Characterization of Positive Allosteric Modulators of the Mu Opioid Receptor

by

Kelsey E. Kochan

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Doctoral Committee:

Professor John Traynor, Chair
Assistant Professor William Birdsong
Associate Professor Emily Jutkiewicz
Assistant Professor Courtney Townsel

Kelsey Kochan

kkochan@umich.edu

ORCID iD: 0000-0002-6340-3822

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Dedication

To Myself,

For persevering through all obstacles and living life authentically

To my nieces, Audree, Kayleigh, Alayna, and Amelia,

For inspiring me to set an example of what women can do despite societal expectations

To Brett,

For the endless love and support that kept me going

To Zelda,

Woof woof woof bark bark bark

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I couldn't have done this without any of you.

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List of Abbreviations, Acronyms, and Symbols

β-FNA	beta-funaltrexamine
ANOVA	analysis of variance
bpm	beats per minute
BR	breath rate
brpm	breaths per minute
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary cells
CI	confidence interval
CPP	conditioned place preference
Cryo-EM	cryogenic electron microscopy
DAMGO	[D-Ala_2, N-$MePhe_4$, Gly-ol]-enkephalin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DOR	delta opioid receptor
EC_{50}	50% effective concentration
EDTA	Ethylenediaminetetraacetic acid
FBS	fetal bovine serum
G protein	guanine nucleotide binding protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine-5'-triphosphate
GTPγS	guanosine-5'-O-(3-[^{35}S]thio)triphosphate
HEK293	human embryonic kidney 293 cells
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HR	heart rate
KOR	kappa opioid receptor
M2R	muscarinic acetylcholine receptor 2
MDS	molecular dynamics simulations
Met-Enk	methionine-enkephalin
mg/kg	milligram/kilogram
MOR	mu opioid receptor
NAM	negative allosteric modulator
NLX	naloxone
NMDAR	N-methyl-D-aspartate receptor
NMR	nuclear magnetic resonance spectroscopy
NOPr	nociceptin receptor

NSAID	non-steroidal anti-inflammatory drug
OUD	opioid use disorder
PAM	positive allosteric modulator
SAM	silent allosteric modulator
SAR	structure-activity relationship
SEM	standard error of the mean
SpH-MOR	superecliptic phluorin-mu opioid receptor
TM	transmembrane
WWTW	warm water tail withdrawal

Abstract

Activation of the mu-opioid receptor (MOR) is responsible for the beneficial analgesic actions of opioid drugs as well as their unwanted actions, including constipation, respiratory depression, and misuse liability. Opioid analgesics that act at MORs are the traditional standard for the clinical management of pain but come with significant risks for adverse events. Thus, an alternative approach for managing pain with improved safety remains an unmet need. One approach utilizes positive allosteric modulators (PAMs) of MOR that interact with a separate location on the receptor from endogenous and traditional (orthosteric) opioid drugs. Previous *in vitro* studies presented that PAMs enhance the potency and/or efficacy of orthosteric opioids. Additionally, PAMs have been shown to enhance endogenous opioid peptide-mediated antinociception with reduced development of side effects than traditional opioids *in vivo*.

One alternative approach suggests MOR agonists that preferentially signal to certain intracellular pathways (specifically a bias towards G-protein over β-arrestin), might show an improved therapeutic index. Additionally, literature suggests that allosteric modulators can influence G-protein-coupled receptor (GPCR) ligand signaling profiles. However, studies have yet to examine this at MOR. In Chapter 2, I compare the ability of orthosteric MOR agonists to activate two signaling pathways; G-protein activation and β-arrestin recruitment in the absence or presence of two structurally distinct MOR PAMs,

