 6 of GTPγ^{35}S binding relative to SNC80 versus 63% ± 7 and 75% ± 6 for TAN-67 and DPDPE respectively, a statistically significant difference. Thus BMS 986187 gives a greater level of G protein activation than DPDPE but a reduced level of internalization. Additionally, BMS 986187

affords a similar level of internalization as TAN-67 but stimulates a higher level of GTPγ^{35}S binding.

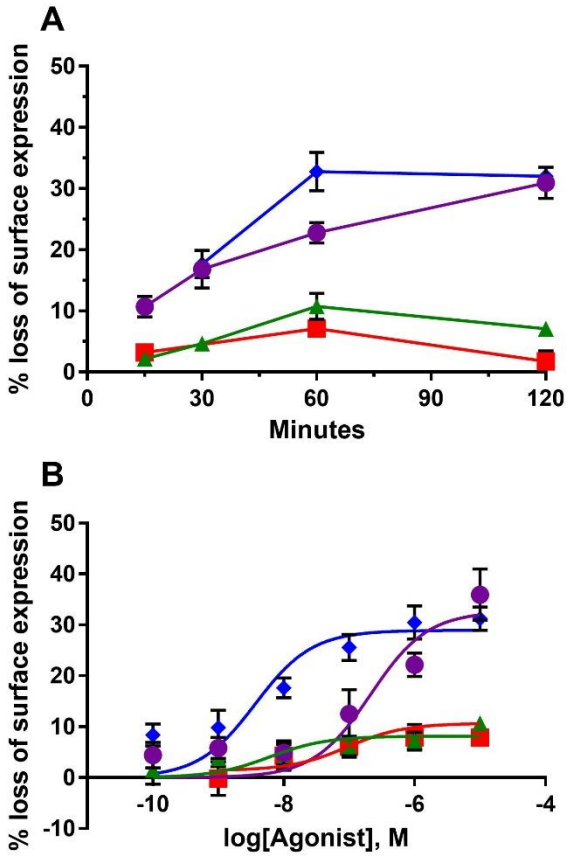

Figure 2.3 DOPr Internalization. Receptor internalization by DOPr ligands in HEK cells expressing FLAG-tagged hDOPr. Preliminary time-course studies (means ± SEM, n = 3 experiments in triplicate) with 10 µM concentrations of ligands (A) were to identify an appropriate time (1 h) to determine (B) concentration-response studies for the different DOPr ligands (means ± SEM, n = 5 experiments in triplicate). The symbols in (A) also refer to (B).

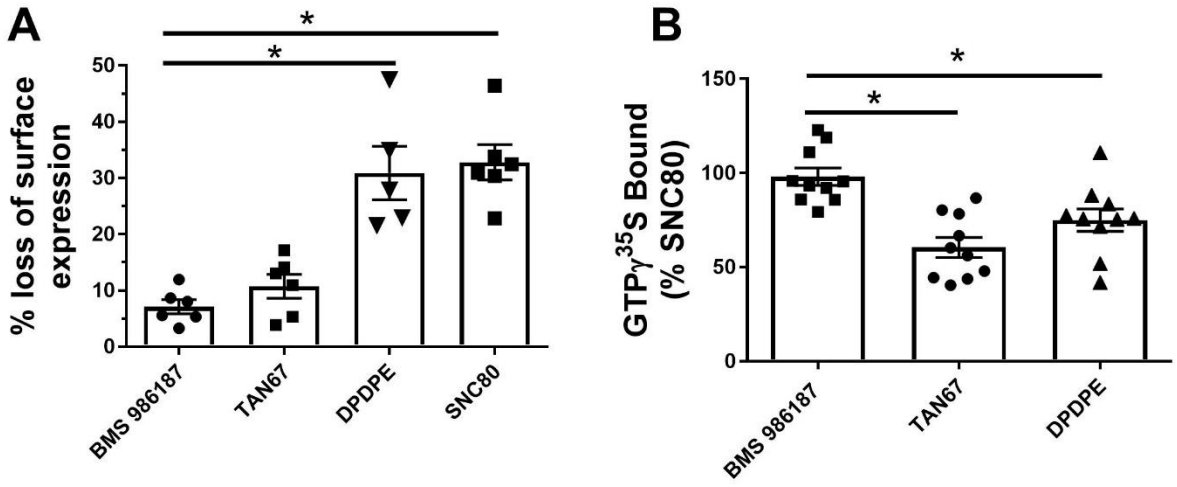

Figure 2.4 BMS 986187 shows biased activation of GTPγ35S relative to receptor internalization. DOPr ligands were evaluated in FLAG-tagged HEK hDOPr cells by measuring (A) receptor internalization or (B) stimulation of GTPγ^{35}S bound. BMS 986187 showed significantly lower internalization relative to DPDPE and SNC80, despite showing significantly greater GTPγ^{35}S binding relative to TAN-67 and DPDPE. GTPγ^{35}S binding is normalized as percent of 10 µM SNC80 to control for variation between the different preparations. All experiments were performed using saturating concentrations of compounds (10 µM). Data are expressed as mean ± SEM of five (internalization) or ten (GTPγ^{35}S) individual experiments, each performed in duplicate.

BMS 986187 is G protein biased relative to β-arrestin 2 recruitment

The fact that BMS 986187 affords greater GTPγ^{35}S stimulation that DPDPE and TAN-67, while causing a low level of DOPr internalization is indicative of ligand bias. Internalization of receptors is largely β-arrestin-dependent therefore in order to explore this further, we directly compared concentration responses for BMS 986187 and SNC80 to recruit β-arrestin 2 to DOPr and stimulate GTPγ^{35}S binding. BMS 986187 and SNC80 stimulated a similar level of GTPγ^{35}S binding in HEK hDOPr cells (Figure 2.5B), in agreement with our previous result (Figure 2.1A) although BMS 986187 was less potent (Table 2.1). In contrast, using the PRESTO-TANGO assay BMS 986187 recruited β-arrestin 2 very weakly up to 100 µM, the limit of solubility (Figure 2.5A). Extrapolation of

the BMS 986187 concentration-response curve assuming a similar maximum to SNC80, afforded an EC_{50} for BMS 986187 of 579 μM. In comparison TAN-67 and DPDPE recruited similar levels of β-arrestin 2 as SNC80, although DPDPE (16.1 ± 8.0 μM) was much less potent than TAN-67 (327 ± 176 nM) or SNC80 (353 ± 141 nM). From these data the relative bias of BMS 986187 for GTPγ^{35}S stimulation over β-arrestin 2 recruitment was evaluated with SNC80 serving as a reference agonist using the log(max/EC_{50}) function as described by Kenakin (Kenakin, 2017) (Table 2.1). This shows BMS 986187 is G protein biased relative to β-arrestin 2 when compared to SNC80, with a bias factor of 82 (Table 2.1). It should be noted that the PRESTO-TANGO assay employs a chimeric DOPr receptor with a Vasopressin receptor tail, although the effect of this modification should be eliminated using SNC80 as a reference ligand (Kenakin, 2017). Utilizing this assay, we found DPDPE to be biased towards G protein compared with SNC80 with a calculated bias factor of 11 (Table 2.1). This is similar to the bias of DPDPE compared to SNC80 of 6, calculated using the same equation from data in Chiang et al., 2016, who employed a complementation assay for β-arrestin recruitment in CHO cells. Moreover, the bias of BMS986187 fits with the change in ligand order when comparing internalization with GTPγ^{35}S binding (Fig 2.4), and the PRESTO-TANGO assay has been previously used in studies of receptor bias (Che *et al.*, 2018).

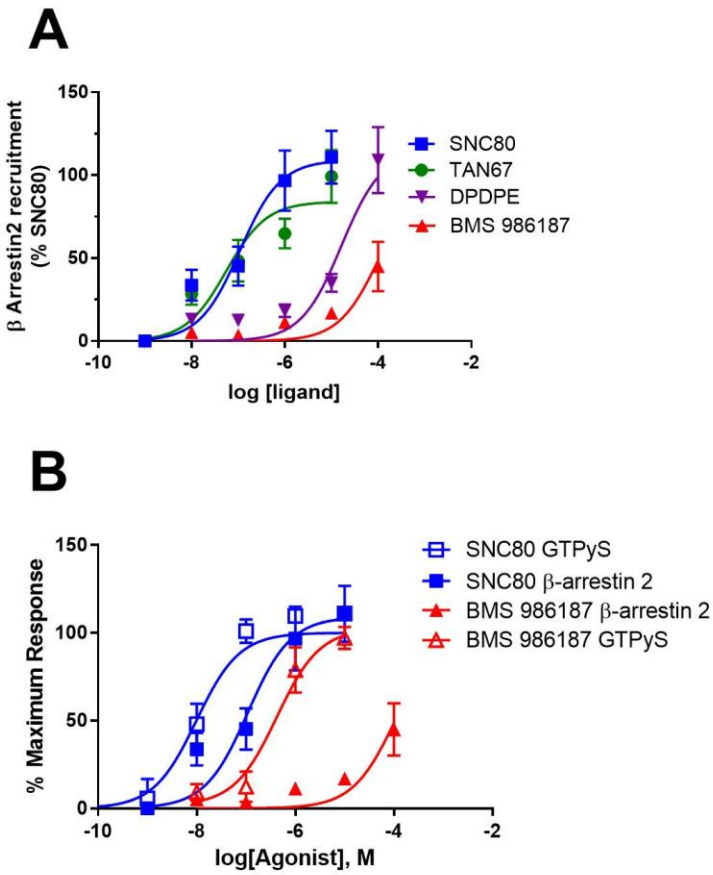

Figure 2.5 BMS 986187 is G protein biased over β-arrestin 2. BMS 986187 bias was evaluated between β-arrestin 2 recruitment (A) and GTPγ^{35}S binding (B) relative to the standard orthosteric agonist SNC80. Normalization was performed to control for sources of variation across preparation and to allow for comparison across both assays. β-arrestin assays were performed in HTLA cells transiently transfected with hDOR-TANGO and GTPγ^{35}S assays in membranes from HEK cells expressing FLAG-tagged hDOPr as described in the methods. Data are presented as the mean of five (GTPγ^{35}S) or seven (β-arrestin 2) independent experiments, each performed in duplicate and expressed as mean ± S.E.M.

To confirm the low degree of β-arrestin 2 recruitment to DOPr by BMS 986187, we performed confocal microscopy in FLAG tagged HEK DOPr cells transfected with 0.4 µg β-arrestin 2 GFP as shown in Figure 2.6. Cells were incubated for 5 min with 10 µM of either SNC80 or BMS 986187, a saturating concentration for G protein activation. β-arrestin 2 localization was then quantified as a ratio of fluorescent intensity at the cell membrane divided by the cytoplasmic intensity using line scan analysis. Consistent with the findings in Figure 5B and published literature (Chiang, Sansuk and van Rijn, 2016), SNC80 afforded statistically significant translocation of β-arrestin 2 to the plasma membrane, however, localization in cells treated with BMS 986187 was not significantly different from baseline. This suggests the maximal β-arrestin 2 recruitment in response to BMS986187 is much less than recruited by SNC80, so we re-calculated the bias factor assuming the 100 µM data point in Fig. 2.5A (the point of solubility) was the maximal effect; this calculation yielded a bias factor of 34 (Table 2.1).

BMS 986187 shows low levels of ERK 1/2 activation

Agonists at opioid receptors have been shown to signal through ERK 1/2 via both G protein and β-arrestin mediated pathways and previous work has shown that BMS 986187 acting as a PAM can increase the potency of orthosteric DOPr agonists in promoting ERK 1/2 phosphorylation (Burford et al., 2015). However, BMS 986187 (10 µM) alone failed to elicit significant ERK1/2 phosphorylation relative to vehicle in HEK hDOPr cells, whereas SCN80 (1 µM) afforded statistically significant phosphorylation of ERK/12 (Figure 2.7).

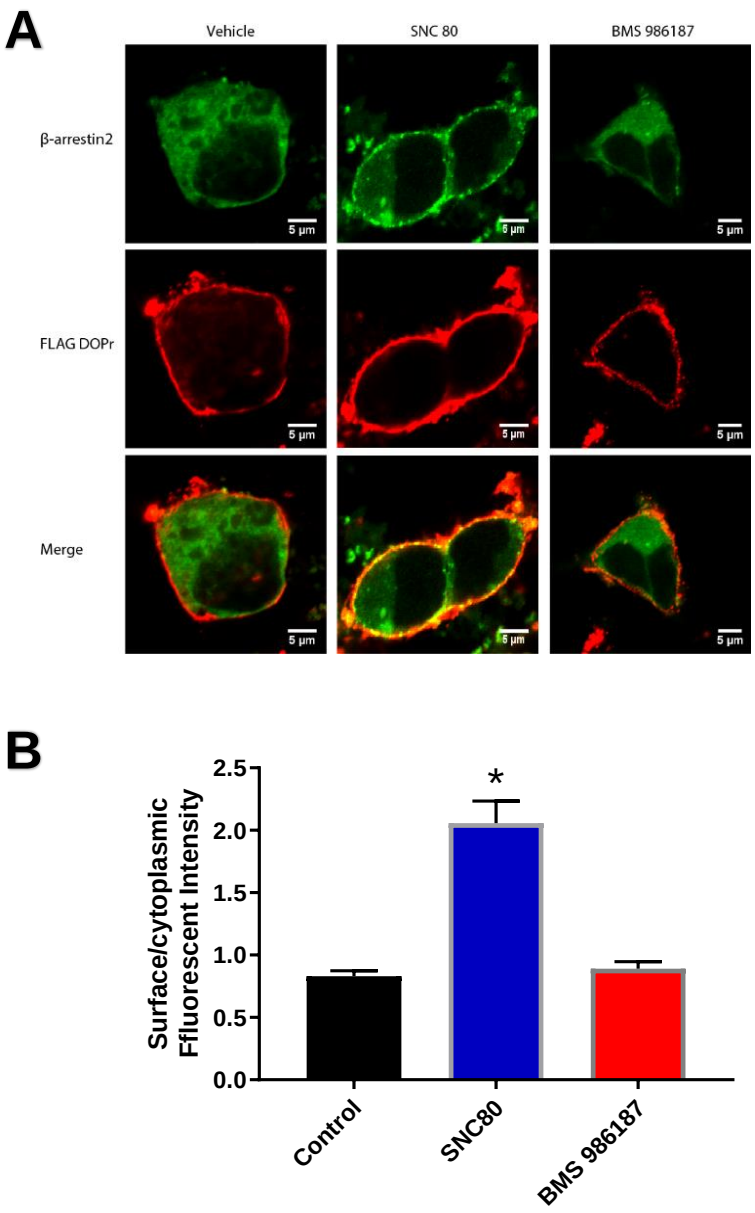

Figure 2.6 Effect of SNC80 and BMS 986187 on β-arrestin 2 Recruitment. FLAG-tagged HEK hDOPr cells were transfected with 0.4 µg β-arrestin 2 GFP and treated with 10 µM of either SNC80 or BMS 986187 for five min and imaged using confocal microscopy. FLAG-tagged DOPr is represented in the red channel with the green channel representing β-arrestin 2. (A) Representative images and (B) β-arrestin 2 recruitment expressed as surface/cytoplasmic GFP intensity. Data represent the means ± S.E.M. of 34 cells per condition from five independent drug treatments.

As expected, we observed SNC80- promoted DOPr internalization (Figure 2.7A), but also saw internalization at later time points in approximately 50 % of cells treated with BMS 986187 (Figure 2.7A). A similar percentage of vehicle treated cells showed DOPr internalization, although to a lesser degree than BMS 986187, suggesting that the modulator is promoting constitutive internalization of DOPr in these cells (Trapaidze *et al.*, 2000; Ong *et al.*, 2015). This agrees with the BMS-986187-induced enhancement of DOPr internalization shown in Figure 2.3.

BMS 986187 induces low levels of DOPr phosphorylation and desensitization

DOPr phosphorylation, arrestin recruitment and receptor internalization are initiated by a phosphorylation event at Ser363 in the C-tail of DOPr (Kouhen *et al.*, 2000; Qiu, Loh and Law, 2007). As BMS 986187 promotes only a low level of receptor internalization or β-arrestin 2 recruitment, we hypothesized this was due to inefficient phosphorylation of this residue. To assess this, we performed western blot analysis of Ser363 phosphorylation of FLAG-tagged DOPr expressed in HEK 293 cells treated with various DOPr agonists for one h as shown in Figure 2.8. Consistent with the internalization data, BMS 986187 did not induce significant phosphorylation of this residue compared to the vehicle control. Similar findings were seen with TAN-67, whereas the higher internalizing agonists DPDPE and SNC80 caused a marked degree of phosphorylation deemed statistically different from vehicle.

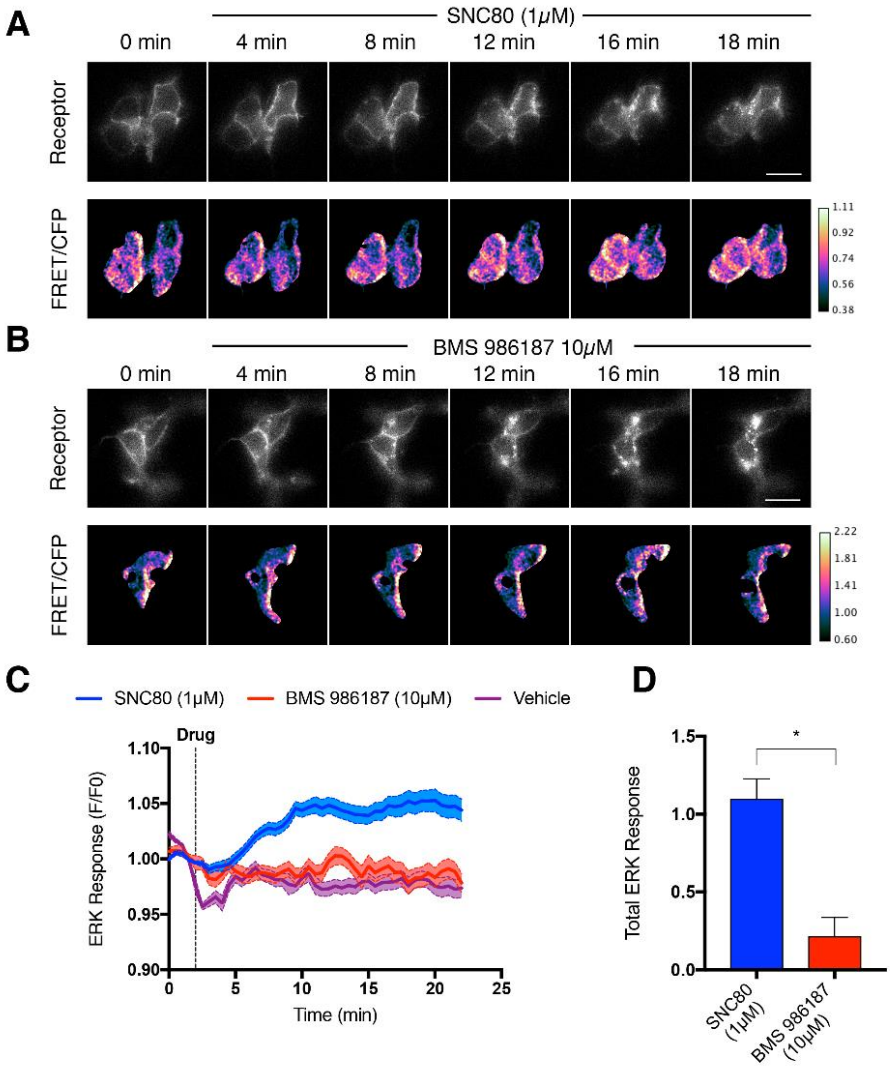

Figure 2.7 BMS 986187 elicits low ERK 1/2 phosphorylation. **(**A) Example montage of cytoplasmic ERK response in FLAG-tagged hDOPr expressing HEK 293 cells in response to 1μM SNC80. Top row: FLAG-tagged DOPr labeled with Alexa647-conjugated M1 Antibody. Bottom row: ratio of FRET/CFP fluorescence of expressed cEKAR sensor. Agonist added at 2.5 min. Scale bar is 20μm, frames every 4 min. (B) Representative montage of cytoplasmic ERK response measured in DOPr-expressing HEK 293 cell in response to 10μM BMS 986187. (C) Average ERK response over time of DOPr-expressing HEK 293 cells treated with either 1μM SNC80 (n=47 cells), 10 μM BMS 986187 (n=34 cells) or vehicle (n=39 cells). Responses are represented as fractional change over baseline. Solid line is the mean response, shaded region inside dotted lines represents ± SEM. (D) SNC80 produced a significantly higher total ERK response compared to BMS 986187. Total response is measured as area under curve of treatment condition minus area under curve of vehicle. Error bars represent means ± SEM.

Since BMS 986187 induced only a low level of receptor phosphorylation, arrestin recruitment and internalization, we predicted there would be a reduced DOPr desensitization, measured as a loss of receptor signaling, when cells were treated with BMS 986187 compared with SNC80. CHO hDOPr cells were incubated for varying times with equipotent concentrations (as determined by GTPγ^{35}S binding, Figure 2.1) of 10 µM BMS-986187 or 500 nM SNC80. Membranes homogenates were then prepared and GTPγ^{35}S binding determined following a challenge with a maximal concentration (10 µM) of SNC80. Membranes from cells pretreated with SNC80 or BMS 986187 showed a reduction in the maximal GTPγ^{35}S response, but the loss was more rapid for SNC80 (Figure 2.9), such that a one h pretreatment with SNC80 resulted in a 86% loss compared to only a 39 % loss with BMS 986187, a statistically significant difference. Likewise, pre-incubation of cells for 30 min with BMS 986187 caused a lesser effect on the concentration response curve for SNC80 (maximum response = 68 ± 7%; EC_{50} = 7.4 ± 1.6 nM) than pre-incubation with SNC80 (maximal response = 31 ± 7%; EC50 = 25 ± 3.5 nM).

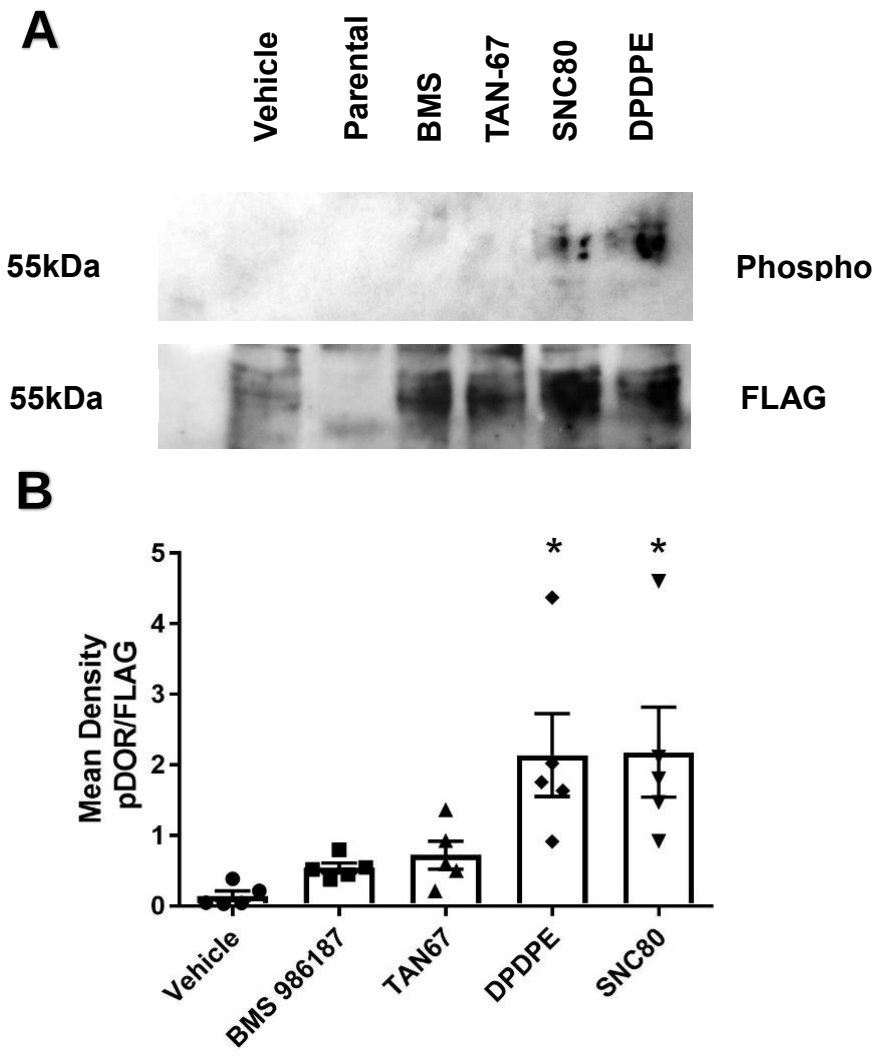

Figure 2.8 BMS 986187 poorly phosphorylates Ser363 on DOPr. (A)Western Blot of FLAG-tagged HEK hDOPr membranes incubated with 10 μM concentrations of various DOPr ligands probed for phosphorylated Ser363 (B) Data are expressed as a ratio of phosphorylated receptor to total receptor (FLAG). Each column is the mean of five independent experiments ± S.E.M.

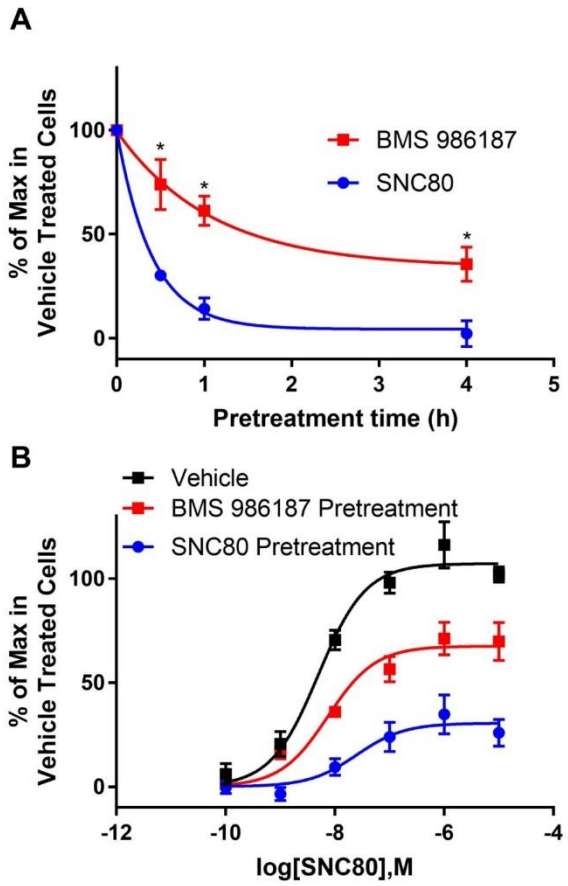

Figure 2.9 BMS 986187 treatment produces significantly less loss of agonist activity at DOPr than SNC80. (A) CHO cells expressing the hDOPr were pretreated with either 500 nM SNC80 or 10 µM BMS 986187 for the indicated times and then membrane homogenates were prepared as described in the methods. The level of GTPγ^{35}S binding induced by 10 µM SNC80 in the membranes was determined and plotted against the time of cell pretreatment with SNC80 or BMS-986187. (B) CHO cells expressing the hDOPr were pretreated as above with SNC80 or BMS 986187 for 30 mins, membranes homogenates prepared and SNC80 concentration response curves for stimulation of GTPγ^{35}S binding were constructed. Data are expressed as % of maximum binding in untreated cells ± S.E.M from five independent experiments, each performed in duplicate.

Discussion

The data presented indicate that BMS 986187 is a biased allosteric agonist at the DOPr receptor, giving a maximal response in the GTPγ^{35}S assay of equivalent magnitude to that seen with the orthosteric full agonist SNC80, albeit BMS 986187 is considerably less potent. In contrast to this strong response observed in G protein activation, BMS 986187 does not significantly recruit β-arrestin 2. This is a consequence of low levels of receptor phosphorylation and leads to a low level of receptor internalization and desensitization. When compared to SNC80, BMS 986187 is significantly biased toward G protein activation relative to recruitment of β-arrestin 2. This is the first evidence of an allosteric agonist displaying bias at an opioid receptor.

The direct agonist activity of BMS 986187 in the GTPγ^{35}S assay in HEK DOPr cells agrees with the results from the original report of PAMs at the DOPr using adenylyl cyclase inhibition in CHO cells as a readout (Burford et al., 2015). The allosteric nature of the observed agonism was suggested by the inability of BMS 986187 to compete with the orthosteric ligand ^{3}H-diprenophine (Burford et al., 2015). In the present study we confirm that BMS 986187 agonist activity occurs via binding to an allosteric site because the orthosteric antagonists NTI and naloxone only partially inhibit the ability of BMS986187 to stimulate GTPγ^{35}S binding, and increasing concentrations of the NTI do not give parallel shifts in the BMS 986187 concentration-response curve. This verifies that agonism can be mediated by sites on the receptor other than the orthosteric site and demonstrates an indirect interaction between the allosteric and the orthosteric sites. While these results in transfected HEK cells are encouraging, DOPrs are expressed at

supraphysiological levels, which may not translate to relevant *in vivo* agonism (Langmead and Christopoulos, 2006; Kelly, 2013).

Using mouse brain homogenates, we verified that the level of G protein activation elicited by BMS 986187 is similar to the full agonist, SNC80. However, these data also indicated that BMS 986187 is not completely selective for DOPr, since the same assay performed using mouse brain homogenates from DOPr knockout mice, still afforded a low level of GTPγ^{35}S stimulation. Previous work has indicated that BMS 986187 can act as a PAM for the MOPr and kappa opioid receptors, although no significant direct ago-PAM activity has so far been detected at either of these receptors (Livingston *et al.*, 2018). Alternatively, BMS 986187 could be acting at another, so far unidentified GPCR.

Despite stimulating a higher level of GTPγ^{35}S binding than the DOPr peptidic agonist DPDPE, BMS 986187 produced significantly lower receptor internalization. Thus, maximal G protein stimulation was in the order BMS 986187 = SNC80 > DPDPE > TAN-67 whereas maximal internalization was in the order SNC80 = DPDPE > TAN-67 = BMS 986187. This striking change of order of maximal effect across the two different signaling outputs using the same cell line indicates bias resulting from BMS 986187 occupancy of its allosteric binding site. We confirmed this finding by calculating the degree of bias for the signaling preference of BMS 986187-occupied DOPr for G protein activation versus β-arrestin 2 recruitment compared to SNC80 as a reference ligand. The very low level of β-arrestin 2 recruitment by BMS 986187 was confirmed by the lack of observable recruitment of GFP-labelled β-arrestin 2 to the plasma membrane in DOPr expressing HEK cells.

Comparing β-arrestin recruitment using the Presto Tango assay with GTPγ^{35}S binding we find DPDPE to be G protein biased at DOPr expressed in HEK cells, relative to SNC80. This agrees with studies that indicate SNC80 is a “super recruiter” of β-arrestin 2 relative to DPDPE at DOPr in CHO cells, whereas both have similar activity as inhibitors of AC (Chiang et al., 2016) and with predictions from studies *in vivo* that SNC80 is a “high-internalizing agonist” when compared to the low internalizing delta agonist ARM390 and partial agonists such as TAN67 (Pradhan et al., 2009, 2010; Pradhan et al., 2012). In contrast, bias calculations (Charfi et al., 2015) based on data obtained at the DOPr expressed in HEK cells (Charfi et al., 2013), suggest SNC80 is highly biased towards AC inhibition relative to internalization when compared to DPDPE, although these data also show that SNC80 recruits much more β-arrestin than DPDPE. Overall, these findings highlight the importance of understanding the relativity of bias, where the chosen reference ligand can have a significant impact on the direction, magnitude and interpretation of observed results. Here, we chose to use SNC80 as a reference ligand as it is a “standard” DOPr ligand characterized in many *in vitro* and behavioral assays (Jutkiewicz *et al.*, 2005; Danielsson *et al.*, 2006; Pradhan *et al.*, 2010; Chu Sin Chung *et al.*, 2015; Dripps *et al.*, 2017). On the other hand, we have shown DPDPE to be 11-fold biased towards G protein relative to β-arrestin recruitment using SNC80 as a reference, Consequently, if DPDPE is used as a reference ligand the bias of BMS 986187 towards G protein activation is reduced to 9-fold.

BMS 986187 also failed to afford DOPr-mediated phosphorylation of ERK1/2 in the MAP kinase pathway, despite showing significant G protein activation. This implies phosphorylation of ERK 1/2 *via* the allosteric site on DOPr may be a β-arrestin 2 mediated

process, a ligand dependent effect observed at other GPCRs (Shukla *et al.*, 2008). However, this contrasts with the finding that BMS 986187 when acting as a PAM for DOPr promotes ERK1/2 phosphorylation (Burford et al., 2015). This apparently conflicting data implies that the BMS 986187-occupied DOPr may signal differently depending on whether the orthosteric site is occupied. We are currently investigating the effect of BMS 986187 on ERK1/2 phosphorylation using a variety of DOPr agonists. PAM activity arises at opioid receptors by a negative indirect action with the Na^+ ion binding site. Na^+ holds the receptor in inactive conformations (R) and loss of Na+ ion binding allows the receptor to adopt ensembles of active receptor (R*) states (Pert, Pasternak and Snyder, 1973; Liu *et al.*, 2012; Livingston and Traynor, 2014). PAMs with greater efficacy to displace Na^+ ion would then be predicted to show allosteric agonism by the same process. However, driving receptor activation through an allosteric site, there is no a priori reason why an allosteric agonist could not show functional selectivity at DOPr by promoting a different ensemble of R* conformations than an orthosteric ligand. Indeed, such an effect has been observed at the mAChr where allosteric agonists promoted bias when comparing G protein activation and ERK 1/2 phosphorylation (Gregory *et al.*, 2010).

Recruitment of β-arrestin and DOPr internalization requires sequential phosphorylation of several Ser and Thr residues in the C terminal tail of the receptor by G protein receptor kinases (GRKs) (Stoffel, Pitcher and Lefkowitz, 1997; Ferguson, 2001; Qiu, Loh and Law, 2007). Mutagenesis studies have indicated that an important initial phosphorylation site in the DOPr phosphorylation cascade is Ser363 (Kouhen *et al.*, 2000). BMS 986187 produced a low level of Ser363 phosphorylation relative to DPDPE and SNC80 and similar to the low internalizing agonist TAN-67. This explains why the

allosteric agonist shows significantly less desensitization than SNC80. However, it is perhaps surprising given the low level of phosphorylation and β-arrestin 2 recruitment that BMS-986187 caused DOPr desensitization. It is possible that over extended periods, even with limited phosphorylation and β-arrestin 2 recruitment, BMS 986187 is able to drive significant desensitization. Alternatively, the BMS 986187-occupied DOPr is in different conformational states to an orthosteric agonist-occupied DOPr and so may employ different desensitization mechanisms. In this regard it should be noted that mutants of DOPr expressed in HEK cells showing no detectable phosphorylation still desensitize over time (El-Kouhen et al., 2000). Nonetheless, the low level of phosphorylation observed establishes that the bias driven by BMS 986187 results from reduced phosphorylation of the receptor, even though BMS 986187 does recruit G protein and presumably G protein receptor kinases. This signifies that DOPr conformations induced in the presence of BMS 986187 differ from those adopted in the presence of SNC80. A greater understanding of these conformations would provide insight into the driving force behind G protein versus β-arrestin mediated signaling for both orthosteric and allosteric ligands.

In conclusion, BMS 986187 is a biased allosteric agonist of the DOPr. Biased allosteric agonism at DOPr could represent a novel strategy for treating chronic pain and depression while potentially avoiding limiting factors such as rapid tolerance development and induction of convulsions.

Table 2.1. Summary of Bias Calculations. Calculations were performed from data generated in Figure 5, as described in the methods to determine ΔΔlog(max/EC_{50}) (Kenakin, 2017). *In order to extrapolate an accurate EC_{50} value for BMS 986187-mediated arrestin recruitment, the E_{max} was constrained to 1, equivalent to the level of recruitment by SNC80 or 0.5, assuming the maximum value is that observed at the point of solubility (Figure. 5B).

	β-arrestin 2		Bias (Toward G protein)			GTPγ^{35}S	
	Max	EC_{50} (nM)	Arrestin/G protein Fold EC_{50} Shift	ΔΔlog(Max/EC_{50})	Bias Factor	EC_{50} (nM)	Max
SNC80	1	353 ± 141	18.6	0	1	19.0 ± 6	1
BMS 986187	1* 0.5*	578,500 ± 419,100 238,179 ± 188,400	1787 737	1.91 ± 0.7 1.53 ± 0.82	81 34	323 ± 96	0.92 ± 0.03
DPDPE	1	16,100 ± 805	85	1.03 ± 0.67	11	189 ± 25	0.85 ± 0.09

Methods

Materials
Animals

All animal care and experimental procedures complied with the US National Research Council's Guide for the Care and Use of Laboratory Animals (Council, 2001). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Male mice were used for all experiments. C57BL/6N (RRID:MGI:5659255) mice were obtained from Envigo (formerly Harlan, Indianapolis, IN). The *Oprd1*^{tm1Kff}/J mouse strain (Oprd1tm1Kff/J, RRID:IMSR_JAX:007557) was obtained from The Jackson Laboratory (Bar Harbor, Maine, https://www.jax.org/strain/007557; Filliol et al., 2000). Mice were group-housed with a maximum of five animals per cage in clear polypropylene cages with corn cob bedding and nestlets as enrichment. For breeding of the *Oprd1*^{tm1Kff}/J mice heterozygote pairs were employed. Mice had free access to food and water at all times. Animals were housed in pathogen-free rooms maintained between 68 and 79^{o}F and humidity between 30 and 70% humidity with a 12 h light/dark cycle with lights on at 07:00 h.

Cell Lines

Human Embryonic Kidney (HEK293, RRID:CVCL_0045) cells stably expressing a tTA-dependent luciferase reporter and a β-arrestin 2-TEV fusion gene (HTLA cells; Thermo-Fisher Scientific) were maintained in DMEM supplemented with 10% FBS, 1% penicillin and 100 µg/ml streptomycin, 2 µg/ml puromycin and 100 µg/ml hygromycin B at 37^{o}C and 5% CO_2. HEK293 (ATCC Cat# CRL-1573, RRID:CVCL_0045) cells expressing N-terminally FLAG tagged human-DOPr (HEK-hDOPr) were cultured in DMEM containing 10% FBS and 1% penicillin and streptomycin and maintained in 0.8

mg/ml G418. HEK 293 cells stably expressing an N-terminally FLAG-tagged variant of hDOPr for ERK 1/2 imaging studies were generated as previously described and maintained in DMEM supplemented with 10% FBS (Shiwarski *et al.*, 2017). CHO (ATCC Cat# CCL-61, RRID:CVCL_0214) cells stably expressing wild-type human-DOPr (CHO-hDOPr) were grown in DMEM containing 10% FBS and 1% penicillin and streptomycin and maintained in 0.4 mg/ml G418 as previously described (Burford et al., 2015)

Membrane Homogenate Preparations.

Cells were harvested and membrane homogenates prepared as previously described (Clark *et al.*, 2003). Briefly, cells were washed with ice-cold phosphate buffered saline, pH 7.4 and detached from plates by incubation in harvesting buffer (0.68 mM EDTA, 150 mM NaCl, and 20 mM HEPES at pH 7.4) and pelleted by centrifugation at 200g for 3 minutes. Cells were resuspended in ice-cold 50mM Tris (pH 7.4), homogenized using a Tissue Tearor (Dremel; Mount Prospect, IL, USA), and centrifuged at 20,000g at 4^{o}C for 20 min. The pellet was then resuspended, homogenized, and centrifuged a second time. This final pellet was resuspended in ice-cold 50 mM Tris (pH 7.4) and homogenized using a glass dounce to give a protein concentration of 0.5-1.5 mg/mL and stored at -80°C. Protein concentration was determined using the bicinchoninic acid quantification method (BCA) with BSA serving as the standard.

For brain homogenates, mice (8 to 12 weeks of age) were euthanized by cervical dislocation. Whole brain tissue, from the optic chiasmus forward, was removed immediately and chilled in ice-cold 50 mM Tris base, pH 7.4. Membrane homogenates were prepared as previously described (Lester and Traynor, 2006). Final membrane

pellets were resuspended in 50 mM Tris base, pH 7.4, aliquoted, and stored at −80 °C. Protein content was determined using BCA assay with BSA as the standard.

Stimulation of GTPγ^{35}S Binding

Agonist stimulation of GTPγ^{35}S binding was measured as described previously (Clark *et al.*, 2003). Homogenates of HEK cells expressing FLAG-tagged-hDOPr, CHO cells expressing wild-type hDOPr or mouse brain (15-20 µg/well) were incubated in "GTPγS buffer" (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) containing 0.1 nM GTPγ^{35}S, 30 µM guanosine diphosphate (GDP) and varying concentrations of BMS 986187, or DOPr agonists for 1h in a shaking water bath at 25°C. The reaction was terminated by vacuum filtration through GF/C filters using a Brandel harvester and washed five times with ice-cold GTPγS buffer. Filters were dried, and following the addition of EcoLume scintillation cocktail, counted in a Wallac 1450 MicroBeta Liquid Scintillation and Luminescence Counter (Perkin Elmer). The level of GTPγ^{35}S binding was expressed as fmols bound/mg protein or by comparison with the full DOPr agonist SNC80 at 10 µM to account for variability between membrane preparations.

DOPr Internalization

As described previously (Bradbury, Zelnik and Traynor, 2009), FLAG-tagged HEK-hDOPr cells were plated at a density of 0.5 x 10^6 cells per well in poly-D-Lysine coated, 24-well plates. When cells reached 80% confluency they were treated with vehicle (1% DMSO) or indicated drugs and rocked at room temperature for the indicated times. Cells were then washed three times with ice-cold tris-buffered saline (TBS) and fixed with 3.7% paraformaldehyde in TBS at room temperature for 15 min. After fixing, cells were washed three times with cold TBS and blocked at room temperature with 1% BSA in TBS for 60

min. Following block, cells were washed two times with TBS and incubated with FLAG M2-Alkaline Phosphatase Antibody at a 1:625 dilution for 60 min. Cells were then washed five times with TBS and treated with p-nitrophenylphosphate for 8 min. The reaction was stopped with 3N NaOH and 200 µl from each well was transferred to a 96-well plate for reading at 405 nm on VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA). The percentage of internalized receptors was determined as loss of surface receptors using the following equation [1–(Drug O.D.–Background O.D./ Control O.D.–Background O.D.) x 100]. Background was determined as the absorbance of non-transfected HEK cells and control was the absorbance of cells incubated in the absence of drug.

β-Arrestin 2 Recruitment

Confocal Microscopy

Recruitment of β-arrestin 2 in FLAG-tagged HEK DOPr cells was performed as described previously (Bradbury, Zelnik and Traynor, 2009). Briefly, cells were seeded into 24-well plates containing Poly-D-Lysine coated glass coverslips. Cells were transfected using Lipofectamine 2000 with 0.4 µg of β-arrestin 2-GFP cDNA and incubated for 48 h, then treated with either vehicle, 10 µM SNC80 or 10 µM BMS 986187 for 5 min. Following fixation with 3.7% paraformaldehyde, cells were incubated with M2 Mouse Anti-FLAG primary antibody followed by AlexFluor 594 Goat Anti-Mouse secondary antibody. Images were obtained using a NikonA1R Confocal Microscope and quantified using Image J software (National Institutes of Health) (ImageJ, RRID:SCR_003070).

Presto-Tango Arrestin Recruitment

For the PRESTO-TANGO assay HTLA cells at 15,000 cells/well were transfected with plasmids (20ng) encoding FLAG-tagged hDOPr-TANGO (OPRD1-TANGO; Thermo-Fisher Scientific) using Lipofectamine 2000 and plated in Greiner Bio-One cell culture micro-plates. After 24 h, cells were treated with the indicated drug at the indicated concentrations. After 48 h, One-Glo solution was added to each well and luminescence was measured using a Pherastar plate reader (BMG Labtech, Germany). Data were normalized to percent of standard full agonist (10 µM SNC80) to account for variability between assays in plating and transfection efficiency.