BMS-986187 or BMS-986122. Orthosteric agonists included in this study have been previously reported as neutral (morphine, methadone, and Met-Enkephalin), G-protein biased (SR17018), or potentially β-arrestin 2 biased (fentanyl). Both BMS-986187 and BMS-986122 shifted the G protein activation concentration-response curve to the left similarly across the MOR-agonists. In contrast, BMS-986187 enhanced the potency for β-arrestin recruitment ranging from 10-178 fold, whereas BMS-986122 elicited much smaller shifts with all orthosteric agonists (1-7 fold). In the absence of PAM, we report the signaling bias for DAMGO and methadone as neutral, morphine, Met-Enkephalin, and SR17018 as G protein, and fentanyl as β-arrestin 2 biased. BMS-986187 significantly enhanced the β-arrestin 2 bias for fentanyl as well as the G protein bias for SR17018. BMS-986122 reduced the β-arrestin 2 bias for fentanyl and increased the G protein bias for methadone. In addition, both PAMs increased receptor internalization induced by DAMGO, morphine, and methadone, but not fentanyl. Overall, these studies provide evidence that PAMs can differentially influence the degree and direction of downstream signaling of MOR depending on the orthosteric agonist present.

While previous studies determined the ability of PAMs to enhance endogenous opioid effects, we do not yet know the pharmacology of PAM function in the presence of exogenous opioids, for example morphine, *in vivo*. Furthermore, it is unknown if PAMs enhance all of the effects induced by exogenous opioids, such as antinociception, constipation, respiratory depression, and reward. If PAMs only enhanced the desired opioid-induced effects, they would be clinically applicable for opioid sparing, that is combining with opioid drugs to reduce the doses of opioids needed for clinical pain relief.

In Chapter 3, I study the ability of MOR PAM, BMS-986122, to enhance the effects of three clinically relevant opioids, morphine, methadone, and fentanyl. I show that BMS-986122 enhances MOR opioid agonist-induced antinociception, but does not alter constipation or respiratory depression. Additionally, BMS-986122 shows a slight attenuation of fentanyl-induced reward. Overall, these data provide a rationale for further development of MOR PAMs to be used as opioid sparing agents in the clinic.

Chapter 1 : General Introduction

The Prevalence of Pain in the United States

Pain is one of the most commonly experienced medical ailments worldwide (Mills et al., 2019). In the United States, chronic pain affects 21% of adults, equating to almost 52 million Americans (CDC, 2023). This number has been steadily rising following an increased focus on pain in disabled veterans in the 1940s and 1960s (Bernard et al., 2018). Chronic pain is a debilitating condition that prevents patients from engaging in everyday activities such as driving, working, and enjoying activities that are a normal source of joy. As such, this condition is commonly linked to comorbidities such as depression, neurodegenerative disorders such as Alzheimer's disease, risk of suicide, and significant risk of developing substance use disorders (Ditre et al., 2019; Dahan et al., 2014).

Pain is inherently a complex condition to diagnose and treat, as there are physiological, emotional, cultural, and social implications to how pain is perceived and managed by the patient. With this understanding, multiple modalities for relieving pain are necessary to as well as teasing apart the connection between these implications and identifying the varying levels of pain experienced. Common early pain therapies included acupuncture, acetylsalicylic acid (aspirin) from willow bark, and opium from poppy plants (Patil et al., 2016; Collier, 2017; Desborough & Keeling, 2017). The isolation of morphine and commercialization of hypodermic needles lead to an increase in the efficacy and

availability of pain therapies in the 1900s. Following World War II, pain management was predominately pharmacological, with the use of analgesics and anesthetics becoming a new standard of care (Bernard et al., 2018). This is primarily a result of how effective opioids are at blocking the pain stimulus. From a physician's perspective, why wouldn't you use an extremely effective treatment that will work for patients experiencing any pain? Unfortunately, this perspective was before we had a significant understanding of how much impact the adverse effects associated with opioid use would have on public health.

The United States Opioid Epidemic

The continued use of opioids as the gold standard for the clinical management of pain over many years has had serious ramifications for the United States. On the surface, the pharmaceutical industry was booming in the early 2000s, with annual prescription rates rapidly increasing, the development of new formulations of opioids (e.g. Oxycontin®), and industry groups marketing these drugs specifically as non-addicting (Rummans et al., 2018). Due to the efficacy and downplayed safety concerns of taking opioids, regulation surrounding prescribing and using opioids wasn't called into question until overdose deaths became increasingly visible across the US. The rapid increase in opioid overdose deaths before the 2010s was largely due to either a combination of opioids with other drugs (cocaine, benzodiazepines, antidepressants, etc.) or due to heroin use alone (NIDA, 2023). Since 2013, the prevalence of fentanyl and synthetic opioids increased significantly, inducing a steep uptick in opioid overdose deaths (NIDA, 2023). Most of the increases in fentanyl deaths over this time were due to illicit fentanyl

being mixed with other drugs such as heroin, either with or without the consumer's knowledge (DEA, 2016).