ERK 1/2 Phosphorylation

HEK 293 cells stably expressing an FLAG-tagged hDOPr were transiently transfected with the extracellular signal-regulated kinase activity reporter cEKAR (Fritz *et al.*, 2013). ERK activity in response to SNC80 and BMS 986187 was assessed as previously described (Weinberg *et al.*, 2017). Briefly, cells were plated at low density, allowed to grow for two days, and then serum starved for 4 h. Cells were labeled with Alexa Fluor 647 anti-mouse M1 antibody for 10 min. Single-cell fluorescence for cyan fluorescent protein (CFP; 405 nm excitation, 470/50 emission filter), FRET (405nm excitation, 530lp emission filter), and M1 (647 nm excitation, 700/75 emission) was collected every 30 s for 22.5 min, with addition of drug (1 µM SNC80 or 10 µM BMS 986187) occurring after 2.5 min of no-treatment baseline. The ratio of FRET to CFP fluorescence was calculated for each cell on a frame-by-frame basis and normalized to the average ratio during baseline. For calculating total response, the mean area under the curve was taken for the vehicle condition, and that mean was subtracted from the individual area under the curve for each cell in the treatment conditions. Each experiment

was conducted using the same batch of transiently transfected cells from the same stable cell line and passage number and carried out on the same day under all conditions (vehicle, SNC80, BMS-986187) to ensure that any non-responding cells were represented equally across treatment conditions.

Western blot for phosphoSer363

As described previously (Bradbury, Zelnik and Traynor, 2009), HEK cells stably expressing FLAG-tagged hDOPr were plated at a density of 0.5 x 10^6 cells per well in poly-D-Lysine coated, 24-well plates and experiments were performed when cells were at 80% confluency. Cells were treated with vehicle (1% DMSO), TAN-67, DPDPE, SNC80 or BMS 986187 for 1 h. Cells were then rinsed with phosphate buffered saline (PBS) and lysates were collected with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS)) plus protease inhibitor cocktail, 2 mM EDTA, 100 mM NaF, 100 mM phenylmethanesulfonyl fluoride, and 10 mM sodium orthovanadate. Lysates were then sonicated briefly and centrifuged at 10,000 g for 10 min. Equal amounts of protein samples were diluted in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.0008% bromophenol blue) and β-mercaptoethanol, loaded onto 10% polyacrylamide gels. Following transfer to nitrocellulose, membranes were blocked for 1 h with 5% non-fat dried milk in PBS then incubated with 1:1000 dilution of rabbit anti-phosphorylated δ-opioid receptor antibody overnight at 4˚C. Membranes were washed and incubated with 1:10000 HRP-goat anti-rabbit IgG for 1 h. To probe total FLAG-DOPr, the membranes were stripped using mild stripping buffer (distilled water, pH 2.2, 1.5% glycine, 0.1% SDS, 1% Tween 20), washed, then blocked with 5% non-fat dried milk for 1 h. Following block, membranes were

incubated with 1:1000 mouse-anti-FLAG for 1 h 5% non-fat dried milk in TBS-Tween containing 1mM $CaCl_2$. Membranes were washed and treated with 1:10000 HRP-goat-anti-mouse IgG for 1 h. Following wash, membranes were treated with 1:1 SuperSignal chemiluminescent substrate and bands were detected using the EpiChemi3 darkroom (UVP, Upland, CA, USA). Band intensity was quantitated using Image J (National Institutes of Health) and normalized to total hDOPr, as determined by FLAG staining, to account for any differences in total protein.

Receptor Desensitization

Desensitization was determined by incubating hDOPr CHO cells with either vehicle or drug for indicated time periods at 37˚C. Following incubation, cells were washed five times with PBS and membranes were prepared as described above. For the time course of desensitization, maximum GTPγ^{35}S binding was measured using 10 μM SNC80 in vehicle treated cells; drug treated conditions were expressed as percent of this maximal binding. For concentration response, GTPγ^{35}S binding elicited by SNC80 was measured in membranes pre-treated with either 500 nM SNC80, 10 μM BMS 986187 or vehicle for 30 min and expressed as fmol bound/mg of protein.

Analyses and statistical analyses

The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). All *in vitro* assays were a mean of at least 5 separate preparations, except where stated, and each was run in duplicate or triplicate as given in the relevant figure legend, to ensure the reliability of the single values. None of the *in vitro* biochemical experiments were performed or analyzed blinded

Data were graphed as individual experiments for analyses unless otherwise stated and statistical analysis was performed using Graphpad Prism 6.5. Concentration-effect curves were analyzed using a three-parameter curve fit with Hill Slopes set to 1.0. Maximal values were not constrained; minimum values were constrained to zero if contained in the 95% confidence intervals. For internalization, the GTPγ^{35}S assay, and confocal microscopy, one-way ANOVA was performed and Tukey post hoc test for multiple comparisons applied if F was significant. The desensitization time course was analyzed by two-way ANOVA. Bias calculations were performed as described by Kenakin, (2017) as follows: For each ligand and respective response, individual experimental curves were used to calculate log(max/EC50). The difference in log(max/EC50) between arrestin recruitment and GTPγ^{35}S, Δlog(max/EC50), was then calculated. Individual results were combined to give means ± SEM values shown in Table 1. Finally, the differences between the Δlog(max/EC50) values for the reference ligand (SNC 80) and test ligand were calculated to give a ΔΔlog(max/EC50) values, the antilog of which is the bias factor.

For all analyses significance was set at 5% (0.05 p value)

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Chapter 3 : Allosteric Modulators of the Mu Opioid Receptor Promote Divergent Signaling Bias

Summary

Positive allosteric modulation and biased agonism at the mu opioid receptor are two emerging paradigms for designing safer analgesics. Evidence at other class A GPCR's indicates allosteric modulators can induce bias in orthosteric ligands. However, no studies to date have examined this phenomenon at the mu opioid receptor. Studies described in this chapter sought to investigate this using two structurally distinct mu positive allosteric modulators, BMS 986187 and BMS 986122, by comparing ability of the non-biased mu-opioid agonist DAMGO to activate G protein compared with β-arrestin 2 recruitment. The results demonstrated that BMS 986187 promoted β-arrestin 2 bias whereas BMS 986122 promoted G protein bias. An alteration of bias was also seen using orthosteric ligands that are β-arrestin 2 biased (fentanyl), bias neutral (morphine, methadone) and G protein biased (SR17018). Despite promoting similar enhancements of G protein signaling, BMS 986187 promoted up to 100-fold enhancements in β-arrestin 2 recruitment, where BMS 986122 elicited modest shifts (1-7 fold). The β-arrestin 2 bias produced by BMS 986187 was observed in the absence of functional Gi proteins and/or presence of GRK 2/3 inhibition Overall, this set of studies provides evidence of divergent signaling bias promoted by two distinct allosteric ligands downstream of the mu opioid

receptor and highlights G protein independent β-arrestin 2 recruitment as a possible mechanism underlying this bias.

Introduction

The burden of pain continues to increase globally. Pain affects roughly 20% of the population and remains the largest cause of disability. Opioids represent the most clinically efficacious class of compounds for treating acute pain. However, opioid-related side effects, including respiratory depression and abuse liability, have led to the current opioid epidemic in the United States (Jones *et al.*, 2018). Treating pain without opioid-related side effects remains the holy grail of opioid pharmacology.

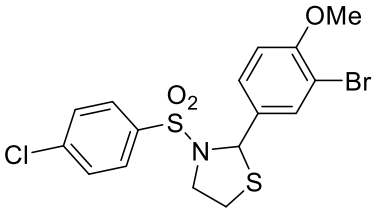
Two novel strategies to target G protein coupled receptors, such as the mu opioid receptor, include biased agonism and allosteric modulation (Foster and Conn, 2017). Biased agonism describes the preferential engagement of one signaling pathway over another (e.g., G protein activation over β-arrestin 2 recruitment). In the case of the mu opioid receptor, there is increasing evidence that G protein biased signaling may result in fewer side effects, including respiratory depression and constipation (Bohn *et al.*, 1999, 2000; Raehal, Walker and Bohn, 2005; Schmid *et al.*, 2017). Therefore, there are numerous ongoing efforts to design compounds that preferentially engage G proteins, instead of β-arrestins (DeWire *et al.*, 2013; Manglik *et al.*, 2016; Schmid *et al.*, 2017).

Allosteric modulation is a process whereby a ligand binds to a receptor at a site distinct from the orthosteric ligand. From this site, ligands can modulate affinity and/or efficacy of orthosteric ligands as well as induce active receptor conformations in the absence of an orthosteric ligand. Ligands that enhance orthosteric ligand binding affinity and/or efficacy in this manner are named positive allosteric modulators (PAMs) A

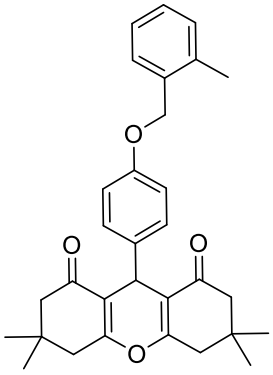
theoretical benefit of this approach is that mu opioid receptor PAM's could enhance the activity of endogenous neuropeptides released during pain states, such as the endorphins and enkephalins. Contrasting the global receptor activation elicited by exogenous opioids, PAM's would be predicted to only enhance endogenous pain modulation at distinct regions in the body where endorphins and enkephalins are released, limiting off-target activation of mu-OR and side effects. In addition, there is increasing evidence that allosteric modulators are able to influence the signaling bias of G protein coupled receptors (Davey *et al.*, 2012; Wootten, Christopoulos and Sexton, 2013; Cook *et al.*, 2015; Khajehali *et al.*, 2015).

BMS 986122 and BMS 986187 are allosteric modulators of the mu, delta, and kappa opioid receptors (Livingston *et al.*, 2018), though with differing activities across the three receptors, but bind to a conserved site on the receptors. Previous work has shown that BMS 986187 can promote an active conformation of the delta opioid receptor, in the absence of an orthosteric ligand, that is biased toward G protein activation over β-arrestin 2 recruitment (Stanczyk *et al.*, 2019). In addition, BMS 986187 was seen to have a cooperativity factor of 2 for β-arrestin 2 recruitment and a cooperativity factor of 1 for G protein activation when DAMGO was used as an orthosteric ligand, where cooperativity indicates the positive allosteric interaction between allosteric modulator and orthosteric ligand for a given response (Livingston *et al.*, 2018). Given the potential implications of biased signaling and allosteric modulation of the mu opioid receptor in designing safer pain therapeutics, we sought to examine how these two structurally distinct allosteric modulators influence downstream signaling of orthosteric, mu opioid ligands.

The findings show that BMS 986122 promotes G protein bias for a range of ligands while BMS 986187 promotes β-arrestin 2 bias. This divergent signaling between the modulators was further supported by the ability of BMS 986187 to enhance G protein independent β-arrestin 2 recruitment. Overall, this highlights the complex interplay between allosteric regulation and biased signaling and presents a framework for testing differential bias *in vivo*.



BMS 986122



BMS 986187

Figure 3.1 Structures of BMS 986122 and BMS 986187

Results

Structurally distinct modulators produce divergent signaling profiles

Evidence suggests BMS 986187 and BMS 986122 act on the mu opioid receptor through a shared or overlapping binding site (Livingston *et al.*, 2018). However, given their diverse structural nature, we determined the efficacy (defined as fold-shift in orthosteric ligand potency) these PAMs have on orthosteric ligand ability to activate G protein signaling or recruit β-arrestin 2. Allosteric modulators can exhibit probe dependence, a variable cooperativity depending on the orthosteric ligand used (Wootten,

Christopoulos and Sexton, 2013). Therefore, we collected concentration-response curves in GTPγ^{35}S binding and β-arrestin 2 recruitment assays using the following compounds in the presence or absence of either allosteric modulator: methadone, DAMGO, morphine, and fentanyl (Fig 3.2). The EC_{50} and Max values for the respective curves are presented in table 2.1. BMS 986187 promoted β-arrestin 2 shifts ranging from 6 to 242-fold while eliciting 7 to 24-fold G protein shifts. BMS 986122 promoted β-arrestin 2 shifts ranging from 1 to 6-fold while eliciting 7 to 30-fold G protein shifts.

In addition, we observed a switch in the potency rank order for methadone and DAMGO when comparing the BMS 986122 and BMS 986187 treated conditions. Methadone agonism in the presence of 986187 has a potency rank order of β-arrestin 2 (EC_{50}= 0.89) > G protein activation (EC_{50}= 15 nM), whereas the opposite is true with BMS 986122: G protein (EC_{50}= 7.0 nM) > β-arrestin 2 (EC_{50}= 33 nM). This same effect was observed with DAMGO, with BMS 986187 co-treatment resulting in β-arrestin 2 (EC_{50}= 3.0 nM) > G protein (EC_{50}= 10 nM) while BMS 986122 co-treatment resulted in G protein (EC_{50}= 18 nM) > β-arrestin 2 (EC_{50}= 50 nM). Although a potency switch was not observed for fentanyl and morphine, similar results were obtained. BMS 986187 led to a 100-fold shift in the potency of fentanyl to recruit β-arrestin 2 (1.3 nM vs 0.013 nM, EC_{50}) while promoting only a 7.5-fold shift in G protein activation (75 nM vs 10 nM). Conversely, BMS 986122 co-treatment led to a 2.6-fold shift in the potency of fentanyl to recruit β-arrestin

2 (1.3 nM vs 0.5 nM) while promoting an 8-fold shift in G protein activation (75 nM vs 9.4 nM).

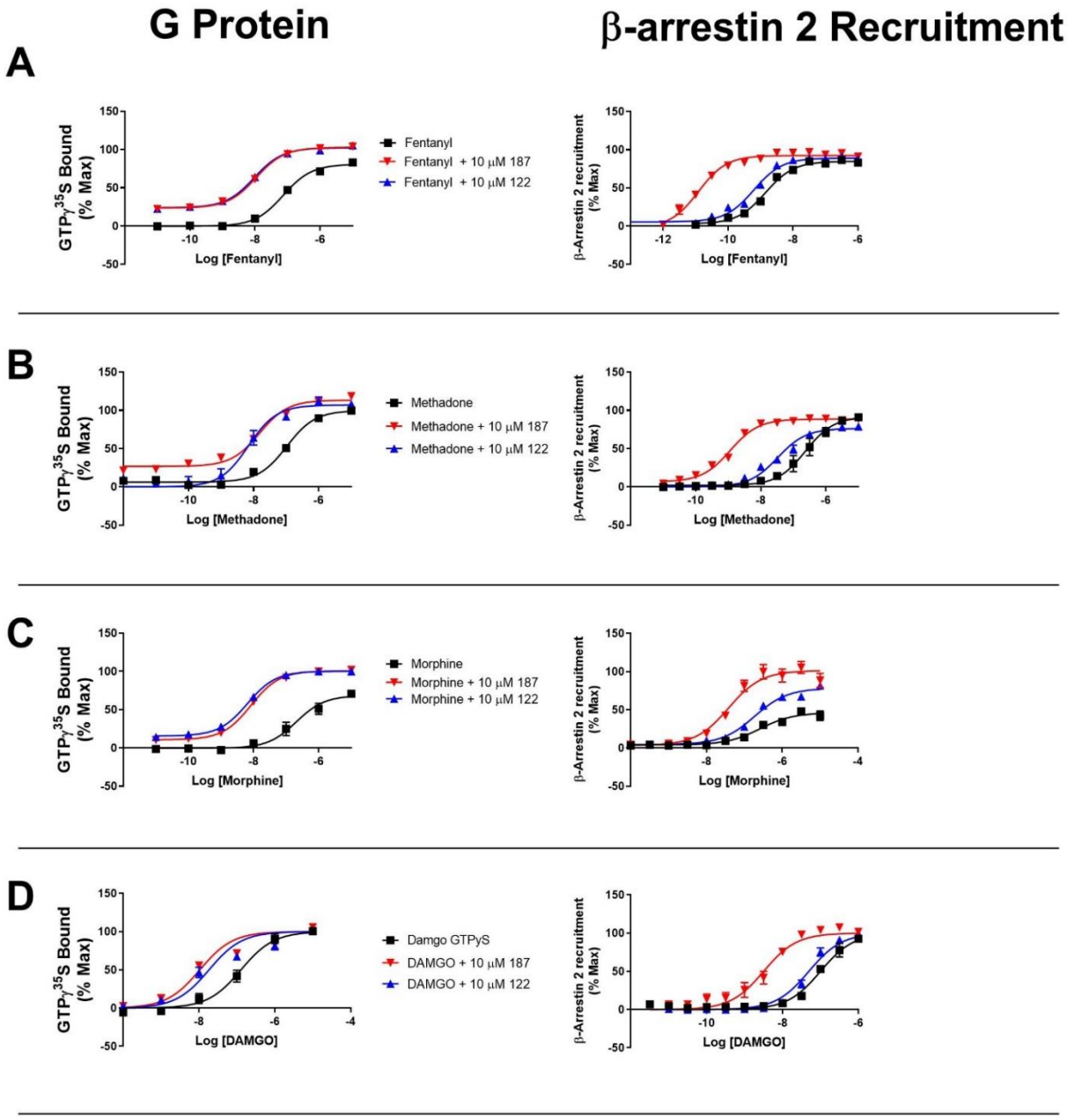


Figure 3.2 Allosteric Modulators show differential signaling bias. Concentration-response curves for Fentanyl (A), Methadone (B), Morphine (C) or DAMGO (D) in the presence or absence of 10 μM BMS 986187 or BMS 986122 at stimulating GTPγ^{35}S binding (left) or β-arrestin 2 recruitment (right). Data are presented as the mean ± S.E.M of 3-5 independent experiments performed in duplicate and normalized to percentage of 10 μM DAMGO (% Max).

Table 3.1. Summary of potency and bias factors for orthosteric ligands. Values reported are the mean of 3-5 independent experiments performed in quadruplicate. 95% confidence intervals are reported in parentheses.

Drug	G protein		β-Arrestin 2 Recruitment		ΔΔLog(Max/EC50)		
	EC50	Emax	EC50	Emax	DAMGO Reference	Self Reference	Between Modulators
DAMGO	127 (90-183)	1	101(83-120)	1	0	0	
122	18 (10-34)	1	50(43-56)	1	0.66 (1.12-0.19)	0.66 (1.12-0.19)	1.17 (1.68 - 0.65)
187	10 (5.8-20)	1	3.1(2.4-4.1)	1	-0.51 (-0.01-(-1.01))	-0.51 (-0.01 -(-1.01)	
Fentanyl	75 (63-89)	0.81 (0.78-0.83)	1.3 (1-1.5)	0.84 (.83-.86)	-1.85 (-1.48-(-2.21))	0	
122	9.4 (7.0-12.6)	1	0.5 (.42-.58)	0.88 (.86-.9)	-1.3 (-0.85 - (-1.72))	0.56 (0.86-0.25)	1.6 (1.9 - 1.28)
187	10 (7.7-13.4)	1	0.013 (0.011-0.015)	0.92 (.91-.94)	-2.9 (-2.5-(-3.3))	-1.06 (-0.76-(-1.35))	
Morphine	201 (94-474)	0.68 (0.59-0.78)	257 (150-438)	0.46 (.41-.51)	0.23 (1.07 - (-0.61))	0	
122	6.5 (4.6-9.0)	1	186 (154-225)	0.78 (.75-.81)	1.5 (1.86-1.13)	1.27 (2.03 - 0.5)	0.88 (1.11-0.63)
187	8.3 (6.5-11)	1	38 (27-54)	1 (.95-1.1)	0.63 (0.97-0.28)	0.4 (1.15 -(-0.3))	
Methadone	82 (57-120)	1	216 (150-310)	0.9 (.84-.98)	0.43 (1.08-(-0.21))	0	
122	7 (4.1-11)	1	33 (26-40)	0.76 (.73-.79)	0.8 (1.4-0.2)	0.37 (1.17 - (-0.42))	2.07 (2.78-1.36)
187	15 (10-22)	1	0.89 (0.75-1)	0.88 (.86-.9)	-1.27 (-0.72-(-1.82))	-1.7 (-0.95- (-2.44))	

BMS 986187 engenders β-arrestin 2 bias, BMS 986122 engenders G protein bias

The change in potency rank order observed in Fig 3.2 is indicative of signaling bias. To quantify these data, bias was determined using the ΔΔLog(max/EC_{50}) approach as previously described (Kenakin, 2017) by comparing signaling responses normalized to a reference ligand. However, with this approach the direction and magnitude of bias greatly depends on the reference condition used. For this reason, bias was calculated using three different references: DAMGO to compare across groups (Fig 3.2A, Table 3.1), each orthosteric ligand against itself to compare within groups (Fig 3.2B, Table 3.1) and BMS 986187 + orthosteric ligand conditions to compare between modulators (Fig 3.2C, Table 3.1).

Relative to DAMGO alone, we determined that fentanyl was β-arrestin 2 biased in the absence of allosteric modulators, with a ΔΔlog(Max/EC_{50}) value of -1.85, morphine and methadone were unbiased (0.23, 0.43; non-significant). BMS 986187 shifted bias for all ligands except morphine towards β-arrestin 2 signaling (ΔΔlog(Max/EC_{50}) values in parentheses) DAMGO (-0.51), Fentanyl (-2.9), Morphine (0.63), Methadone (-1.27). Conversely, in the presence of BMS 986122 signaling was shifted in favor of G protein: DAMGO (0.66), Fentanyl (-1.3), Morphine (1.5), Methadone (0.8). Significant changes in ligand bias were observed for both DAMGO and methadone, where bias changed from neutral to β-arrestin 2 in the presence of BMS 986187 or G protein biased in the presence of BMS 986122. In the case of fentanyl, BMS 986122 reduced β-arrestin 2 bias whereas BMS 986187 increased it. Surprisingly, both ligands promoted morphine G protein bias,

although the ΔΔlog(Max/EC_{50}) was more significant with BMS 986122 (1.5) compared to BMS 986187 (0.63).

To gain a more direct representation of PAM impact on bias we compared each compound against itself in the absence or presence of each modulator. Using this approach, the divergent signaling properties observed by the two allosteric modulators are exemplified with BMS 986187 and BMS 986122 driving β-arrestin 2 and G protein bias, respectively (Table 3.1, Figure 3.3).BMS 986187 promoted statistically significant bias in DAMGO (-0.51), fentanyl (-1.06) and methadone (-1.7). Conversely, BMS 986122 engendered statistically significant bias in DAMGO (0.66), fentanyl (0.56) and morphine (1.27).

Finally, we calculated the net bias between orthosteric ligands in the presence of either modulator. For this calculation, orthosteric ligand and BMS 986187 co-treatment was used as the reference condition, and a G protein bias for BMS 986122 co-treatment with the orthosteric ligand was determined relative to this condition. This approach highlighted the strong divergence in signaling preferences elicited by these two compounds. Strikingly, there was significant bias between modulator conditions for every orthosteric ligand tested in the following rank order of bias: methadone (117.5)> fentanyl (39.8)> DAMGO (14.8)> morphine (7.6). This highlights the extreme bias that can be elicited between two structurally unique mu opioid PAM's

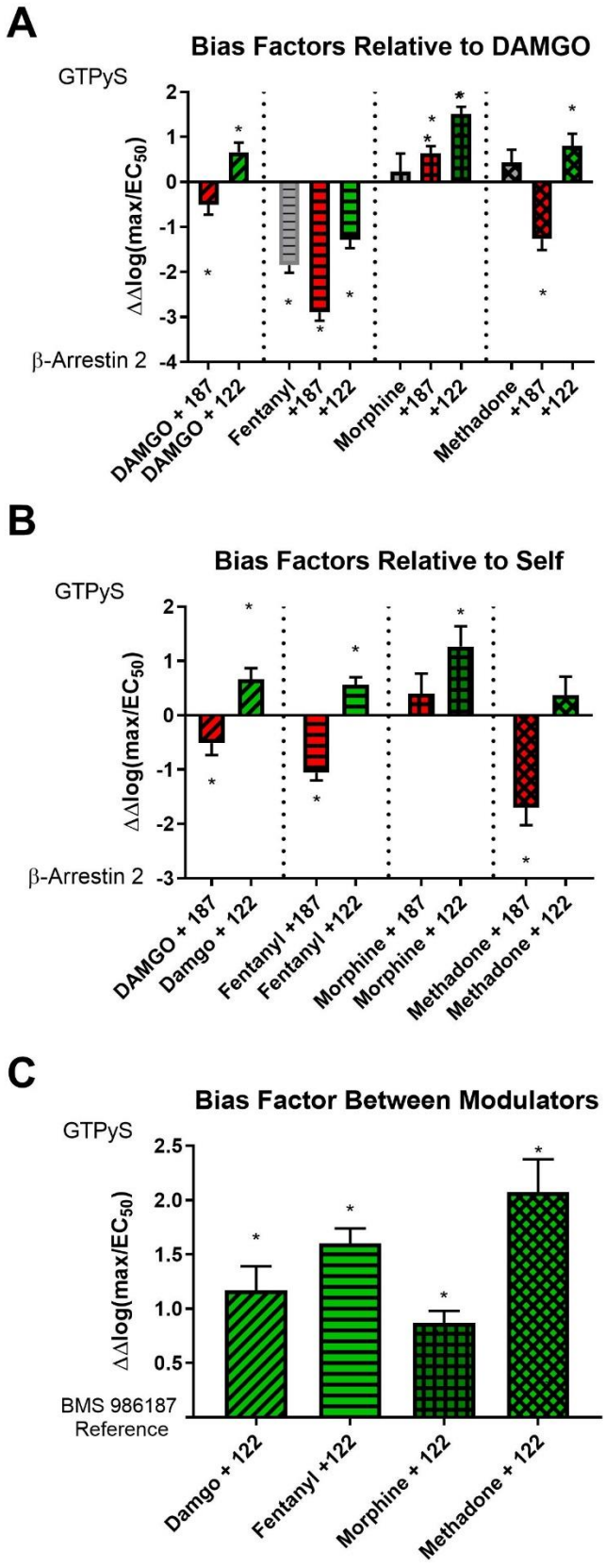


Figure 3.3
Allosteric modulators produce divergent signaling bias.
Bias factors for the listed conditions when comparing GTPγ^{35}S and β-arrestin 2 recruitment. (A) DAMGO alone used as reference ligand. (B) Each orthosteric ligand alone serves as a reference for the modulator conditions and (C) BMS 986187 co-treatment condition serves as a reference for comparing BMS 986122 co-treatment. Data presented are calculations from 3-5 independent experiments for each response performed in duplicate ± pooled S.E.M. Where significance is defined as 95% CI that does not contain zero.

BMS 986187 eliminates G protein bias in SR17018

BMS 986187 can engender β-arrestin 2 bias in both neutral (DAMGO, morphine, methadone), and β-arrestin 2 biased ligands (Fentanyl). To determine if BMS 986187 could reverse an existing G protein bias we chose to investigate SR17018, the most G protein biased ligand reported in the literature to our knowledge (Schmid *et al.*, 2017). Previous studies demonstrated that this compound had a bias factor ranging from 40 to 80-fold. In our hands, when tested in GTPγ^{35}S binding and β-arrestin 2 recruitment assays, SR17018 had a potency of 143 (90-230, 95% C.I.) and 7,100 (6,600-7,700, 95% C.I.) nM, respectively (Figure 3.4). This represents a 50-fold separation between GTPγ^{35}S binding and β-arrestin 2 recruitment potencies and confirms the previously reported G protein preference for this compound. However, in the presence of BMS 986187, GTPγ^{35}S binding and β-arrestin 2 recruitment potencies were increased to 133 (79-230, 95% C.I.) and 152 (135-171, 95% C.I.) nM, respectively, showing no difference in the potency across the two assays. Additionally, the maximum stimulation in GTPγ^{35}S binding increased from 47% (43-51, 95% C.I.) to 101% (96-106, 95% C.I.). In contrast, When SR17018 was examined in conjunction with BMS 986122 in the GTPγ^{35}S binding it gave a potency value of 49 nM (33-72, 95% C.I.) and a maximum of 72% (68-76, 95% C.I.). However, the potency and maximum values determined for β-arrestin 2 recruitment remained unchanged. It should be noted that the baseline GTPγ^{35}S binding levels in the presence of modulators but absence of orthosteric ligand were elevated by BMS 986187 (47%) and BMS 986122 (16%). This is suggestive of Ago-PAM activity, however, as evidenced by Fig 3.2 this effect was variable across assays and is likely a result of high-receptor expression and efficient G protein coupling.

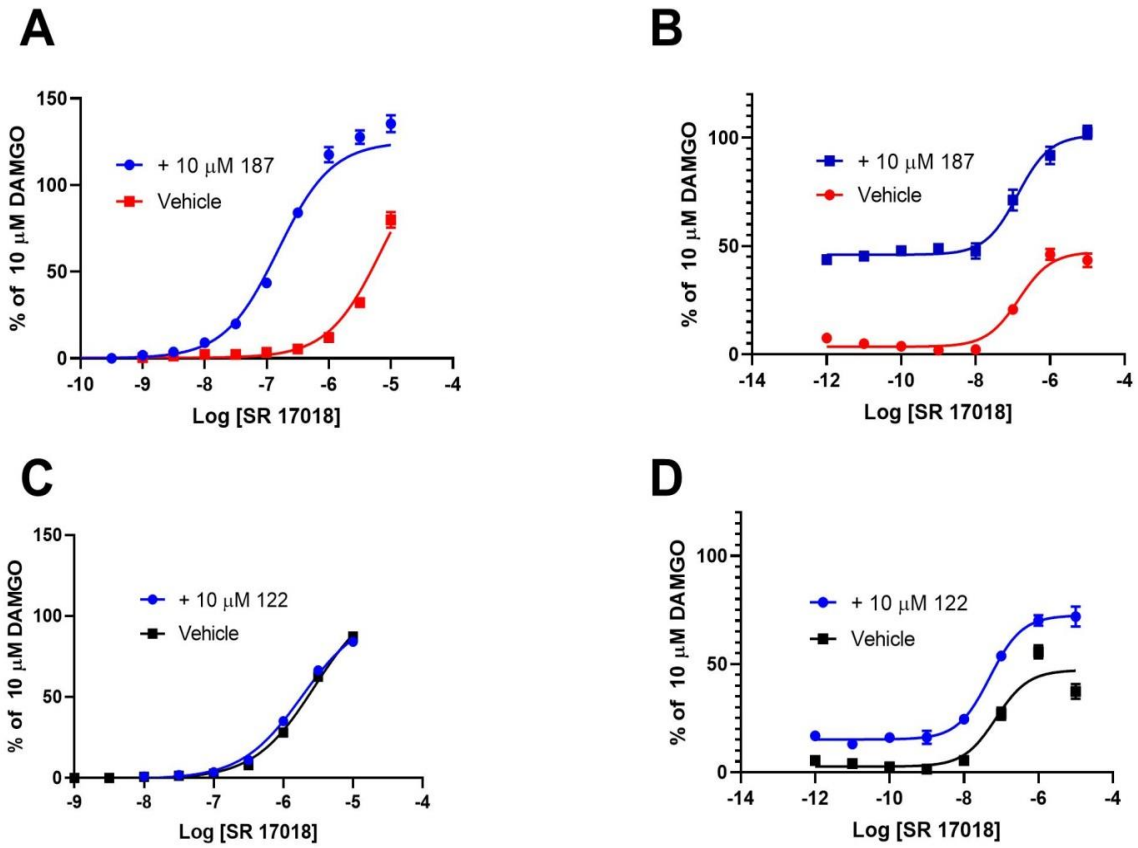


Figure 3.4 Allosteric modulation of G protein biased ligand. (A) β-arrestin 2 recruitment of SR17018 with or without 10 μM BMS 986187. (B) GTPγ^{35}S binding of SR17018 with or without 10 μM BMS 986187. (C) β-arrestin 2 recruitment of SR17018 with or without 10 μM BMS 986122. (D) GTPγ^{35}S binding of SR17018 with or without 10 μM BMS 986122. All experiments are normalized as percentage of 10 μM DAMGO and are the mean ± S.E.M of 2-5 experiments performed in duplicate.

BMS 986187 enhances G protein-independent β-arrestin 2 recruitment

BMS 986187 shifts β-arrestin 2 recruitment for orthosteric agonists far beyond the degree of enhancement in G protein activation. Thus, we hypothesized that BMS 986187 was enhancing β-arrestin 2 recruitment through non-G protein mediated pathways. To test this, agonist recruitment of β-arrestin 2 was measured in cells treated with pertussis toxin (PTX) to make them devoid of Gi/o activity (Fig 3.5A) as shown by a lack of ability to inhibit adenylate cyclase. Cells treated with pertussis toxin still showed significant agonist-stimulated β-arrestin 2 recruitment, albeit a lower max and potency (Fig. 3.5). DAMGO potency was shifted from 183 nM (161-209, 95% C.I.) to 617 nM (446-944, 95% C.I.) while the maximum activation was decreased from 100% to 80% (70-97, 95% C.I.). Fentanyl potency was shifted from 48 nM (41-55, 95% C.I.) to 128 nM (113-144, 95% C.I.) while the maximum was decreased from 90% (85-93, 95% C.I.) to 50% (48-51, 95% C.I.). Surprisingly, Morphine potency trended toward an increase from 305 nM (125-752, 95% C.I.) to 115 (98-137, 95% C.I.) while the maximum decreased from 43% (37-5, 95% C.I.1) to 14% (13-15, 95% C.I.). These data suggest that β-arrestin 2 recruitment to the receptor is occurring, at least to some degree, in a Gi/o independent manner.

This Gi/o independent recruitment was enhanced in the presence of BMS 986187, both in potency (Fig 3.5C, D) and in efficacy (Fig 3.5B). DAMGO potency increased from 617 nM (446-944, 95% C.I.) to 16 nM (11-22, 95% C.I.) while the maximum remained unchanged. Fentanyl potency increased from 128 nM (113-144, 95% C.I.) to 8 nM (6-10, 95% C.I.) while the maximum increased from 50% (48-51, 95% C.I.) to 60% (57-63, 95% C.I.). In the case of morphine, BMS 986187 addition in PTX treated conditions led to a potency increase from 115 nM (98-137, 95% C.I.) to 32 nM (27-40, 95% C.I.) while the

maximum increased from 14% (13-15, 95% C.I.) to 42% (40-44, 95% C.I.). These data indicate that BMS 986187 is capable of driving β-arrestin 2 recruitment in a Gi/o independent manner.

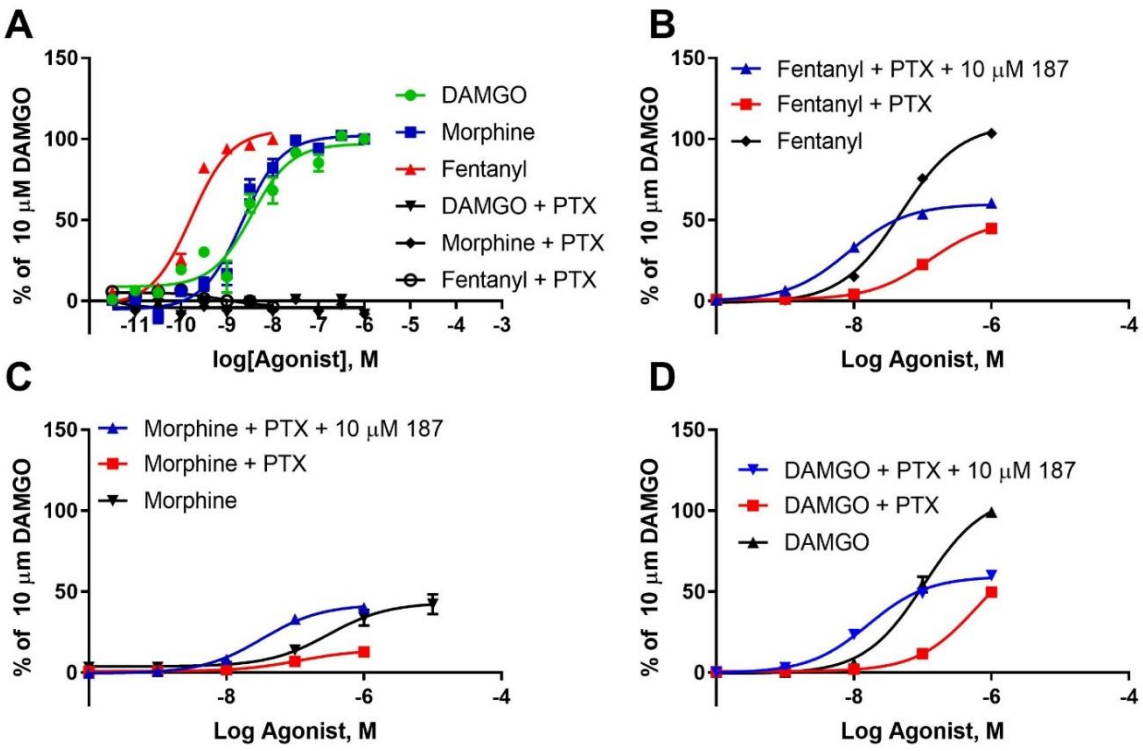


Figure 3.5 BMS 986187 mediated β-arrestin 2 bias is driven by G protein independent mechanism. (A) Adenylyl Cyclase inhibition assay in cells (CHO Pathhunter cells) treated with or without pertussis toxin (PTX). Concentrations response curves for β-arrestin 2 recruitment (CHO Pathhunter cells) were evaluated in vehicle and PTX treated cells with and without 10 μM BMS 986187 for fentanyl (B), morphine (C) and DAMGO (D). Data presented are the mean ± S.E.M of 5 independent experiments performed in duplicate and normalized to percentage of 10 μM DAMGO.

BMS 986187 enhances Gi/o independent, GRK 2/3 dependent β-arrestin 2 recruitment

β-arrestin 2 recruitment mediated via Gi/o proteins is known to occur, in part, through recruitment of GRK2/3 and subsequent phosphorylation of the receptor. Therefore, we determined whether this protein was also dispensable for BMS 986187 mediated β-arrestin 2 recruitment. Fentanyl was chosen for its high potency for β-arrestin 2 recruitment and evaluated in the presence or absence of PTX treatment, BMS 986187, and co-treatment with Compound 101, a GRK 2/3 inhibitor (Thal *et al.*, 2011) (Fig 3.6). In non-PTX treated cells the potency of fentanyl for β-arrestin 2 recruitment was 6.7 nM (5.1-8.7, 95% C.I.) with a maximum of 100% when normalized to 10 μM DAMGO. Upon treatment with compound 101, the potency was unchanged at 9 nM (6.5-12, 95% C.I.), while the maximum was decreased to 37% (34-40, 95% C.I.). This decrease in maximum was partially rescued by the addition of BMS 986187 to 49% (47-51, 95% C.I.), while the potency was increased to 1.5 nM (1.2-1.9, 95% C.I.) (Fig 3.6A). In PTX treated cells, the potency of fentanyl for β-arrestin 2 recruitment was 19 nM (17-20, 95% C.I.), with maximum of 56% (54-58, 95% C.I.). Surprisingly, the potency increased slightly with the addition of compound 101 pretreatment to 11 nM (9-14, 95% C.I.) while the maximum decreased further to 20% (19-21, 95% C.I.). Similar to the non-PTX treated conditions, the addition of BMS 986187 increased the maximum to 29% (29-30, 95% C.I.) and the potency to 1.5 nM (1.3-1.7, 95% C.I.). These results indicate that BMS 986187 is able to enhance both Gi/o independent and GRK 2/3 independent β-arrestin 2 recruitment to the mu opioid receptor.

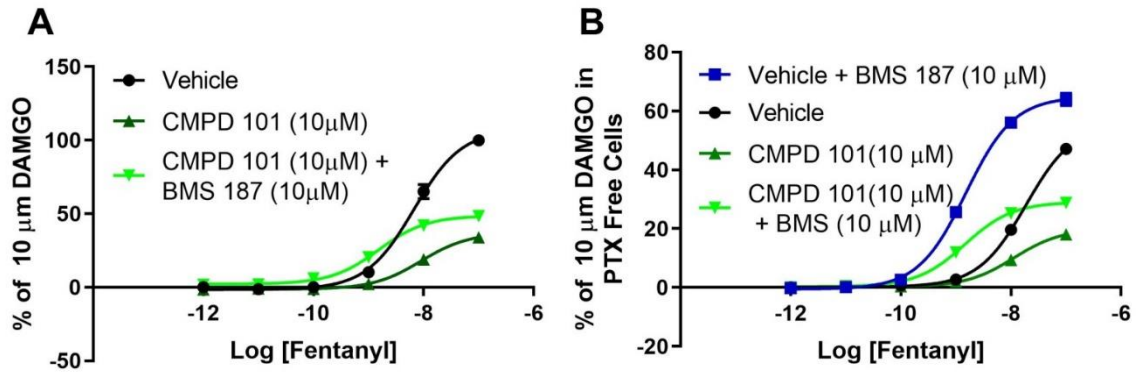


Figure 3.6 BMS 986187 enhances G protein and GRK 2/3 independent β-arrestin 2 recruitment. Concentration-response curves in CHO Pathhunter cells for fentanyl induced β-arrestin 2 recruitment in vehicle (A) or PTX (B) pretreated cells. Data presented are the mean ± S.E.M of five independent experiments performed in quadruplicate normalized to percentage of 10 μM DAMGO in non-PTX treated cells seeded at equal density.

Divergent signaling leads to distinct receptor internalization profiles

The striking differential effect on bias between BMS 986122 and BMS 986187 raises the question of whether these compounds would maintain divergence in internalization which is known to occur in a primarily β-arrestin 2-mediated manner. We hypothesized that BMS 986187 would drive significantly more receptor internalization compared to BMS 986122 when paired with an orthosteric ligand. Internalization was measured as loss of receptor surface expression and evaluated for all ligands across concentrations of 10, 100 and 1000 nM (Fig 3.7).

Comparing internalization induced by orthosteric ligand alone, BMS 986187 led to an increase morphine, fentanyl, DAMGO and methadone elicited internalization. Significant differences were observed in DAMGO, Methadone and Morphine internalization. DAMGO (100nM) treatment reduced surface expression to 58% ± 2, however, BMS 986187 co-treatment (10 μM) further decreased surface expression to 33% ± 4. methadone (1 μM) decreased surface expression to 56% ± 10, whereas BMS

986187 co-treatment reduced surface expression to 35% ± 3. Finally, morphine treatment did not change surface expression significantly at any concentration, but BMS 986187 co-treatment reduced surface expression to 47% ± 5. In comparison, BMS 986122 treatment led to an increase in orthosteric ligand-induced internalization at higher concentrations (1 µM), but to a lesser extent than BMS 986187. BMS 986122 significantly decreased 1 µM morphine-induced internalization to 52% ± 6.

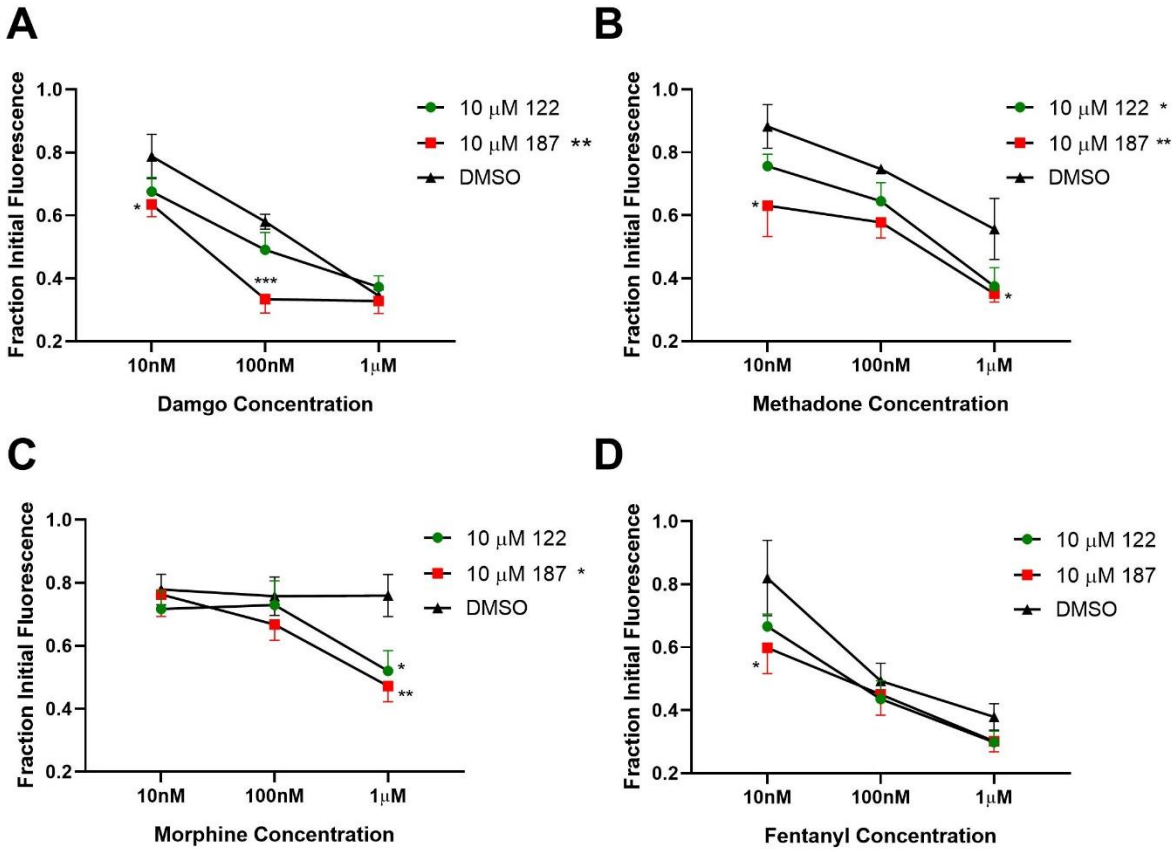


Figure 3.7 BMS 986187 and BMS 986122 effects on receptor internalization. Receptor internalization was evaluated in HEK 293 stably expression the mu opioid receptor for DAMGO (A), Methadone (B), Morphine (C) or Fentanyl (D) in the presence or absence of 10 µM BMS 986122 or BMS 986187. Data presented are the mean of 3-5 individual experiments ± S.E.M. *P<0.05 as determined by two-way ANOVA with Dunnett's post-hoc test.