In 2017, the US Government declared the opioid epidemic a public health emergency. Despite political and regulatory interventions including drug monitoring programs, physician prescription reporting, and drug scheduling changes, deaths by opioid overdose continue to increase (Jones et al., 2018). Since 1999, more than 932,000 people have died from a drug overdose in the US, with opioids being the main driver (CDC, 2020). Additionally, overdose deaths involving synthetic opioids such as fentanyl increased by 56% from 2019-2020, accounting for 82% of all overdose deaths (CDC, 2021).

While overdose deaths are a significant issue, three million Americans suffer from opioid use disorder (OUD). A diagnosis of OUD requires meeting two or more of the eleven criteria (APA, 2013), such as continued use despite life disruption, need for increased doses of the drug, withdrawal occurring when the dose is decreased, and continued use despite physical or psychological challenges. In a clinical setting, tolerance to these drugs can be achieved within days, and withdrawal can be severe, especially if the patient was taking high doses for an extended period of time (Degenhardt et al., 2019). The increase in OUD cases can be partially attributed to the over-prescription of opioids, the downplay of the abuse liability of the drugs, and the substantial marketing released by pharmaceutical companies starting back in the 1990s (Azadfard et al., 2023). The industry has since taken a hit with four US-based companies paying $26 billion to settle

several lawsuits claiming their involvement in the opioid crisis (NPR, 2022). As a result, pharmaceutical companies have little to no investment in involving themselves in the development of new opioids or pain therapies.

The opioid epidemic has had a broad impact on all communities across the US. While initial reports suggested that OUD and overdose deaths were primarily focused on white, rural demographics, records indicate that Black individuals in urban areas faced severe impacts that went largely underreported (Griffith et al., 2018). In fact, Michigan was deemed a hotspot for opioid overdose deaths, and further analysis suggests the urban area of Detroit showed a significant increase in heroin and fentanyl overdose deaths compared with all other areas in Michigan (Gondré-Lewis et al., 2022). Additionally, the rate of drug overdose in Black communities nationwide was the higher than any other demographic (SAMHSA, 2020). With the rates of overdose deaths and OUD cases continually rising across all demographics nationwide despite regulatory intervention, it is clear that we need a safer pharmacological alternative to traditional opioids.

The development of novel pain therapies presents as a challenge for two main reasons: opioids are the best and most effective at relieving pain, and pharmaceutical companies want no involvement in the development of new drugs that have any effect on the opioid system. Several attempts to manipulate the opioid system to separate the beneficial from adverse effects of opioid drugs have been made with minor success. As it stands, the opioid crisis is not slowing down, companies are hesitant to develop novel

therapies, and healthcare providers are subjectively limiting prescriptions to avoid repercussions. While OUD and overdose deaths persist, the need to manage moderate to severe pain is still present. As such, it is important to continue to study alternative methods for managing pain and eliminating the opioid crisis as a public health emergency.

Traditional Opioids as the Standard of Care:

Opioids function by mimicking endogenous opioids such as endorphins, enkephalins, and dynorphins, that act at opioid receptors. There are three classical opioid receptors, mu, delta, and kappa. These receptors belong to the seven transmembrane Class A G protein-coupled receptor (GPCR) family. Activation of GPCRs propagates signals via a downstream cascade that amplifies as the signal travels further downstream. Opioid receptors couple to the Gi/o subfamily of heterotrimeric G proteins, which inhibit neurotransmission following activation. G proteins are comprised of α and $\beta\gamma$ subunits that dissociate and interact with downstream effector proteins upon receptor activation. The α subunit suppresses the production of cyclic adenosine monophosphate (cAMP) through direct inhibition of adenylyl cyclase. The $\beta\gamma$ subunit can interact with multiple proteins to hyperpolarize neurons, such as potassium and calcium channels, as well as several protein kinase pathways that contribute to downstream cell signaling (mitogen-activated protein kinases and extracellular signal-regulated kinases). Additionally, active $\beta\gamma$ subunits recruit G protein receptor kinases (GRK) to the plasma membrane, facilitating phosphorylation of the C terminus of the GPCR. This phosphorylated state of the receptor recruits β arrestin proteins beginning the process of receptor desensitization, internalization, and degradation or recycling back to the membrane. However, β arrestins