Discussion

In this study, we have demonstrated that BMS 986187 promotes β-arrestin 2 recruitment bias compared to G protein activation, whereas BMS 986122 produces the opposite effect. These results are particularly striking since we have previously shown these two structurally distinct modulators work via a conserved allosteric site (Livingston *et al.*, 2018). In addition, we have previously shown BMS 986187 to be a G protein biased Ago-PAM at the DOPr (Stanczyk *et al.*, 2019). This divergence adds to the mounting evidence that ligands engender discrete receptor ensembles, and this can depend on the ligand, or in this case, ligands complexed with the receptor.

When evaluating bias, we chose to use DAMGO as an enkephalin-derivative, fentanyl and morphine for clinical relevance, and methadone because it is the most responsive to the effect of mu opioid allosteric modulators (Livingston and Traynor, 2014). Additionally, we tested SR 17018 as it is the most G protein biased ligand in the reported literature at the mu opioid receptor (Schmid *et al.*, 2017). When evaluating these ligands alone, relative to DAMGO, fentanyl was seen to be significantly biased towards β-arrestin 2 recruitment. This agrees with previous reports and is correlated with fentanyl having a small therapeutic window (Schmid *et al.*, 2017).

When the chosen orthosteric ligands were evaluated in conjunction with either BMS 986122 or BMS 986187, a significant bias was observed (Fig 3.3). BMS 986122 promoted G protein bias for all orthosteric ligands tested, relative to DAMGO alone. Conversely, BMS 986187 promoted β-arrestin 2 bias relative to DAMGO alone for DAMGO, methadone, and fentanyl, but promoted G protein bias for morphine. This divergence within BMS 986187 treatments suggests the bias engendered can depend

greatly on the orthosteric probe. This is not surprising given the probe dependence previously observed at the mu opioid receptor as well as other GPCRs (Livingston and Traynor, 2014). Future work will explore whether this bias holds across other morphinan compounds.

Evaluating bias relative to DAMGO is a somewhat arbitrary measure. In order to evaluate how these allosteric ligands impacted the bias relative to the paired orthosteric ligand alone, we compared bias within each orthosteric ligand tested with and without each PAM. From this perspective, BMS 986122 promoted G protein bias in all ligands except for methadone and BMS 986187 promoted β-arrestin 2 bias in all ligands except for morphine. Comparing within each orthosteric ligand provides an understanding of the relative change between the given orthosteric ligand alone and allosteric/orthosteric ligand complex. This also provides a clearer picture of the expected relative therapeutic window when testing orthosteric ligands with and without a PAM *in vivo*.

In order to evaluate the magnitude of separation of bias induced by BMS 986122 and BMS 986187, BMS 986187 + orthosteric ligand was used as a reference point. From this reference, BMS 986122 + orthosteric ligand co-treatments were significantly G protein biased. These data give the most descriptive picture of the divergent bias promoted by these two compounds.

We predicted based on previous results that BMS 986187 would promote β-arrestin 2 bias in orthosteric ligands. However, the magnitude of the shift elicited in the β-arrestin 2 recruitment assay was striking. In all cases except morphine, BMS 986187 elicited a shift in β-arrestin 2 recruitment that surpassed the fold-shift in potency observed for G protein activation. Adding weight to this phenomenon, BMS 986122 elicited shifts

showed the opposite effect, suggesting this was not an assay-dependent effect and rather an allosteric ligand-dependent one.

Conventional mu opioid receptor signaling cascades indicate that G protein activation promotes βγ-dependent GRK 2/3 recruitment, leading to receptor phosphorylation and subsequent β-arrestin 2 recruitment (Al-Hasani and Bruchas, 2013 for review). However, for β-arrestin 2 recruitment to be more potent than G protein activation, its recruitment must exist in some capacity independent of this cascade. This is supported by data obtained in PTX-treated cells. Not only can β-arrestin 2 recruitment occur independently of functional Gi/o proteins, but this recruitment can be enhanced by the presence of BMS 986187, both in potency and efficacy. Moreover, using the GRK 2/3 inhibitor, compound 101, we provide strong evidence that β-arrestin 2 recruitment can happen in a GRK 2/3 independent manner and that this is also enhanced by BMS 986187.

The data from PTX-treated cells and compound 101 treatment provide multiple key insights. Firstly, compound 101 treatment led to a larger decrease in β-arrestin 2 recruitment (37%) than depletion of Gi/o signaling via PTX (55%). This suggests that GRK 2/3 functions in both a G protein dependent and independent fashion, a phenomenon that has been described at the D2 receptor (Pack *et al.*, 2018). Additionally, β-arrestin 2 recruitment in compound 101 treated cells is further decreased in the presence of pertussis toxin. This suggests that G protein activation promotes β-arrestin 2 recruitment in both a GRK 2/3 dependent and independent fashion. In support of this, recent evidence has shown that β-arrestin 2 recruitment, while heavily depending on GRK 2/3, can happen independently at the mu opioid receptor (Miess *et al.*, 2018)

Finally, the mechanism by which BMS 986187 drives β-arrestin 2 bias appears to be at least partly through enhancement of these processes as indicated by the enhancement of β-arrestin 2 recruitment in the absence of Gi/o signaling and GRK 2/3 function. It is unclear which signaling molecules BMS 986187 is indirectly enhancing to lead to β-arrestin 2 recruitment in the absence of Gi/o proteins and GRK 2/3 function, although there are a few possibilities which all may coexist. First, beyond GRK isoforms 2/3 there are GRK 5/6 and PKC enzymes which have been shown to play a role in receptor desensitization for class A GPCRs (Kelly, Bailey and Henderson, 2008). Previous work in locus coeruleus neurons suggests that GRK 5/6 plays a minimal role in receptor phosphorylation, β-arrestin 2 recruitment and desensitization, however, it is possible this role will depend on cell type and relative GRK isoform expression (Lowe *et al.*, 2015). Second, certainly it would seem that BMS 986187 promotes a conformation more favorable to β-arrestin 2 binding. This is supported by the fact that β-arrestin 2 affinity is only enhanced 2-3-fold in the presence of receptor phosphorylation (Gurevich and Gurevich, 2006).

When evaluating loss of receptor expression from the cell surface, the data presented mostly agreed with that from the β-arrestin 2 recruitment assay. With regards to DAMGO and methadone, BMS 986187 afforded a greater loss in cell surface expression than BMS 986122 despite promoting nearly identical shifts in G protein activation, consistent with the observed enhancement of β-arrestin 2 recruitment. In addition, both BMS 986122 and BMS 986187 elicited increases in surface expression loss when paired with morphine. This is consistent with the β-arrestin 2 recruitment data where both compounds enhanced morphine efficacy, BMS 986187 to a larger extent.

However, the fentanyl internalization data is not readily explained by the β-arrestin 2 recruitment data. When combined with fentanyl, the apparent bias observed when comparing β-arrestin 2 with G protein activation was absent. In fact, both BMS 986187 and BMS 986122 enhanced the decrease in cell surface expression to similar levels despite over a ten-fold difference in the β-arrestin 2 recruitment potency between the two modulators. One possibility is that fentanyl is already β-arrestin 2 biased and neither modulator leads to a G protein bias, simply a reduction in β-arrestin 2 bias for BMS 986122, a change that may not be significant enough to impact the loss of surface expression. Another possibility is that β-arrestin 2 recruitment and internalization, while clearly correlated, can show some divergence in profiles. Accumulating evidence shows differential β-arrestin 2 engagement at the plasma membrane can influence downstream consequences and this may explain some of the discrepancies observed (Shukla *et al.*, 2008; Ranjan *et al.*, 2017; Eichel *et al.*, 2018; Latorraca *et al.*, 2018). Finally, these assays are performed in two different cells types and systems. Where one has endogenous β-arrestin 2, the other (Pathhunter), uses an overexpressed system reliant upon enzyme fragment complementation. It is likely in the Pathhunter cells β-arrestin 2 binding is irreversible which may account for greater enhancement in potency; however, we would have expected this phenomenon to generalize to all tested ligands, which it did not.

In conclusion, this study provides the first evidence of biased allosteric modulation of the mu opioid receptor using BMS 986122 and BMS 986187. Despite a proposed similar binding site, these compounds lead to divergent signaling bias downstream of the receptor. This is supported by the fact that ligands binding to the same orthosteric site

can show biased signaling despite overlapping binding interactions. Furthermore, we highlight the ability of the mu opioid receptor to recruit β-arrestin 2 in the absence of Gi/o signaling and GRK 2/3 function, recruitment that is enhanced by BMS 986187. This adds further evidence to conformation-driven ligand bias downstream of the mu opioid receptor. Additionally, this underscores the complex signaling that can be generated by the same receptor when different ligand complexes are introduced. Future studies will need to examine whether these in-vitro biases translate on an in-vivo level.

Methods

Cell Lines

For GTPγ^{35}S assays, CHO cells stably expressing wild-type human-DOPr (CHO-hDOPr) were grown in DMEM containing 10% FBS and 1% penicillin and streptomycin and maintained in 0.8 mg/ml G418 as previously described (Burford et al., 2015). CHO OPRM1 Pathhunter cells from DiscoverX were maintained in 0.8 mg/ml G418 and 0.3 mg/ml Hygromycin B per manufacturer instructions.

For internalization assays, the murine μ opioid receptor was N-terminally tagged with a pH-sensitive GFP (Sankaranarayanan *et al.*, 2000) to generate SpH-MOR. HEK293 stably expressing SpH-MOR were generated using G418 (Invitrogen) as previously described (Soohoo and Puthenveedu, 2013). Cells were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Gibco) and grown at 37℃ with 5% carbon dioxide.

Membrane Homogenate Preparations

Cells were harvested and membrane homogenates prepared as previously described (Clark *et al.*, 2003). Briefly, cells were washed with ice-cold phosphate-buffered saline, pH 7.4 and detached from plates by incubation in harvesting buffer (0.68 mM

EDTA, 150 mM NaCl, and 20 mM HEPES at pH 7.4) and pelleted by centrifugation at 200g for 3 minutes. Cells were resuspended in ice-cold 50mM Tris (pH 7.4), homogenized using a Tissue Tearor (Dremel; Mount Prospect, IL, USA), and centrifuged at 20,000g at 4^{o}C for 20 min. The pellet was then resuspended, homogenized, and centrifuged a second time. This final pellet was resuspended in ice-cold 50 mM Tris (pH 7.4) and homogenized using a glass dounce to give a protein concentration of 0.5-1.5 mg/mL and stored at -80°C. Protein concentration was determined using the bicinchoninic acid quantification method (BCA) with BSA serving as the standard.

Stimulation of GTPγ^{35}S Binding

Agonist stimulation of GTPγ^{35}S binding was measured as described previously (Clark *et al.*, 2003). Homogenates of CHO cells expressing wild-type hDOPr (15-20 µg/well) were incubated in "GTPγS buffer" (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) containing 0.1 nM GTPγ^{35}S, 30 µM guanosine diphosphate (GDP) and varying concentrations of orthosteric agonist with BMS 986122, BMS 986187 or Vehicle for 1h in a shaking water bath at 25°C. The reaction was terminated by vacuum filtration through GF/C filters using a Brandel harvester and washed five times with ice-cold GTPγS buffer. Filters were dried, and following the addition of EcoLume scintillation cocktail, counted in a Wallac 1450 MicroBeta Liquid Scintillation and Luminescence Counter (Perkin Elmer). The level of GTPγ^{35}S binding is expressed as a percentage of the full MOPr agonist, DAMGO, at 10 µM to account for variability between membrane preparations.

β-arrestin 2 Recruitment

β-arrestin 2 recruitment was determined using the commercially available Pathhunter assay by DiscoverX. CHO-βArrestin-hMOR cells were plated at a density of

5,000 cells per well in 384-well white polystyrene cell culture plates (Grenier) using Assay Complete Cell Plating Reagent (DiscoverX) 24 hours prior to drug treatment. The following day cells were treated with indicated drug conditions for 60 minutes at 37^0 Celsius. Following drug incubations, cells were treated with β-galactosidase substrate provided in Pathhunter Detection Kit (DiscoverX), incubated for 60 minutes at room temperature and luminescence was detected using Envision Plate Reader (Perkin Elmer).

Inhibition of forskolin-stimulated cAMP assay

Inhibition of forskolin-stimulated cAMP was evaluated in CHO-Mu Pathhunter cells using the LANCE Ultra Kit per manufacturer's instructions (PerkinElmer). Briefly, cells were plated at a density of 500 cells per well (5 µl per well) in white-walled OptiPlate 384-well plate (PerkinElmer). Following plating, drug dilution containing 6 µM forskolin (Sigma) in manufacturer provided stimulation buffer was added at a volume of 5 µL and incubated at room temperature for 30 minutes. Following incubation, the reaction was terminated by the addition of 5 µL Eu-cAMP tracer solution and ULight-anti-cAMP solution, respectively, and sealed at room temperature for 1 hour. After 1 hour, FRET was detected using an Envision plate reader (PerkinElmer).

Pertussis Toxin Experiments

For Pertussis toxin experiments, cells were treated for 24 hours with 100ng/ml Pertussis Toxin (Tocris). Disruption of Gi/o signaling determined by an absent signal in cAMP assay.

Internalization assay

HEK293 cells stably expressing SpH-MOR were seeded at a density of 6x10^5 cells/well in a 24-well glass bottom dish (CellVis). Cells were allowed to grow for 48 hours

in order to reach 100% confluency. Media was removed and replaced with a carbon dioxide-independent medium made up of Leibovitz media (Gibco) and 1% fetal bovine serum (Gibco). Images were collected using a CSU-X1 spinning disk confocal unit (Yokogawa), a 10X objective on a Ti-E inverted microscope (Nikon), excitation with a 488nm laser line (Andor), a 525/30 emission filter (Semrock), and an iXon 897 EMCCD camera (Andor). For each well, 3 fields of view were selected. Images were acquired every two minutes, with each field of view in every well on the plate imaged once within that time-frame. After a 4-minute (2 frame) baseline, drugs were added simultaneously to one column (4 wells) of the plate. Imaging was paused briefly after completing that column to add drugs to the next column. This was repeated for each of the 6 columns to ensure that drug addition timing matched across the experimental conditions. Images were acquired for 20 minutes after drug addition.

For analysis, images were calibrated to remove background fluorescence, and then integrated density of fluorescence intensity was calculated for each frame. Intensities were normalized to the mean of the two baseline frames, and then fluorescence change from baseline was calculated. All calculations were performed using Fiji (Schindelin *et al.*, 2012) and a custom-written macro. Frames where cells went out of focus were not included in analysis, and fields where cells left the field of view were discarded. The remaining fields of view for each well were averaged together to produce one experimental replicate. Each condition was replicated at least 3 times, with each replicate occurring on a different day of imaging.

Statistical Analysis

Bias calculations were performed as described by Kenakin, (2017) as follows: For each ligand and respective response, individual experimental curves were used to calculate log(max/EC50). The difference in log(max/EC50) between β-arrestin 2 recruitment and GTPγ^{35}S, Δlog(max/EC50), was then calculated. Individual results were combined to give means ± 95% CI values shown in Table 1. Finally, the differences between the Δlog(max/EC50) values for the reference ligand and test ligand were calculated to give a ΔΔlog(max/EC50) values, the antilog of which is the bias factor. Bias was quantified using multiple measures. First, using DAMGO treatment alone as the reference by which all other conditions were compared. Next, the bias was compared for each orthosteric ligand relative to the orthosteric ligand in the presence of either allosteric modulator. For these conditions, orthosteric ligand alone was set as the reference point. Finally, bias engendered by BMS 986122 and BMS 986187 relative to each other was calculated for each orthosteric ligand. In this case, the orthosteric ligand + BMS 986187 was used as the reference and the difference in bias between that condition and orthosteric ligand + BMS 986122 is reported. Bias is considered significant where 0 is not contained in the 95% C.I. for the ΔΔlog(max/EC50). For internalization assay, two-way ANOVA was employed with Dunnett's post-hoc test to compare allosteric modulator conditions with orthosteric alone results. For internalization assay, two-way ANOVA was employed with Tukey's post-hoc test.

For all analyses significance was set at 5% (0.05 p-value).

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Chapter 4 : Structure-activity Relationships of Allosteric Modulators of the Mu Opioid Receptor

Summary

Positive allosteric modulation of the mu opioid receptor is a promising new strategy to the ever-growing problem of acute and chronic pain. Unlike orthosteric ligands, which bind to a conserved activation site on opioid receptors, allosteric modulators bind to a topographically distinct site where they can modulate the affinity and or efficacy of bound orthosteric ligands. From the perspective of pain, allosteric modulators could be employed to enhance the efficacy of endogenous neurotransmitters (endorphins, enkephalins) released during pain states. Thus, eliminating the need for exogenous orthosteric opioids such as morphine and the plethora of associated side effects associated with its use. BMS 986122 (2-(3-Bromo-4-methoxyphenyl)-3-[(4-chlorophenyl)sulfonyl]-thiazolidine) is a positive allosteric modulator of the mu opioid receptor that we have previously shown to enhance orthosteric ligand potency and promote G protein bias, indicating strong therapeutic potential. There have been several studies to date exploring the function of BMS 986122 at a cellular level. However, the structure-activity relationship of this scaffold at the mu opioid receptor is relatively unexplored. In order to further understand the

allosteric pharmacophore and design higher potency compounds, the Vahlteich Medicinal Chemistry Core generated 21 analogs of BMS 986122 and examined structure-activity relationships. Structural changes were evaluated using the GTPγ^{35}S binding assay and β-arrestin 2 recruitment. We were able to identify a series of halogen substitutions that increased both the potency and efficacy of the parent BMS 986122 with GTPγ^{35}S fold shift efficacy increasing from 12-fold to approximately 50-fold. We discovered that some compounds display direct receptor activation and that this activation can function independently from the shift in orthosteric ligand potency. In addition, the identified allosteric agonists have negative cooperativity with the orthosteric antagonist diprenorphine. Overall, this study further highlights the complexity of allosteric-orthosteric interactions.

Introduction

Opioids are the gold standard treatment for pain. In 2018, there were roughly 200-million opioid prescriptions written in the United States alone (Jones *et al.*, 2018). Opioids exert their effects by interacting with the orthosteric site of mu opioid receptors to inhibit neurons responsible for pain transmission (Al-Hasani and Bruchas, 2013). While these therapies are considered efficacious for acute pain, and some forms of chronic pain, they have led to the opioid epidemic in the United States (Jones *et al.*, 2018).

One emerging strategy for targeting pain is the use of positive allosteric modulators at the mu opioid receptor (Burford, Traynor and Alt, 2015). Positive allosteric modulators have the capacity to enhance orthosteric ligand efficacy/affinity and possess limited activity in the absence of orthosteric ligand (Wootten, Christopoulos and Sexton, 2013). For opioid receptors, this presents a two-fold benefit. The body releases enkephalins and

endorphins, endogenous ligands for opioid receptors, in pain states. This would theoretically be in regions of the body responsible for pain transmission, potentially sparing regions involved with constipation and euphoria. Rather than promoting systemic opioid receptor activation, allosteric ligands will act to enhance receptor activity where only orthosteric ligands are present, potentially sparing regions involved with constipation and euphoria. Contrasting systemic opioid receptor activation when exogenous compounds such as morphine are administered. The second benefit of allosteric ligands for targeting pain is the ability to maintain temporal regulation. A major limitation for opioids in chronic pain is that prolonged activation of opioid receptors leads to desensitization and development of tolerance, where high doses of opioid are required for the same level of analgesia. However, allosteric modulators might only enhance receptor activation in a pulsatile manner, dependent on release of endogenous neurotransmitters. By limiting prolonged receptor activation this holds the promise of significantly less tolerance developing over time.

Previous work from our lab identified BMS 986122 and BMS 986187 can drive distinct and divergent signaling profiles when paired with orthosteric ligands. While BMS 986122 led to G protein bias for several ligands, BMS 986187 instead promoted preferential engagement of β-arrestin 2 recruitment. Accumulating evidence suggests that G protein bias is favorable for a reduction in side effects commonly associated with opioids (Bohn *et al.*, 1999, 2000; Raehal *et al.*, 2011; Schmid *et al.*, 2017). For this reason, we chose BMS 986122 as a lead scaffold and generated 21 unique analogs to assess structure-activity relationships. Significant activity was gained by the introduction of halogen groups and activity was lost by the introduction of increasing heterocycle

structures. In addition, it was found that many of the compounds activated the receptor directly from an allosteric site and inhibited binding of the competitive antagonist diprenorphine. Collectively, this work sheds significant light on the SAR of BMS 986122 and adds insight to the complex interplay between the orthosteric and allosteric site on the mu opioid receptor.