can also act as signaling molecules and allow receptors to signal even when translocated internally (Nuber et al., 2016; Barsi-Rhyne et al., 2022).

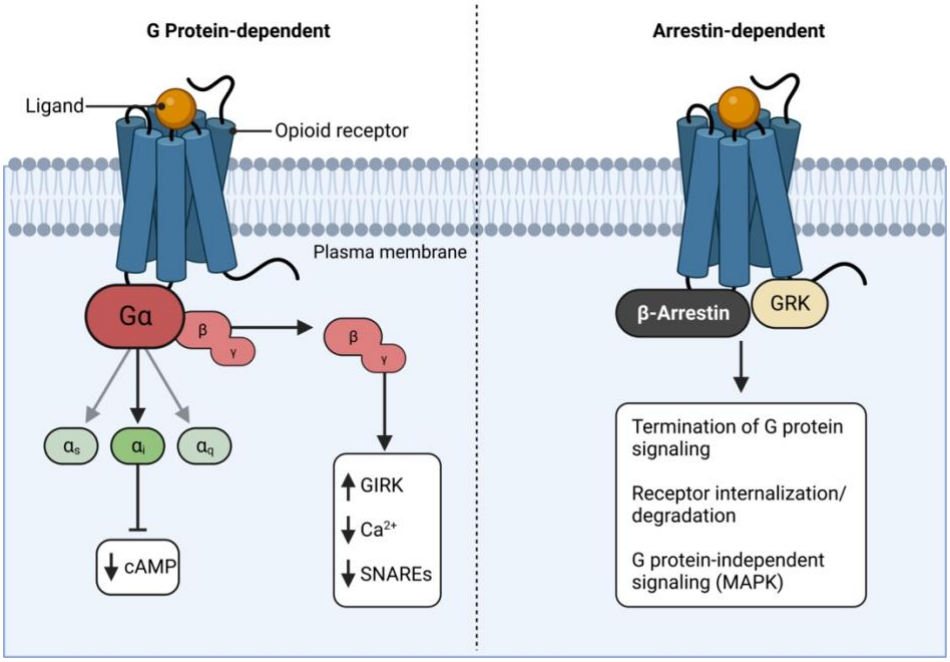

Figure 1.1. Canonical GPCR signaling through G protein or arrestin dependent mechanisms. (Created with BioRender.com)

Clinically used opioids, such as morphine, methadone and fentanyl, function by interacting with the mu opioid receptor (MOR). Activation of MOR not only leads to pain relief, but also the adverse effects associated with opioid use, such as constipation, physical dependence, euphoria, tolerance, and the fatal effect of respiratory depression. All of these effects are due to the activation of MOR, which highlights the danger of using opioids regularly for extended periods of time. However, the efficacy of these drugs in relieving moderate to severe pain is necessary, as current alternatives are not as effective, particularly following surgical procedures. Delta and kappa opioid receptor (DOR, KOR) activation can also produce mild analgesia but induce significant on-target

effects such as convulsions (DOR) and dysphoria (KOR); there are no clinically available selective DOR or KOR agonists in the US.

Table 1.1. Opioid-induced effects at the three main opioid receptors.

Receptor	Effect
Mu opioid receptor (MOR)	Analgesia, tolerance, dependence, euphoria, abuse liability, sedation, constipation, respiratory depression
Delta opioid receptor (DOR)	Mild analgesia, convulsions, antidepressant-like effects
Kappa opioid receptor (KOR)	Mild analgesia, dysphoria, depression, sedation

Thus, MOR remains the best target for developing novel strong analgesics. Several methods to separate the beneficial from adverse MOR effects have been studied over the years at the preclinical level, including compounds that act at more than one receptor, for example MOR and DOR, and compounds that act at multiple opioid receptors. These compounds have been proposed to promote antinociception with less induction of tolerance by combining agonist and antagonist effects or reducing receptor desensitization (Anand et al., 2016; Anand et al., 2018) However, in this thesis I will concentrate on two newer methods, biased agonism and allosteric modulation.

Biased Agonism as an Alternative Method of Pain Management

It has been suggested that ligands can differentially activate certain pathways downstream of MOR to induce specific outputs. This phenomenon is referred to as biased agonism, and the most common example is the separation of downstream signaling of

GPCRs into two main pathways; G protein and β arrestin (Gillis et al., 2020). The significance of this theory lies in the idea that some MOR agonists may preferentially activate one pathway over the other, specifically G protein over β arrestin, to only promote analgesia, and not adverse effects. This idea is based on several studies showing that ligands with a bias towards the G protein pathway may correlate to the production of less severe adverse effects, such as tolerance, constipation, and respiratory depression.

One specific group has extensively studied the effects of opioids in β arrestin knockout animals and observed enhanced antinociceptive effects with reduced development of side effects (Bohn et al., 1999; Raehal et al., 2005). Furthermore, studies have suggested that ligands with a G protein-biased signaling profile display a better therapeutic window, or the separation between doses of opioids that produce antinociception or respiratory depression at 50% of maximal effect (Schmid et al., 2017). One particular compound, SR-17018, showed a significant G protein bias compared to other test compounds. Additionally, the discovery and development of G protein-biased ligands continued into clinical trials. To date only one compound has made it through clinical trials, TRV130 (Oliceridine®) injection is approved for post-operative pain management in the clinic, although its use is limited, due to failure to show significantly reduced adverse effects (respiratory depression) compared to morphine during phase 3 testing (Viscusi et al., 2019).

More recently, the biased agonism hypothesis has become heavily contested, with significant questions arising regarding reproducibility and validity. For example, there are

several quantification methods that rely on the efficacy and potency of a ligand and are sensitive to changes in the assay used to measure a functional signaling pathway as well as the cell line used in these assays. As a result, there a several studies with conflicting results surrounding the signaling bias of traditional opioid agonists (e.g. fentanyl, morphine, methadone, endogenous ligands). The methods used for quantifying signaling bias also rely on the comparison to a “neutral” ligand, or a ligand that does not produce any significant bias. Usually, studies use the met-enkephalin derivative DAMGO as this reference ligand, but some report that it may produce a bias (Rivero et al., 2012). Thus, reported bias measures largely depend on the “neutral” ligand, experimental methods, and cell lines used. Additionally, the correlation between *in vitro* bias and *in vivo* behavior has not been reliably reproduced. Regardless of the caveats with the biased agonism theory, the premise may be a useful tool for teasing apart how MOR signals and how the system could be manipulated to alter behavioral outputs.