Results

The structure-activity relationship of BMS 986122 (CCG257868) and related analogs was evaluated using three methods. In order to evaluate efficacy, DAMGO concentration response curves were evaluated in the presence or absence of 10 μM allosteric modulator compound in the β-arrestin-2 recruitment assay (Fig 4.1)and GTPγ^{35}S binding assay (Table 4.1, 4.2, 4.3). Additionally, allosteric modulator concentration-response curves with an EC_{20} of DAMGO were evaluated in β-arrestin 2 recruitment assays (Fig 4.2). CCG 257868 elicited a 7-fold shift in GTPγ^{35}S stimulation elicited by DAMGO and a small but significant 2-fold-shit in β-arrestin 2 recruitment. Measuring enhancement of an EC_{20} concentration of DAMGO (30 nM) in β-arrestin 2 recruitment, the potency of 257868 was 15.5 μM (12-22, 95% C.I.) with a maximum of 56% (51-66, 95% C.I.) relative to 10 μM DAMGO. The unsubstituted analog 258163 elicited a 3-fold GTPγ^{35}S shift, 1.2-fold β-arrestin 2 shift, and potency of >50 μM and max <30% when measuring EC_{20} DAMGO enhancement.

Right-hand ring SAR

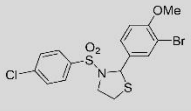
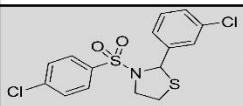
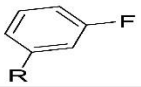
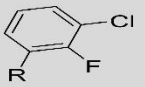
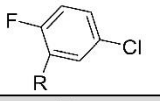
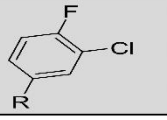
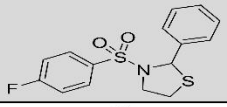
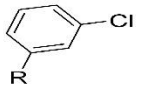
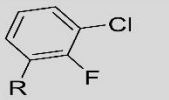
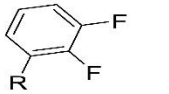
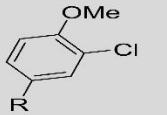
Our initial hypothesis was introduction of halogen groups on the right-hand ring would improve positive allosteric modulator efficacy. Therefore, synthesis efforts focused on the right-hand ring with the 4-chloro left-hand ring and thiazolidine core structure.

Results are summarized in Table 4.1. Compared to the original CCG257868 compound with methoxy and bromo substituents, substitution of either a chlorine (258162) or fluorine (257869) at the meta position led to a significant enhancement of potency and efficacy in all outputs. Using a single 10 μM concentration of the new compounds, the rank order for the shift in the DAMGO concentration curve in the GTPγ^{35}S assay was: 258162 (46-fold) > 257869 (30-fold) > 257868 (7-fold). In the β-arrestin 2 recruitment assay the effects of a 10 μM concentration was much smaller (2-3 fold) with no differences between the compounds. When assayed in the presence of an EC_{20} concentration of DAMGO the compounds showed similar potencies (between and 15 and 28 μM), but the 2-Chloro derivative (258162) afforded a higher response, equivalent to that seen with a maximal concentration of DAMGO.

Using 258162 as a starting point, the impact of additional halogen substitution around the ring was determined via a fluorine walk. Introduction of a fluorine at the 2-position (257985) did not alter the ability of a 10 μM concentration to shift the DAMGO concentration-response curve in the GTPγ^{35}S assay but afforded a significant enhancement in potency over 258162 (11 vs 22 μM), although with a reduced efficacy. However, moving the fluorine to the 5-position (257986) led to a significant reduction in GTPγ^{35}S fold shift for DAMGO (33 vs 1-fold shift) and a loss of potency and efficacy in stimulating DAMGO-induced β-arrestin 2 recruitment. A similar effect in both assays was observed when fluorine was substituted at the 4-position (257963), although in this case efficacy was retained. It should be noted that similar results were obtained for the right-hand phenyl ring SAR for thiazolidine scaffolds with a 4-position fluorine, rather than Cl, on the left-hand ring, although these compounds generally showed higher maximal

responses in the β-arrestin 2 assay. Ultimately, this set of SARs confirmed the 2,3 - halogen substitutions to be to be favorable for allosteric modulator potency and efficacy.

Table 4.1 SAR of right-hand ring modifications. (A) β-arrestin 2 recruitment concentration response curves for allosteric modulators in the presence of an EC_{20} concentration of DAMGO. (B) Fold-shift in DAMGO β-arrestin 2 recruitment elicited by allosteric modulator at 10 μM. (C) Fold-shift in DAMGO GTPγ^{35}S binding elicited by allosteric modulator at 10 μM. Reported with 95% confidence intervals (A) or S.E.M (B,C) in brackets. R indicates core scaffold. *indicates ambiguous 95% confidence intervals due to reaching limit of solubility

CCG#	Structure	(A) β-Arrestin 2 EC50 (μM)	(A) β-Arrestin 2 Emax %	(B) DAMGO Fold Shift β-Arrestin 2	(C) DAMGO Fold Shift GTPγ35S
BMS 986122		16 (12-22)	56 (51-66)	2 ± 0.3	7 ± 4
Thiazolidine Core Series					
258162		23 (20-28)	94 (85-109)	3 ± 0.3	46 ± 11
257869		28 (25-36)	68 (60-82)	3 ± 0.3	30 ± 7
257985		11 (10-13)	71 (68-75)	6 ± 0.6	33 ± 20
257986		>50	40*	1	1
257963		55	121 (91-215)	7 ± 3	1
258169		> 50	24*	1	1
257883		26 (23-29)	98 (91-109)	4 ± 0.4	35 ± 14
258188		7 (7-8)	111 (108-114)	10 ± 1.2	65 ± 40
258642		11 (10-12)	100 (95-105)	6 ± 0.8	56 ± 25
257884		>50	100*	3 ± 0.8	35 ± 10

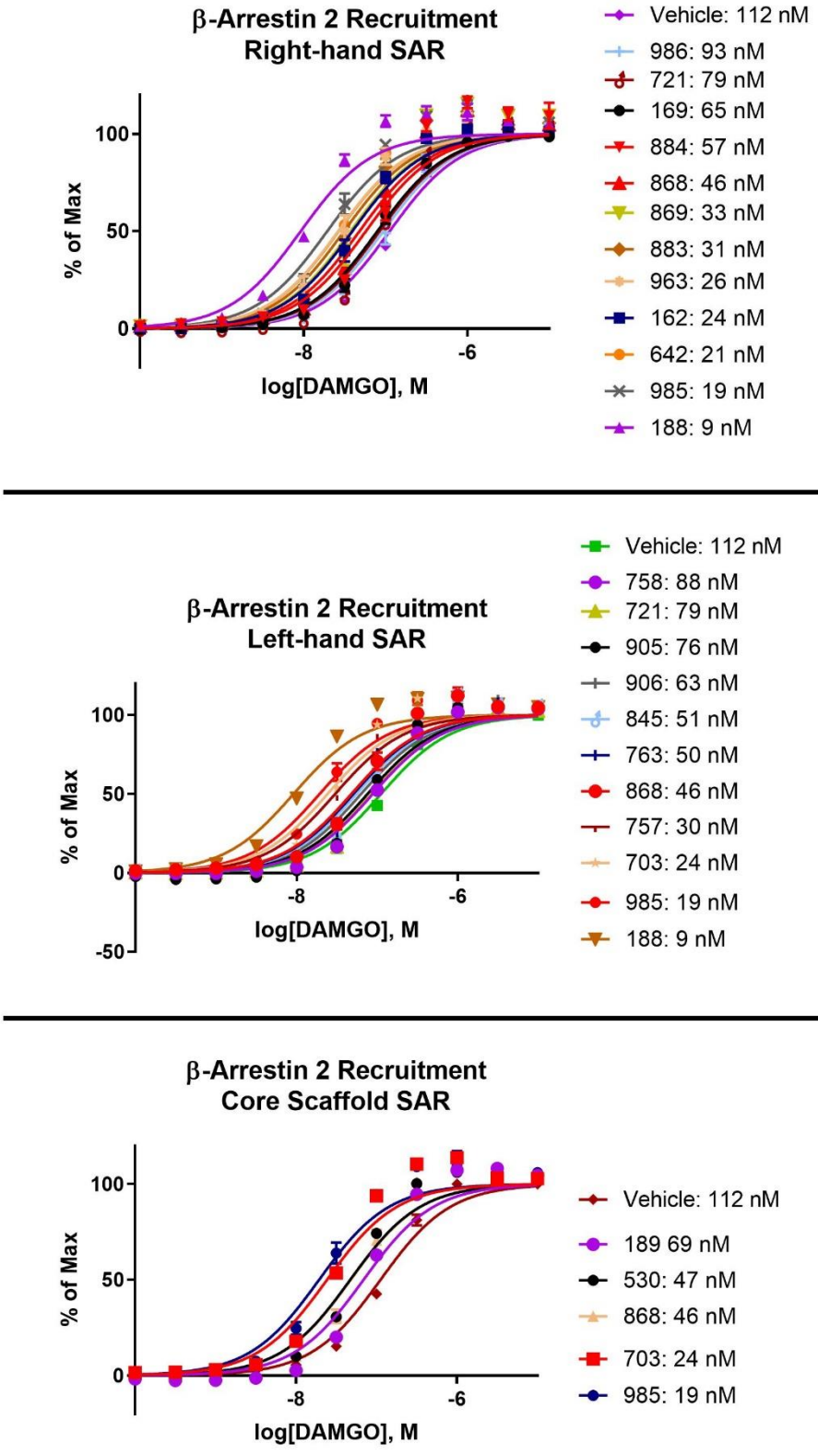


Figure 4.1. β-arrestin 2 fold-shift. DAMGO concentration-response curve in β-arrestin 2 recruitment in the absence or presence of 10 μM allosteric modulator analogs. Data presented are the mean ± S.E.M. 3-5 independent experiments in duplicate. Data are normalized to percent of 10 μM DAMGO (% of Max) Legend represents EC_{50} values for indicated conditions.

Core SAR

Changes to the core thiazolidine heterocycle were evaluated using allosteric modulator concentration response curves in presence of an EC_{20} of DAMGO (30 nM) measuring β-arrestin 2 recruitment as the primary readout (Table 4.2). The five cores evaluated were thiazolidine (257985), Piperidine (258703), Oxazolidine (258189), Pyrrolidine (258530) and Azepane (265127). The right-hand substituents of 3-Chloro and 2-fluoro were kept constant and the 4-Chloro in the left-hand side was kept as in BMS-986122. In potency the cores exhibited the following rank order: 265127 (9.8 μM) = 257985 (11 μM) > 258530 (20 μM) = 258703 (23 μM) > 258189 (50 μM). However, maximal enhancement of the DAMGO response revealed a different rank order: 258198 (100%) = 258703 (96%) > 258530 (78%) = 257985 (71%) > 265127 (32%).

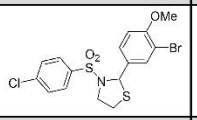
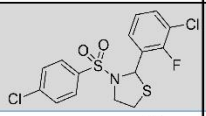
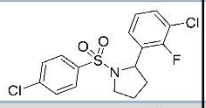
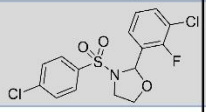
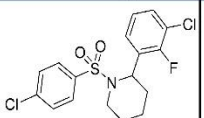
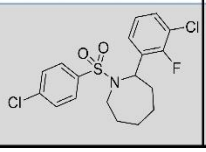
CCG#	Structure	β-Arrestin 2 EC50 (μM)	β-Arrestin 2 Emax %
	CORE SAR		
BMS 986122		16 (12-22)	56 (51-66)
257985		11 (10-13)	71 (68-74)
258530		20 (17-25)	78 (70-91)
258189		>50	100*
258703		23 (17-37)	96 (82-124)
265127		10 (7-14)	32 (28-40)

Table 4.2 SAR of changing thiazolidine core. (A) β-arrestin 2 recruitment concentration response curves for allosteric modulators in the presence of an EC_{20} concentration of DAMGO. (B) Fold-shift in DAMGO β-arrestin 2 recruitment elicited by allosteric modulator at 10 μM. (C) Fold-shift in DAMGO GTPγ^{35}S binding elicited by allosteric modulator at 10 μM. Reported with 95% confidence intervals (A) or S.E.M (B,C) in brackets. R indicates core scaffold. *indicates ambiguous 95% confidence intervals due to reaching limit of solubility

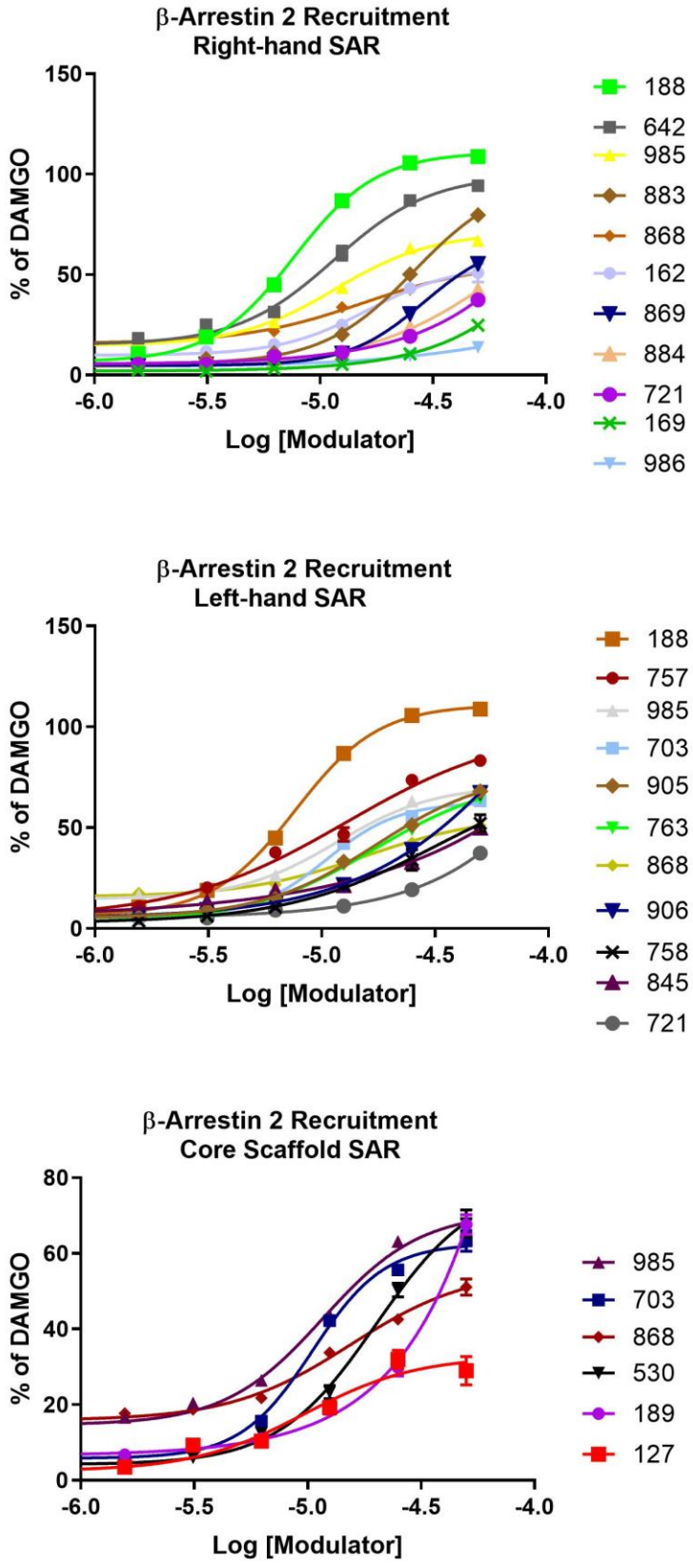


Figure 4.2 Allosteric modulator potency evaluation. Allosteric modulator concentration-response curves for β-arrestin 2 recruitment in the presence of 30 nM DAMGO. Data are presented as percentage of 10 μM DAMGO and are the mean ± S.E.M. of three independent experiments performed in quadruplicate.

Left-Handed Structure SAR

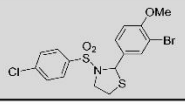
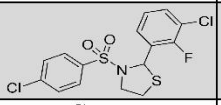
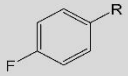
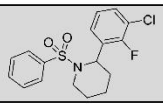
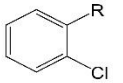
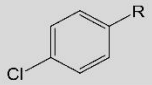
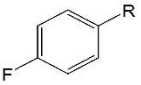
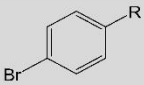
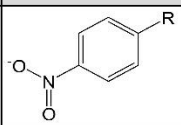
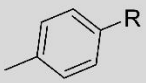
Finally, efforts focused on the left-hand SAR for the thiazolidine scaffold (Table 4.3), keeping the 3-Chloro and 2-Fluoro on the right-hand side (257985). There was a clear relationship between potency and efficacy with a halogen group at the para position. Moving the chlorine from the 4-position (257985) to the 2-position (258721) resulted in a significant loss of modulator potency (11 µM vs 50 µM). In addition, there was a significant reduction in fold-shift observed in the presence of 10 µM of the compounds, in the β-arrestin 2 recruitment (6 vs 2) and GTPγ^{35}S binding (33 vs 1). Substituting a fluorine for the 4-chloro to provide 258188 led to a further enhancement in potency and maximal increase in DAMGO mediated β-arrestin 2 recruitment.

The importance of the 4-position halogen on the left-hand ring was also evident in the piperidine core series where the unsubstituted (258906) and 2-chloro (258845) were significantly less potent and efficacious than the 4-chloro (258703). Potencies to enhance DAMGO mediated β-arrestin 2 recruitment were 97, 50 and 23 µM respectively. β-arrestin 2 fold shifts were similar with an efficacy ranking of 258703 (7-fold)>258845 (4-fold) = 258906 (4-fold). This agreed with GTPγ^{35}S binding fold shifts 258703 (10-fold)> 258845 (8-fold) > 258906 (5-fold).

Based on these findings regarding 4-position halogen importance on modulator efficacy and potency, we tested a series of substitutions at this position in the piperidine scaffold. The substituents represented were fluorine (258757), chlorine (258703), bromine (258763), methyl (258758) and nitro (258905). Modulator potency in β-arrestin 2 recruitment was in the following order: 258757 (12 µM) = 258763 (17 µM) = 258905 (17 µM) = 258703 (23 µM) > 258758 (32 µM). The fold shift afforded by 10 µM of the

modulators for DAMGO mediated β-arrestin 2 recruitment was in the following rank order: 258703 (7-fold) = 258757 (7-fold) > 258763 (4-fold) > 258905 (3-fold) = 258758 (3-fold). Finally, the fold shift observed for GTPγ^{35}S binding had the following rank order: 258905 (11-fold) > 258703 (10-fold) > 258757 (6-fold) > 258763 (5-fold) > 258758 (1-fold).

Table 4.3 SAR of left-hand ring modifications. (A) β-arrestin 2 recruitment concentration response curves for allosteric modulators in the presence of an EC_{20} concentration of DAMGO. (B) Fold-shift in DAMGO β-arrestin 2 recruitment elicited by allosteric modulator at 10 μM. (C) Fold-shift in DAMGO GTPγ^{35}S binding elicited by allosteric modulator at 10 μM. Reported with 95% confidence intervals (A) or S.E.M (B,C) in brackets. R indicates core scaffold. *indicates ambiguous 95% confidence intervals due to reaching limit of solubility.

CCG#	Structure	(A) β-Arrestin 2 EC50 (μM)	(A) β-Arrestin 2 Emax %	(B) DAMGO Fold Shift β-Arrestin 2	(C) DAMGO Fold Shift GTPy35S
BMS 986122		16 (12-22)	56 (51-66)	2 ± 0.3	7 ± 4
Thiazolidine Core Series					
257985		11 (10-13)	71 (68-75)	6 ± 0.6	33 ± 20
258721		> 50	38*	2 ± 0.1	1
258188		7 (7-8)	111 (108-114)	10 ± 1.2	65 ± 40
Piperidine Core Series					
258906		>50	68*	4 ± 0.1	5 ± 1
258845		>50	100*	4 ± 0.7	10 ± 3.8
258703		23 (17-37)	96 (82-124)	7 ± 2	10 ± 2
258757		12 (10-18)	100 (90-116)	7 ± 0.3	6 ± 1
258763		17 (15-20)	76 (70-83)	4 ± 0.3	5 ± 1
258905		17 (15-20)	78 (73-84)	3 ± 0.2	11 ± 5
258758		32*	80*	3 ± 0.2	1

Positive allosteric modulators negatively modulate diprenorphine binding

Initial attempts to characterize the structure-activity relationship for our series of compounds used ^{3}H diprenorphine competition binding assays to determine the modulator's ability to enhance DAMGO affinity. ^{3}H-diprenophine is an orthosteric agonist at the mu opioid receptor. However, these experiments indicated that several of the newer compounds inhibited ^{3}H-diprenorphine binding. To determine whether this interaction was by direct competition for the orthosteric site, or via an allosteric interaction, binding experiments were performed with three compounds: 257986, 257963 and 257985 and contrasted with the orthosteric ligand naloxone (Fig 4.3). At a low (0.3 nM) concentration of ^{3}H-diprenorphine, the compounds decreased binding to levels near or approaching zero, mimicking naloxone. In order to determine if this was due to negative cooperativity, or an orthosteric antagonism, the same experiment was performed with two higher concentrations, 4 nM and 10 nM ^{3}H-diprenorphine. In these experiments, the naloxone competition curve was shifted in a parallel rightward manner. Consistent with orthosteric antagonism calculations of affinity (Ki) values for naloxone remained constant at 1.9 nM (1.5-2.5, 95% CI), 2.1 nM (1.4-3.0, 95% CI) and 2.6 nM (1.8-3.7, 95% CI) for 0.3, 4 and 10nM diprenorphine, respectively. Conversely, when ^{3}H diprenorphine concentrations were increased to 10 nM the allosteric modulators only partially displaced the labeled diprenorphine, reaching a plateau of 58% ± 12 for 257985, 73± 13.4 for 257963 and 59 ± 8.8 for 257986, indicating a negative allosteric modulation (NAM) of the orthosteric antagonist.