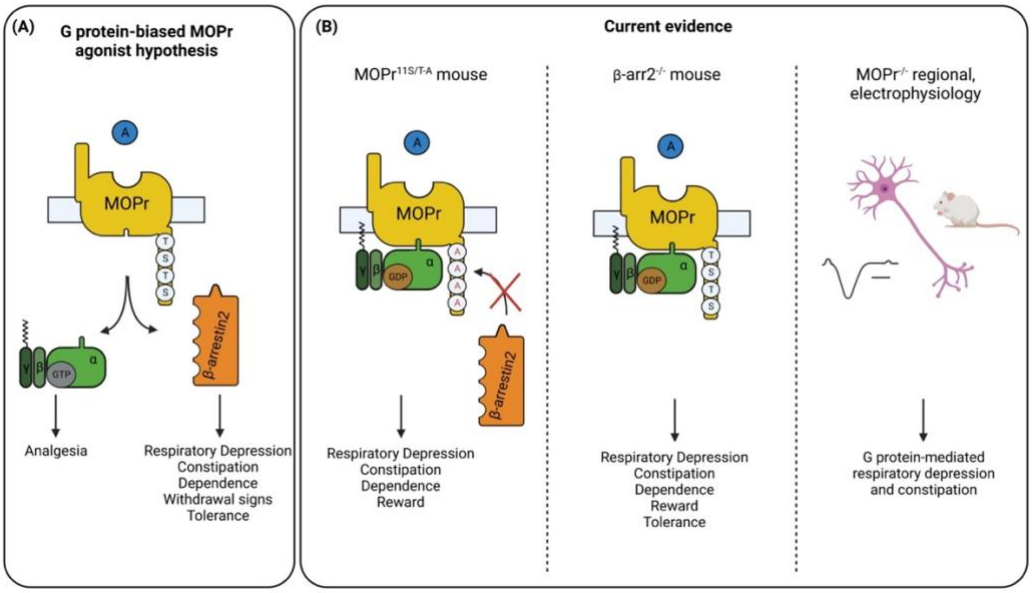

Figure 1.2. G protein-biased agonism theory and current evidence supporting the hypothesis that side effects can also be G protein mediated. (Created with BioRender.com)

Allosteric Modulation of the Mu Opioid Receptor

Another proposed method for pain management is positive allosteric modulation of MOR. Allosteric modulators bind to a site on MOR that is spatially distinct from the so-called orthosteric site where traditional opioids and endogenous peptides bind. As such, these modulators do not compete with opioids for binding sites. The presence of an allosteric modulator can alter orthosteric ligand characteristics, such as affinity, efficacy, and/or potency. The effects induced by an allosteric modulator can be positive, negative, or silent, and these compounds are deemed as PAMs, NAMs, and SAMs, respectively. Additionally, positive allosteric modulators can have their own agonist activity without the presence of an orthosteric ligand, which is referred to as ago-PAM activity (Conn et al., 2014; Noetzel et al., 2012).

Positive allosteric modulators (PAMs) are proposed to have multiple benefits over traditional opioid therapies (Burford et al., 2015). Most importantly, these allosteric modulators have the ability to enhance endogenous opioid activity. As shown in Figure 1.3, endogenous opioids are released in a localized and pulsatile manner. These endogenous ligands are not effective enough to combat severe pain on their own. But in the presence of a PAM, endogenous opioid effects are enhanced to afford pain relief. Critically, the PAMs do this without altering the release patterns of the endogenous opioids. Thus, it is hypothesized that PAMs have the potential to increase the efficacy of endogenous opioids to combat moderate to severe pain without inducing adverse events. This is in stark contrast to administration of exogenous opioids like morphine or fentanyl which activate MORs throughout the body for an extended period of time, managing pain but increasing the risk for serious adverse events.

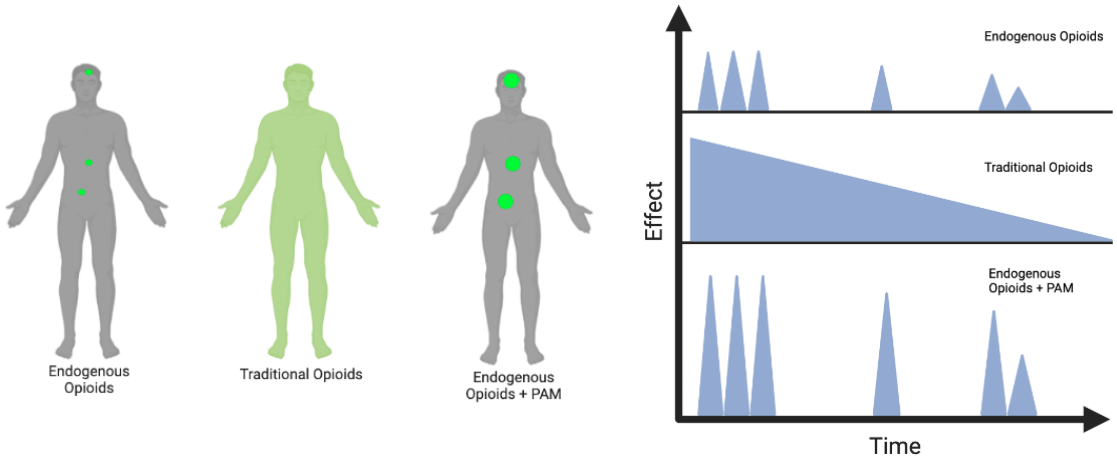

Figure 1.3. PAMs enhance endogenous opioid activity and maintain the spatial (top) and temporal (bottom) regulation of endogenous opioid release, whereas traditional opioids activate MOR all throughout the body for a prolonged period of time.

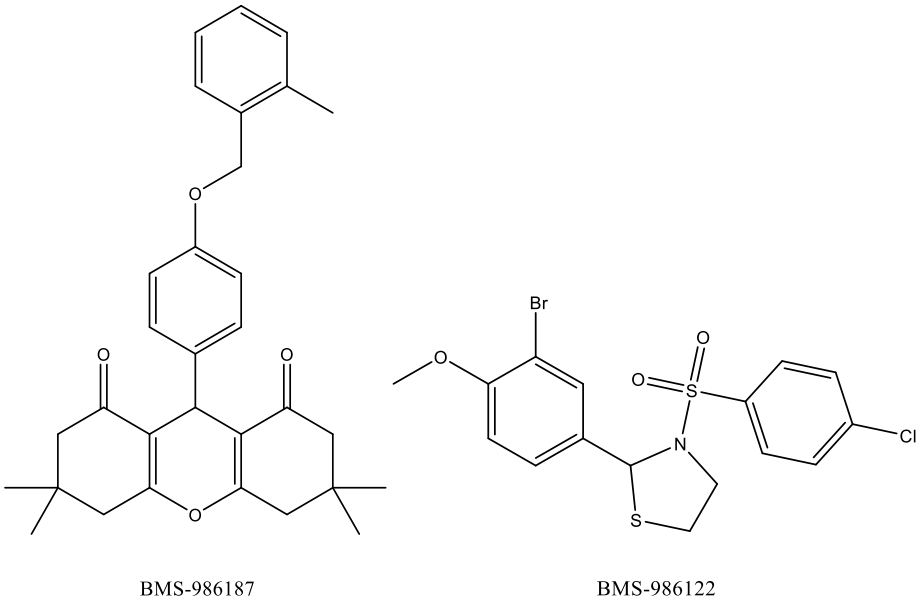

Figure 1.4. Chemical Structures of BMS-986187 and BMS-986122

One PAM, BMS-986122, has been shown to significantly enhance endogenous and exogenous opioid efficacy and potency *in vitro* (Livingston & Traynor, 2018). Additionally, BMS-986122 enhances endogenous opioid-mediated antinociception *in vivo* with reduced development of adverse effects such as constipation, reward, and respiratory depression (Kandasamy et al., 2021).