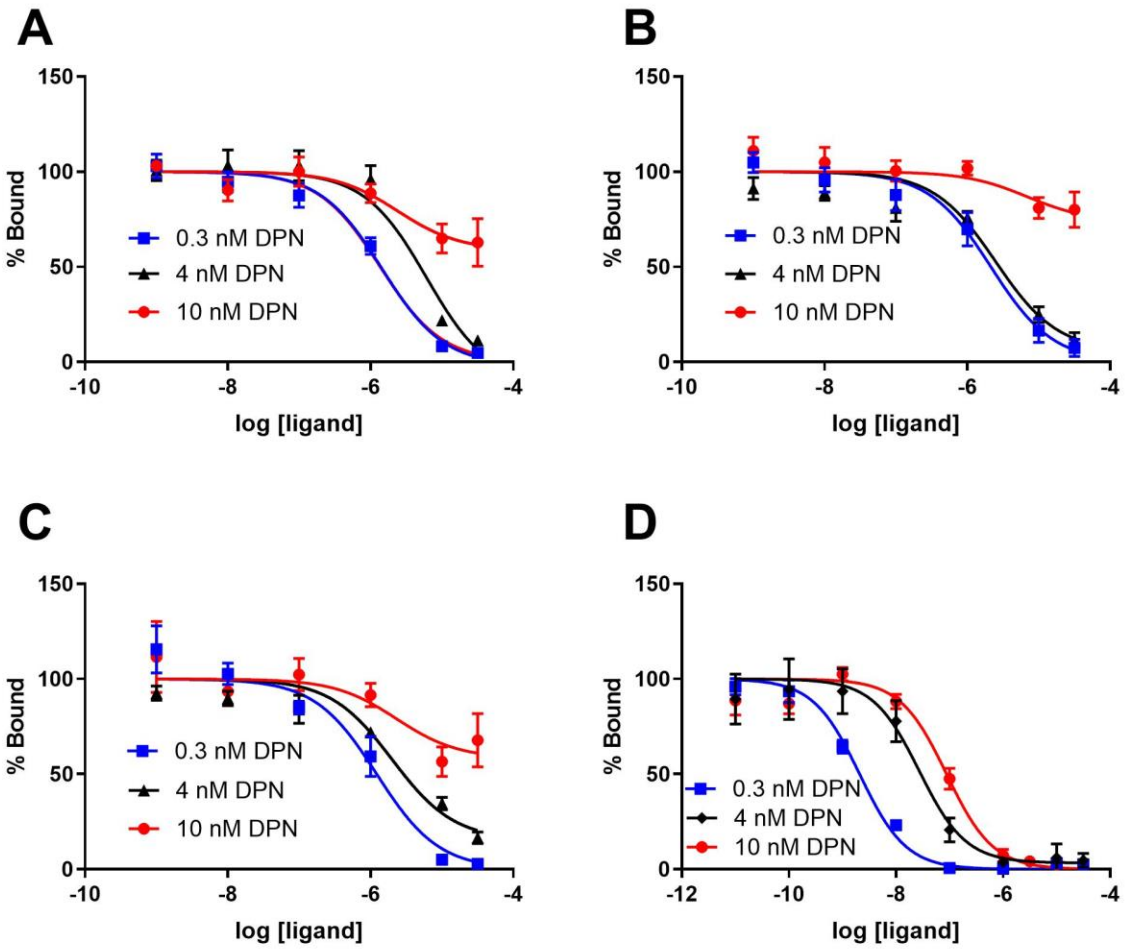


Figure 4.3 Allosteric modulators are negative regulators of diprenorphine binding. Concentration-response curves of CCG 257986 (A), 257963 (B), 257985 (C) and naloxone (D) in competition binding assay with increasing concentrations of radiolabeled diprenorphine. Data presented are normalized to percentage of diprenorphine binding with vehicle. Values shown are the mean ± S.E.M. of five independent experiments each performed in duplicate.

Positive allosteric modulators drive R from allosteric site*

We next determined whether the newer allosteric modulators could elicit GTPγ^{35}S binding in the absence of orthosteric ligand, acting as Ago-PAMs. Based on the data obtained in the binding assay, we hypothesized this action was from the allosteric binding pocket rather than from the orthosteric site. To test this, GTPγ^{35}S binding was performed in the presence of 10 μM 257963, 257985, 257986 or DAMGO and increasing concentrations of the competitive antagonist naloxone (Fig 4.4). The CCG compounds elicited significant GTPγ^{35}S binding in the absence of naloxone: 257963 (48 ± 5%), 257985 (40 ± 10%), 257986 (49 ± 10%) when normalized to 10 μM DAMGO stimulated binding. Consistent with a competitive orthosteric interaction, DAMGO stimulated GTPγ^{35}S binding was reversed upon addition of increasing concentrations of naloxone, however, the allosteric modulator induced GTPγ^{35}S binding showed no significant change upon the addition of increasing concentration of naloxone. when these allosteric compounds were tested for their ability to shift DAMGO concentration-response curves, the allosteric agonism did not correlate with the fold shift observed. While 257985, 257963 and 257986 all showed allosteric agonism, albeit of very low potency, only 257985 showed an appreciable shift (33-fold) in the potency of DAMGO to stimulate GTPγ^{35}S binding.

Bias is conserved in CCG 257868 analogs

Finally, we have previously shown (Chapter 3) that CCG 257868 engenders G protein bias in DAMGO relative to β-arrestin 2 recruitment. In order to determine whether this profile was conserved in newly synthesized analogs, we compared adenylyl cyclase inhibition, GTPγ^{35}S binding and β-arrestin 2 recruitment for DAMGO in

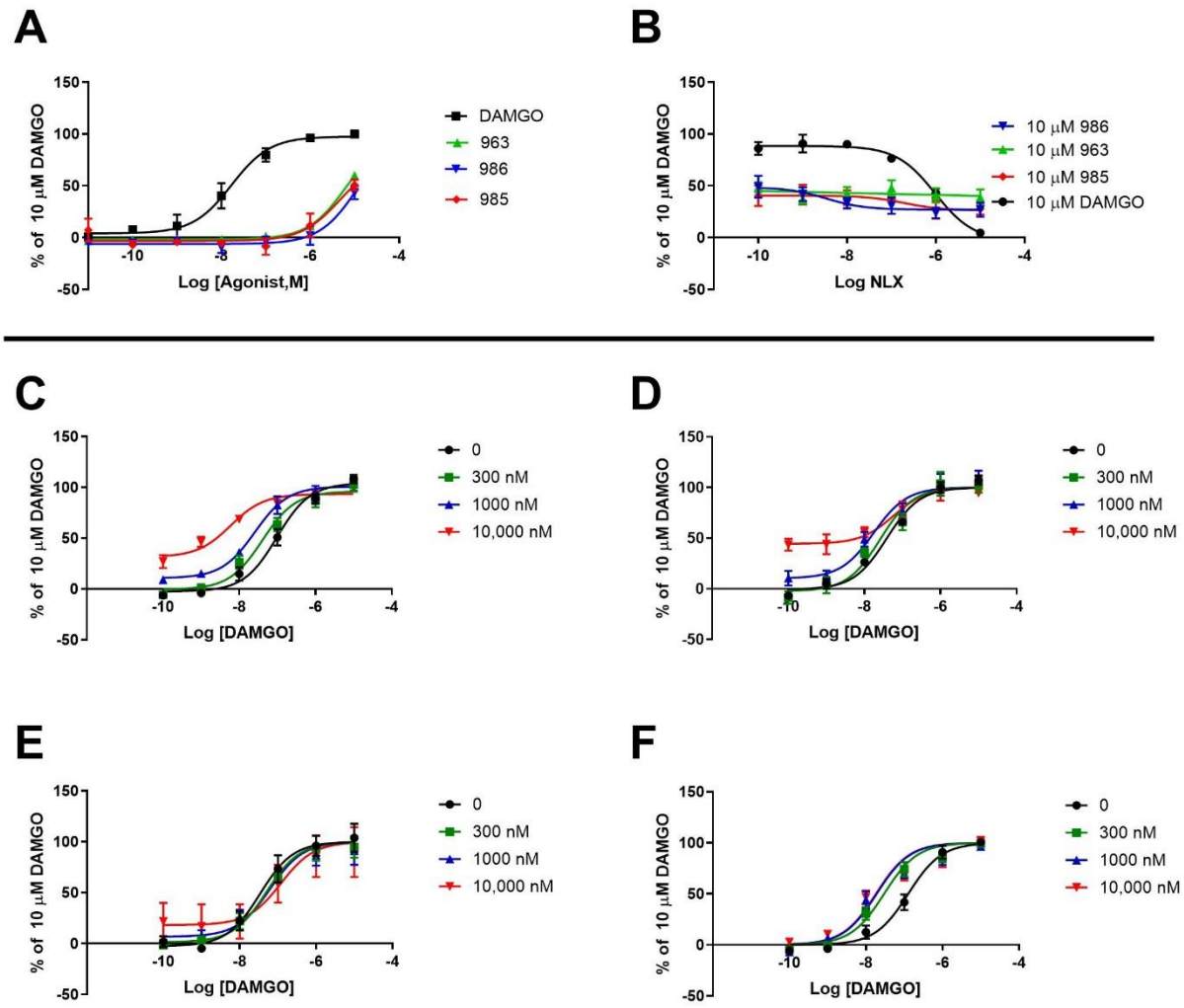


Figure 4.4 Allosteric Agonism is independent of positive modulation. (A) Concentration-response curves of allosteric modulators or DAMGO in GTPγ^{35}S binding assay. (B) GTPγ^{35}S binding concentration response of naloxone in the presence of 10 μM DAMGO or allosteric modulators. (C,D,E,F) GTPγ^{35}S concentration-response curve of DAMGO with or without various concentrations of CCG 257985 (C), 257963 (D), 257986 (E), and 257868 (F). All data presented are the mean ± S.E.M. of 2-5 independent experiments performed in duplicate and are normalized to percentage of 10 μM DAMGO

the absence or presence of 10 µM CCG 258188, selected due to its high efficacy in all tested endpoints (Fig 4.5). Consistent with previous results, CCG 258188 elicited a 163-fold shift in DAMGO concentration-response curve for adenylyl cyclase inhibition, with EC_{50} values of 1 nM (0.7-1.3) in absence of and 0.006 nM (0.003-0.014) in the presence of 10 µM 258188 co-treatment. 258188 also elicited a 30-fold shift in GTPγ^{35}S binding, however, a significantly smaller 12-fold shift was observed for DAMGO in β-arrestin 2 recruitment with EC_{50} values of 112 (100-121) and 9 nM (8-10) in the presence and absence of 10 µM 258188, respectively.

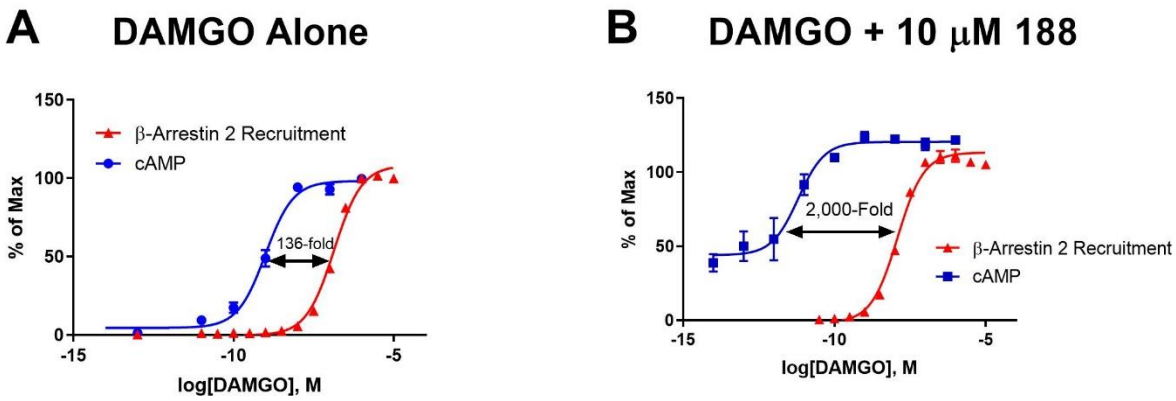


Figure 4.5 G protein bias in conserved in BMS 986122 analogs. Comparison of β-arrestin 2 recruitment and cAMP inhibition potencies for DAMGO in the absence (A) or presence (B) of 10 CCG 257188. Data are represented as the mean ± SEM of five independent experiments performed in quadruplicate and normalized to percentage of 10 µM DAMGO

Discussion

The goal of this work was to identify the structure-activity relationships around the core BMS 986122 structure and provide improved analogs for future drug development. The key lead compounds that were identified are depicted in Fig 4.6, relative to CCG 257868 (BMS 986122). This study defined a set of SARs for allosteric modulators of the mu opioid receptor. Using a stepwise approach, a significant improvement was made in PAM efficacy as determined by GTPγ^{35}S binding and β-arrestin 2 recruitment. A clear result from the SAR conducted was the favorability of halogen substitutions on the 4-position left ring and at the 2 and 3 positions on the right ring. In addition, we highlight the complexity of allosteric regulation of the mu opioid receptors as evidenced by the divergence in allosteric agonism and positive modulation. Ultimately, this chapter provides a foundation for future drug development and elucidation of the allosteric site on the mu opioid receptor

The SAR of the right-hand phenyl substitutions revealed that fluorine substitution at the 2-position was more favorable than chlorine whereas the specific halogen at the 3-position seemed less impactful as long as a halogen was present. Additionally, the introduction of a halogen at any other position led to a significant loss of activity. This suggests that these groups have a specific directional interaction with the receptor and are of high importance for activity. A similar effect was observed for the left-hand ring SAR where a 4' halogen was significantly more favorable than any other position. Future studies will focus on the introduction of bulk at these positions under the assumption that this would help to fill the large allosteric vestibule in the proposed allosteric site highlighted in previous work (Bartuzi, Kaczor and Matosiuk, 2015; Shang *et al.*, 2016).

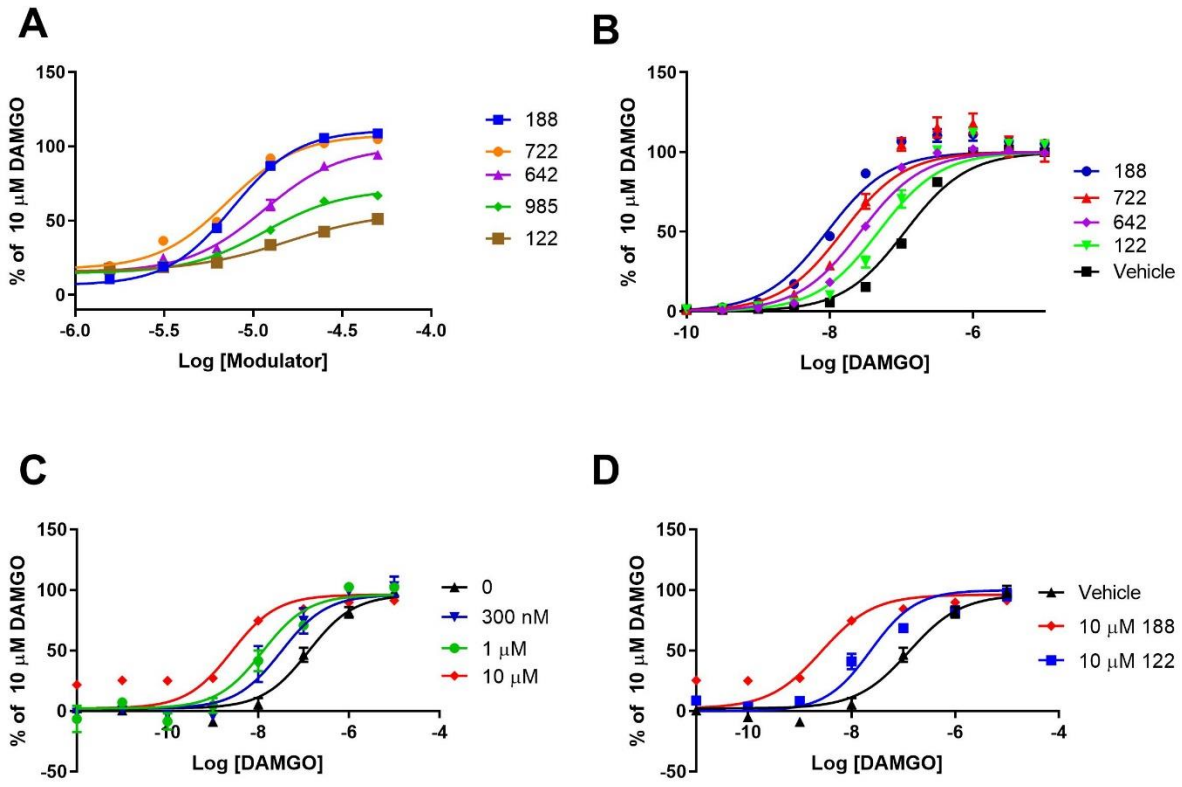


Figure 4.6 SAR campaign key lead compounds. (A) Allosteric modulator concentration-response curve with 30 nM DAMGO for β-arrestin 2 recruitment. (B). DAMGO concentration-response curve for B arrestin recruitment with 10 μM indicated allosteric modulator or vehicle. (C) GTPγ^{35}S binding assay concentration-response curve with increasing concentrations of CCG 257188. (D) GTPγ^{35}S binding concentration-response curve with either 10 μM CCG 257868 (parent compound) or CCG 257188 (lead). All data presented are the mean ± S.E.M of 3-5 experiments performed in duplicate (C, D) or quadruplicate (A, B) normalized to percentage of 10 μM DAMGO.

The thiazolidines represent the most promising core structure based on consideration of potency and efficacy. An interesting observation is that the 7-membered core structure (265127) had the best potency in β-arrestin 2 recruitment, however, this came at a significant reduction in maximum effect to 32%. This suggests that allosteric

modulator affinity and efficacy are not inextricably linked. In contrast, the oxazolidine scaffold (258189), reached a maximum of 100% but was significantly less potent than all compounds tested at 50 μM. Together, these findings demonstrate that the molecular determinants of allosteric modulator affinity and efficacy are distinct. This is supported by the discovery of BMS 986122 where the compound elicited a 7-fold shift in DAMGO stimulated GTPγ^{35}S binding, whereas a different scaffold (BMS 986121) led to a 4-fold shift. However, BMS 986121 was about 4-fold more potent than BMS 986122 (Burford *et al.*, 2013). This presents an important consideration for future development efforts which seek to optimize both efficacy and affinity. The data presented suggest the thiazolidine scaffold is the best combination of affinity and efficacy and future efforts should focus on outer ring substitutions.

Another discovery from these studies was the negative allosteric cooperativity with the orthosteric antagonist diprenorphine. At low concentrations of diprenorphine, CCG 257985, 257963, and 257986 appeared to act as competitive antagonists of this orthosteric antagonist. However, competition binding studies with higher concentrations of ^{3}H-diprenorphine revealed this inhibition was saturable and did not show parallel rightward shifts. If the mechanism was competitive, the competition curves would simply shift parallel to the right, as shown in the naloxone control condition. The ability of these allosteric modulators to negatively regulate ^{3}H-diprenorphine binding is supported by evidence at the delta opioid receptor. We have previously shown that BMS 986187, a structurally distinct Ago-PAM, interacts with antagonists in a negatively cooperative manner from the allosteric site (Chapter 2; Stanczyk *et al.*, 2019). This is further supported by the fact that many of the novel scaffolds are Ago-PAM's at the mu opioid receptor, as

evidenced by the inability of naloxone to reverse GTPγ^{35}S binding elicited by select allosteric modulators at 10 μM. It is likely that these compounds are engendering an active state of the receptor for which ^{3}H-diprenorphine affinity is decreased.

Another interesting divergence is the emergence of allosteric agonism that is not indicative of positive cooperativity with orthosteric ligands. One hypothesis for allosteric modulation of the mu opioid receptor is through the promotion of active state receptor, for which agonists have a higher affinity. However, CCG 257986 and 257963 promote an active state of the receptor but did not exhibit significant cooperativity with DAMGO. This likely highlights the diversity of active states/ensembles. It is possible that the active state promoted by certain allosteric modulators is not more favorable to DAMGO binding to the inactive one. Perhaps certain allosteric modulators trap the receptor in an intermediate state, preventing DAMGO from occupying the preferred fully active receptor conformation. BMS 986122 has been shown to exhibit significant probe dependence, therefore, it is possible that CCG 257986 and 257963 show positive cooperativity with other orthosteric ligands and future experiments can examine this possibility. An understanding of probe dependence with newly synthesized modulators may provide insight into the potential binding site and mechanism of action. It has been previously shown that BMS 986122 shows the greatest cooperativity with ligands that are sensitive to the presence of sodium (Livingston and Traynor, 2015). Confirmation of these findings in BMS 986122 analogs will provide further evidence of the interplay between the conserved sodium site and the allosteric action of BMS 986122 and related analogs.

Finally, we have previously shown that BMS 986122 engenders G protein bias relative to β-arrestin 2 recruitment. This hypothesis was further supported as many of the

tested modulators elicited significantly greater shifts in DAMGO stimulated GTPγ^{35}S binding relative to β-arrestin 2 recruitment. There were some modulators, however, that showed a more neutral or perhaps β-arrestin 2 biased profile. Future efforts will look to investigate this more rigorously and determine the structural determinants for such a bias.

In conclusion, this study provided a clear SAR framework for future allosteric modulator development at the mu opioid receptor. In addition, valuable insight has been gained into the complexities of allosteric regulation at the mu opioid receptors. Specifically, the separation of positive allosteric modulation and allosteric agonism, a phenomenon which warrants future investigation. This phenomenon has been observed at the muscarinic M4 receptor, where SAR studies on LY2033298 reveal allosteric agonists that show no positive cooperativity with acetylcholine (Szabo *et al.*, 2015). Accumulating evidence points to allosteric regulation as being multidimensional. That is, compounds are not simply positive or negative modulators, as this can vary in magnitude and directionality depending on the readout employed and orthosteric probe as well, even within the same scaffold. Future efforts will explore these complexities in more detail with a goal of enhancing the impact of allostery on mu opioid receptor physiology and developing novel therapeutics for the treatment of pain.

Methods

CCG Compounds

All allosteric modulators tested were synthesized (purity of >95% as determined by HPLC) by the Vahlteich Medicinal Chemistry Core (Dr. Andrew White, Dr. Kun Liu, Sherrice Zhang, Dr. Xinmin Gan)at the University of Michigan.

Cell Lines

CHO (ATCC Cat# CCL-61, RRID:CVCL_0214) cells stably expressing wild-type human-DOPr (CHO-hDOPr) were grown in DMEM containing 10% FBS and 1% penicillin and streptomycin and maintained in 0.8 mg/ml G418 as previously described (Burford et al., 2015). CHO OPRM1 Pathhunter cells from DiscoverX were maintained in 0.8 mg/ml G418 and 0.3 mg/ml Hygromycin B per manufacturer instructions.

Membrane Homogenate Preparations

Cells were harvested and membrane homogenates prepared as previously described (Clark *et al.*, 2003). Briefly, cells were washed with ice-cold phosphate-buffered saline, pH 7.4 and detached from plates by incubation in harvesting buffer (0.68 mM EDTA, 150 mM NaCl, and 20 mM HEPES at pH 7.4) and pelleted by centrifugation at 200g for 3 minutes. Cells were resuspended in ice-cold 50mM Tris (pH 7.4), homogenized using a Tissue Tearor (Dremel; Mount Prospect, IL, USA), and centrifuged at 20,000g at 4^{o}C for 20 min. The pellet was then resuspended, homogenized, and centrifuged a second time. This final pellet was resuspended in ice-cold 50 mM Tris (pH 7.4) and homogenized using a glass dounce to give a protein concentration of 0.5-1.5 mg/mL and stored at -80°C. Protein concentration was determined using the bicinchoninic acid quantification method (BCA) with BSA serving as the standard.

Stimulation of GTPγ^{35}S Binding

Agonist stimulation of GTPγ^{35}S binding was measured as described previously (Clark *et al.*, 2003). Homogenates of CHO cells expressing wild-type hDOPr (15-20 µg/well) were incubated in “GTPγS buffer” (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) containing 0.1 nM GTPγ^{35}S, 30 µM guanosine diphosphate (GDP) and varying

concentrations of orthosteric agonist with BMS 986122, BMS 986187 or Vehicle for 1h in a shaking water bath at 25°C. The reaction was terminated by vacuum filtration through GF/C filters using a Brandel harvester and washed five times with ice-cold GTPγS buffer. Filters were dried, and following the addition of EcoLume scintillation cocktail, counted in a Wallac 1450 MicroBeta Liquid Scintillation and Luminescence Counter (Perkin Elmer). The level of GTPγ^{35}S binding is expressed as percentage of the full MOPr agonist, DAMGO, at 10 μM to account for variability between membrane preparations.

^{3}H-Diprenorphine Binding Assay

Assays were performed using CHO-hDOPr membranes by the method as described (Livingston et al., 2018). Displacement of 0.3, 4 and 10 nM ^{3}H-Diprenorphine was measured using increasing concentration of naloxone, CCG 257985, CCG 257986 or CCG 257963.

β-arrestin 2 Recruitment

β-arrestin 2 recruitment was determined using the commercially available Pathhunter assay by DiscoverX. CHO-βArrestin-hMOR cells were plated at a density of 5,000 cells per well in 384-well white polystyrene cell culture plates (Grenier) using Assay Complete Cell Plating Reagent (DiscoverX) 24 hours prior to performing the assay. The following day cells were treated with indicated drug conditions for 60 minutes at 37^{0} Celsius. Following drug incubations, cells were treated with β-galactosidase substrate provided in Pathhunter Detection Kit (DiscoverX), incubated for 60 minutes at room temperature and luminescence was detected using Envision Plate Reader (Perkin Elmer).

Inhibition of forskolin-stimulated cAMP assay

Inhibition of forskolin-stimulated cAMP was evaluated in CHO-Mu Pathhunter cells using the LANCE Ultra Kit per manufacturer's instructions (PerkinElmer). Briefly, cells were plated at a density of 500 cells per well (5 µl per well) in white-walled OptiPlate 384-well plate (PerkinElmer). Following plating, drug dilution containing 6 µM forskolin (Sigma) in manufacturer provided stimulation buffer was added at a volume of 5 µL and incubated at room temperature for 30 minutes. Following incubation, the reaction was terminated by the addition of 5 µL Eu-cAMP tracer solution and ULight-anti-cAMP solution, respectively, and sealed at room temperature for 1 hour. After 1 hour, FRET was detected using an Envision plate reader (PerkinElmer)

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Chapter 5 : Discussion and Future Directions

Summary and Significance

The studies contained in this thesis investigated allosteric regulation of opioid receptors, both from a SAR standpoint and an investigation into downstream signaling consequences. These data are the first investigations looking at the intersection of allostery and biased signaling at opioid receptors and the structure activity relationships that govern function. Ultimately, the work conducted expands the pharmacological toolkit for opioid drug discovery while adding knowledge to our expanding view of ligand-receptor interactions and their downstream cellular consequences.

Chapter 2

I have shown that BMS 986187 is an Ago-PAM at the delta opioid receptor, activating the receptor in the absence of any orthosteric ligand. Acting from an allosteric site, BMS 986187 produces G protein bias relative to β arrestin recruitment. This was supported by multiple lines of evidence measuring receptor internalization, ERK 1/2 phosphorylation, and receptor phosphorylation at the C terminal tail. Ultimately, BMS 986187 promotes G protein-biased signaling stemming from low receptor phosphorylation. From the perspective of the delta opioid receptor, this work is the first evidence of biased allosteric agonism. However, there are multiple studies examining the role of differential signaling at the delta opioid receptor. Development of delta opioid receptor ligands is limited by the on-target side effects these compounds display. Most

notably, animals given certain delta opioid ligands display convulsions and tolerance to the analgesic effects develop rapidly (Jutkiewicz *et al.*, 2005; Danielsson *et al.*, 2006; Pradhan *et al.*, 2012; Lutz, Pierre-Eric and Brigitte, 2014). Therefore, efforts have focused on designing biased ligands to determine whether the beneficial effects stem from separate effectors than the negative side effects. Identifying BMS 986187 as a G protein biased delta opioid receptor ligand serves as a research tool for testing assumptions regarding signaling and as a guide for the mechanistic underpinnings of biased signaling. Beyond the context of the delta opioid receptor, this study provides important considerations as allosteric ligands and bias are becoming more prevalent drug targets at all GPCR's. Indeed, it has been shown for other class A GPCRS that allosteric agonism can be biased, although it is unclear what role this would play in a physiological setting where receptor activation would be a mix of both direct allosteric agonism and receptors co-occupied by allosteric and endogenous ligands (Khajehali *et al.*, 2015; Sengmany *et al.*, 2017). Nonetheless, it is clear that allosteric modulators are a powerful option for promoting unique receptor pharmacology at GPCR's, expanding the drug discovery toolkit for the largest class of drug targets.

Chapter 3

Studies examining the bias promoted by BMS 986187 and BMS 986122 at the mu opioid receptor revealed a divergence between these two unique chemical scaffolds. Despite acting via a putative conserved site (Kathryn E. Livingston *et al.*, 2018), BMS 986187 engendered β arrestin bias when paired with orthosteric ligands whereas BMS 986122 engendered G protein bias. In addition, I was able to show that BMS 986187 arrestin bias is enabled by the ability to enhance β arrestin recruitment even in the

absence of functional Gi/o and/or GRK 2/3 inhibition. This work also highlighted the ability of receptors to recruit β arrestin without involvement of the canonical G protein signaling cascade. A caveat is that this is an overexpressed receptor system, so the question of whether appreciable β arrestin recruitment can happen completely independent of G protein signaling in native tissues remains unknown. However, this line of experimentation provides strong support for the ability of allosteric ligands to promote biased signaling between these two signaling pathways with the enabling mechanism being the non-overlapping factors that govern their recruitment to the receptor.

This work provides valuable considerations on two fronts. First, given the breadth of literature correlating β arrestin recruitment with untoward side effects, the rational candidate for development is BMS 986122 from a drug development standpoint This may lead to a reduction in side effects as has been ascribed to G protein biased profiles. Second, from an opioid receptor physiology standpoint, these data highlight the complex and diverse signaling profiles that can be generated downstream of the mu opioid receptor. This growing understanding is transforming traditional drug discovery efforts at opioid receptors and many other GPCR's. At the time of this writing, the mechanistic basis for bias on a molecular level remains unknown. However, it is clear that activation with different orthosteric, allosteric, or orthosteric-allosteric ligand complexes leads to unique, multi-tonal signaling profiles.

Chapter 4

Finally, I was able to provide SAR information for the activity of BMS 986122 at the mu opioid receptor. This work highlights the impact of major core changes to the parent scaffold as well as halogen substitution on the peripheral benzene rings. We were able

to develop improved compounds relative to BMS 986122 in collaboration with the Vahlteich Medicinal Chemistry Core (University of Michigan). In addition, this work was able to show that allosteric agonism and positive allosteric modulation are not necessarily linked. Hypothetically, it would make sense if the most cooperative ligands show allosteric agonism as they shift the receptor equilibrium more closely to the active conformation, a state the orthosteric agonist presumably prefers. However, this was not necessarily the case with the mu allosteric agonists developed here. One explanation is that the allosteric modulators promote an intermediate receptor conformation but prevent transition to the fully active form preferred by DAMGO. This is supported by a recent study suggesting diverse receptor conformations by a series of mu opioid orthosteric agonists (Kathryn E Livingston *et al.*, 2018). Future investigations should explore the mechanistic separation between allosteric agonism and positive cooperativity with orthosteric ligands. This could be achieved by investigating the molecular determinants that are shared and separate for allosteric agonism and positive cooperativity with orthosteric ligands. In addition, molecular dynamics simulations could provide more robust hypothesis for this divergence on a molecular level.

In line with the theme of this thesis, these studies further support the true complexity of receptor states and activation mechanisms. Ultimately, I was able to identify multiple lead compounds that show significant improvement compared to the parent scaffold BMS 986122 and retain G protein bias, a strong foundation for future drug development efforts.

Future directions

The work presented provides significantly more insight into the signaling consequences of allostery at opioid receptors. In addition, I provide a foundational SAR framework for future scaffold development and binding site inference. However, as is always the case in science, the work presented raises as many questions as answered.

Determine binding site

The binding site(s) for the mu PAMS remains unknown. However, work done to date provides significant evidence for potential binding site. Firstly, we were able to show that both BMS 986122 and BMS 986187 interact with the mu, delta, and kappa opioid receptor, but not the NOP receptor (Kathryn E. Livingston *et al.*, 2018). The NOP receptor is the most structurally dissimilar of the four, and structural analysis will likely reveal multiple potential binding sites that are conserved across mu, delta and kappa, but absent in NOP. Additionally, there have been two papers proposing potential binding sites of the allosteric modulators BMS 986187 and BMS 986122 at delta and mu opioid receptors, respectively based on in silico studies (Fig 5.1). Of note, both studies highlight a similar proposed region near the extracellular loops of the receptor which engages transmembrane domains one, two and seven (Bartuzi, Kaczor and Matosiuk, 2016; Shang *et al.*, 2016). The proposed binding site is in a similar position to reported sites in other GPCRs. However there are subtle differences, thus the allosteric site appears more extracellular compared to maraviroc at CCR5, but located more closely to the orthosteric pocket than LY2119620 at the muscarinic (M2) receptor. (Kruse *et al.*, 2013; Tan *et al.*, 2013).

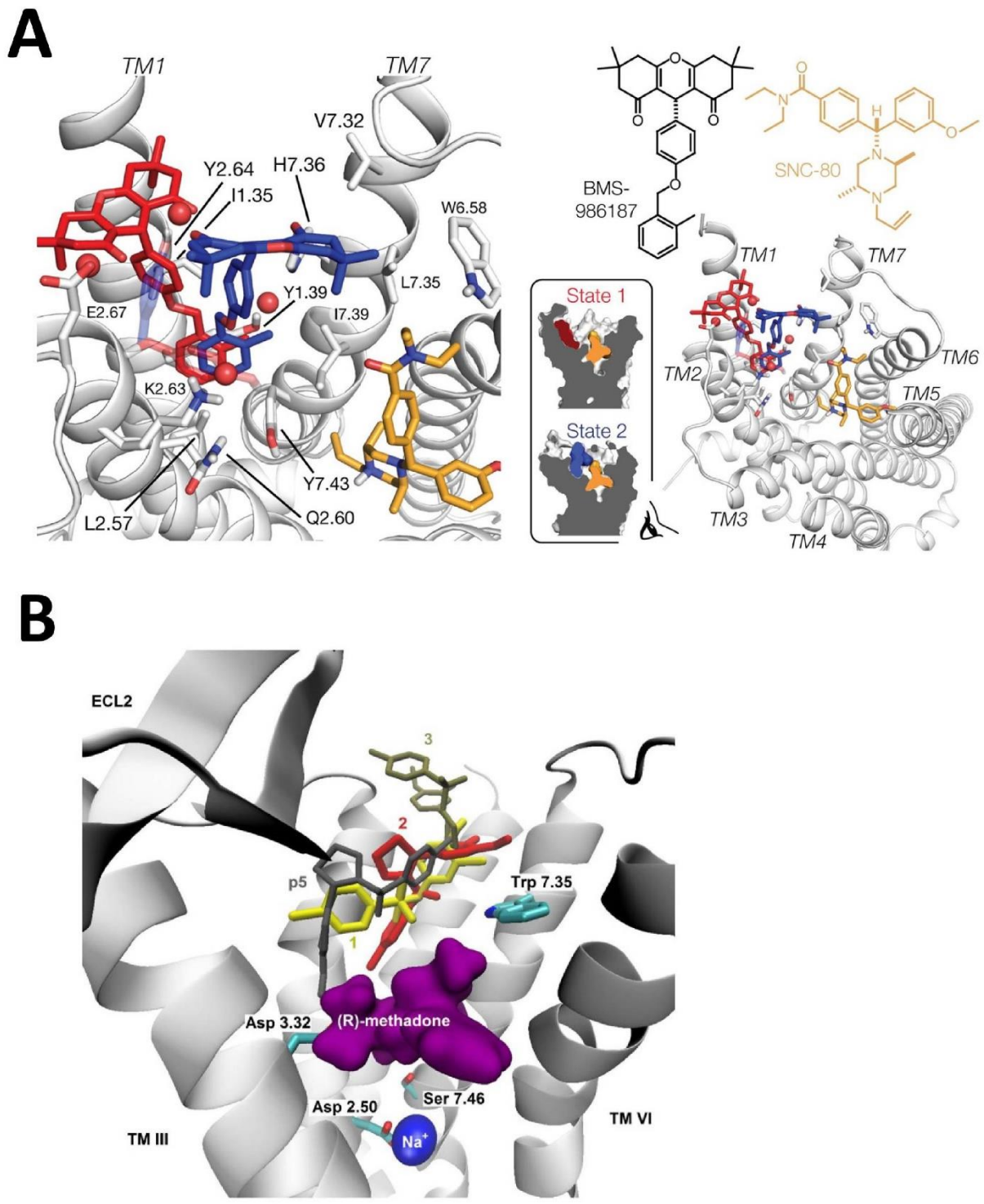

Figure 5.1 Proposed binding sites of BMS 986187 and BMS 986122. (A) Proposed binding site of BMS 986187 in conjunction with SNC 80. Reprinted with permission from ACS Publications (Shang *et al.*, 2016). (B) Proposed binding site of BMS 986122 in conjunction with methadone. Reprinted with permission from ACS Publications (Bartuzi, Kaczor and Matosiuk, 2016)

Taken together with the evidence for a shared/overlapping binding site presented in Livingston et al., 2018, this provides strong indication that indeed this region is at least one site of PAM binding. Finally, the SAR data provided in this chapter should add further insight when paired with the studies outlined above. In particular, previous work has identified Trp 7.35 as a key residue for interaction with the right-hand ring of BMS 986122 (Bartuzi, Kaczor and Matosiuk, 2016). Importantly, the proposed orientation would suggest that the favorable 2 and 3-position halogen substitutions outlined above would engage hydrogen bonding with 7.35.

Future studies to determine the binding site would employ multiple complementary techniques. The most definitive approach, albeit the most difficult, would be to solve a crystal structure of the PAMs in complex with the receptor, using either X-ray analysis (Huang *et al.*, 2015) or cryo-EM (Koehl *et al.*, 2018). The PAMs exhibit low affinity (μM) in the absence of orthosteric ligands. Therefore, it would be most advantageous to attempt crystallography in the presence of an orthosteric ligand which increases PAM affinity (Kathryn E Livingston *et al.*, 2018). Methadone exhibits the highest cooperativity with all PAMs tested to date and is the most logical choice, although its flexibility is not ideal. The allosteric ligand most likely to yield a crystal structure is either CCG 257188 at mu or BMS 986187 at the delta opioid receptor. CCG 257188 has the highest cooperativity of any PAM at mu, while BMS 986187 shows the highest cooperativity at delta relative to any PAM at any opioid receptor.

Another potential approach is the combination of molecular dynamics simulations followed by SAR influenced modeling/docking and mutagenesis. Using molecular dynamics simulations could help identify potential binding sites that were previously

inaccessible to docking methods based on available crystal structures. Then, using the knowledge that BMS 986122 and BMS 986187 occupy a similar site should allow for a narrower sample set of potential binding sites. In addition, the SAR provided in Chapter 4 could further aid in rational modeling/docking as the compounds that test best experimentally should generally show the highest docking scores at the correct binding site. However, this approach is limited by the possibility that the available active-state structures may not be in a conformation with an exposed allosteric site.

Furthermore, future studies should examine the SAR of the CCG analogs tested herein at the delta, kappa and nociceptin receptors. It is possible that certain structural changes engender more activity at one receptor over another. By comparing the structural differences at a proposed binding site, the SAR differences at the four receptors would add further confidence to the correctly identified site. These approached would then be best confirmed with a site-directed mutagenesis approach looking for loss of PAM activity.

Determine mechanism of bias

One of the largest questions for modern GPCR pharmacology is what determines the mechanistic basis of bias. While this thesis, like many other accumulating works, highlights biased signaling and the molecular chaperones involved, it stops short of determining a fundamental mechanism on a receptor-orthosteric ligand-allosteric ligand conformation scale. One of the main reasons bias is nebulous is because receptors and signaling events are not static. In fact, it has been shown that receptor bias can change depending on what time point the functional outputs of interest are measured (Klein Herenbrink *et al.*, 2016). From a scientific perspective, it becomes difficult to control for all variables and determine a final cause. Almost certainly, not all bias is the same. There

are likely many mechanisms by which receptor signaling can be biased and, each example is a variable combination of these mechanisms. The two most obvious hypothetical mechanisms are kinetics and conformation, which are the rate at which the receptor-ligand or receptor-allosteric ligand-ligand complexes form/dissociate and the actual ensemble of receptor states that is promoted by said complex, respectively. It has already been shown that ligand residence time can play an important role on receptor bias (Klein Herenbrink *et al.*, 2016). It is unclear exactly why residence time impacts bias, but it is likely due to the receptor engaging in a specific conformation for an extended period of time.

In line with this, many studies have examined how small changes in receptor conformations change signaling cascades using a combination of BRET based conformation studies, mutagenesis and crystallography. Whether it be G protein subtype selectivity (Lorenzen *et al.*, 2018; Touhara and MacKinnon, 2018), or differential β arrestin engagement (Eichel *et al.*, 2018; Latorraca *et al.*, 2018); receptor conformation plays an enormous role in determining downstream signaling.

Regarding the mechanism of bias at opioid receptors, in particular with allosteric ligands, the most immediate direction would be to analyze the kinetics of receptor signaling for the two different entities (BMS 986187 and BMS 986122) outlined in this thesis. The most straightforward approach would be to examine is changes in K_{on} and K_{off} of orthosteric ligands paired with BMS 986187 and BMS 986122. Both compounds change orthosteric ligand affinity, therefore either K_{on}, K_{off}, or a combination of both are changing, as these are the determining factors for ligand affinity. It is possible that one allosteric ligand promotes a faster on rate while another leads to a slowed off rate. To my

knowledge, no studies have examined the impact of ligand binding kinetics on bias at opioid receptors. It is likely given their varied affinities that the kinetics of allosteric ligand binding are different as well. Regardless, this is presently more difficult to ascertain as it requires a radiolabeled allosteric ligand, which is not currently available, and these experiments would be problematic based on the low affinities of the PAMs even in the presence of orthosteric ligand. Another approach to understanding the influence of allostery on ligand bias would be molecular dynamic simulations. However, to date, these methodologies have proven limited in determining the mechanism of bias with known orthosteric ligands, adding further complexity with an allosteric co-ligand is a significant limitation.

Further explore allosteric bias at delta and kappa opioid receptors

One surprising finding of this thesis work was that BMS 986187 alone produced a G protein bias at the delta opioid receptor while engendering arrestin bias to orthosteric ligands at the mu opioid receptor. Future work should examine whether the G protein bias of BMS 986187 at delta opioid receptor changes in the presence of an orthosteric ligand. In addition, BMS 986187 produces robust positive modulation at the kappa opioid receptor, another unexplored vista for allosteric opioid bias. It has been suggested that G protein biased ligands at the kappa receptor show a reduction in kappa-opioid-mediated dysphoria, while retaining analgesic efficacy (Bohn and Aubé, 2017). Using allosteric modulators to engender bias at other opioid receptors is a potential way to test current hypotheses regarding favorable bias profiles.

Determine in-vivo consequences of bias through allostery

One of the major challenges with biased signaling is translating and validating signaling difference at an *in vivo* level. The recent tour de force by Schmid et al. 2017 outlines the most rigorous methodology to correlate *in vitro* bias with *in vivo* pharmacology. Translating ligand bias in animal models requires control for pharmacokinetic differences. To date, there have been many G protein biased ligands to test *in vivo*, but far fewer β-arrestin 2 biased ligands. Therefore, BMS 986122 and BMS 986187 are perfect tools for interrogating *in vivo* bias. Theoretically, BMS 986122 should expand the therapeutic window of mu-orthosteric ligands, whereas BMS 986187 should reduce it. Moreover, SR 17018 has already been validated *in vivo*. BMS 986187 removes the bias exhibited by this compound *in vitro* and further work should explore whether this manifests *in vivo* in the form of a reduced therapeutic window.

Overall Conclusions

Drugs targeting opioid receptors have the potential to treat a multitude of human diseases. Beyond widespread use in pain management, drugs targeting the different opioid receptors have the potential to be used as antidepressant and addiction treatment medications. However, drug development efforts are hindered by the multitude of serious side effects stemming from opioid receptor activation. Future therapeutics will need to employ novel strategies, such as biased signaling and allosteric modulation, to circumvent these issues This work highlights the intersection of the two strategies at opioid receptors. Thus, in addition to promoting biased signaling alone, allosteric modulators can engender bias onto orthosteric ligands. Building off a growing body of evidence, we were able to select an allosteric modulator scaffold that engenders G protein bias at the mu opioid receptor and significantly enhance its activity through a series of

structural modifications. This work specifically advanced development of a potentially safer pain therapeutic. On a broader scale, it uncovered multiple phenomenon new to opioid receptors. Future work investigating these phenomena will enhance our understanding of opioid receptor function and, more generally, G protein coupled receptors.

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