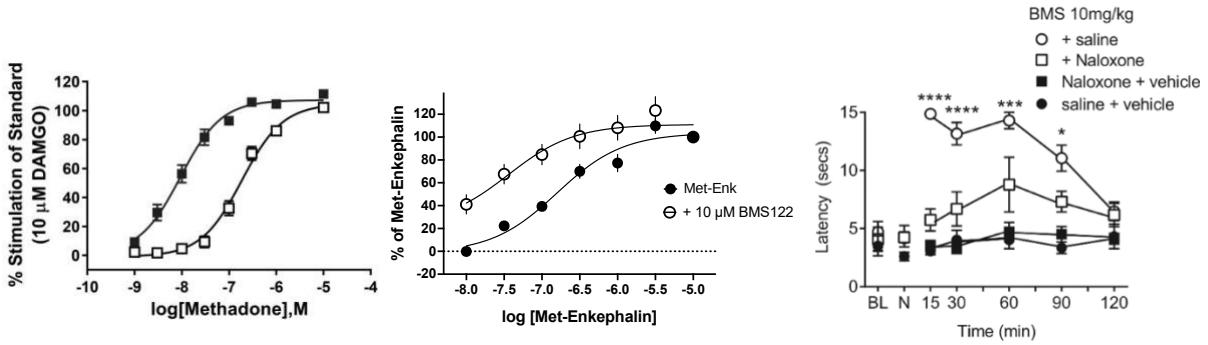

Figure 1.5. Allosteric modulator BMS-986122 enhances opioid-induced activity *in vitro* and *in vivo*. BMS-986122 enhances methadone induced G protein activation *in vitro*, enhances endogenous opioid peptide G protein activation in the mouse periaqueductal gray (PAG) section of the brain, and produces naloxone-reversible antinociception in the mouse warm water tail withdrawal. Figures courtesy of Dr. John Traynor, Dr. Kathryn Livingston, and Dr. Ram Kandasamy.

Another advantage of allosteric modulation is that these compounds have the ability to alter the signaling bias of orthosteric ligands (Davey et al., 2012; Khajehali et al., 2015). This means the modulators have the potential to drive the ligand-receptor response toward a potentially more favorable signaling profile. Two novel PAMs, BMS-986187 and BMS-986122 (Figure 1.4), have been shown to increase the potency of endogenous and exogenous opioids *in vitro* (Fig 1.5) (Livingston et al., 2018). Additionally, BMS-986187 has been shown to have a G protein-biased signaling profile in the absence of an orthosteric ligand at DOR (Stanczyk et al., 2019). It has also been shown that orthosteric ligand cooperativity with PAMs is probe dependent. This phenomenon explains that the enhancement observed in the presence of PAMs is dependent on the orthosteric ligand present. For example, *in vitro* data suggest that methadone is the most sensitive to PAM shifts, showing the largest enhancement in EC_{50} values. Furthermore, these PAMs have been suggested to bind to a similar conserved site on MOR (Livingston et al., 2018), though several binding sites have been proposed

using molecular dynamics simulations and nuclear magnetic resonance techniques (Chan et al., 2023)

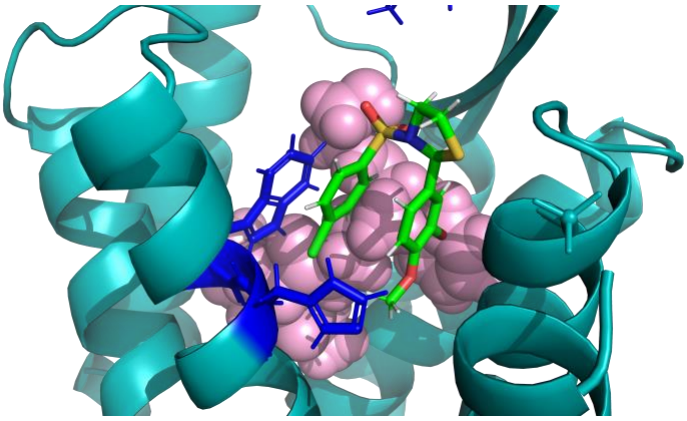

Figure 1.6. Proposed allosteric binding site on MOR using molecular dynamics simulations to identify key residues (blue) for BMS-986122 (green) binding on DAMGO-bound (pink) MOR (teal). The allosteric site is above the orthosteric site. Figure courtesy of Dr. Wallace Chan.

Taken together, these data suggest that positive allosteric modulation of MOR is a useful tool for investigating how ligand signaling at opioid receptors might be controlled. Thus, PAM tool compounds are potentially useful in aiding drug discovery efforts to develop a new class of drugs to treat pain and therefore help combat the opioid epidemic. Such compounds might function on their own or be used in combination with other methods of analgesia.

Opioid Sparing to Combat the Opioid Epidemic

In addition to biased agonism and allosteric modulation, opioid sparing has become a point of interest as a method for reducing opioid use. This method utilizes the combination of non-opioid analgesics with opioids to lower the dose and amounts of opioids needed to manage pain. Opioid sparing allows patients to receive the same

amount of pain relief but with a lower risk for adverse effects like the development of addiction. Several modalities for opioid sparing have been examined, including both pharmacological and non-pharmacological therapies.

Some of the relevant opioid sparing modalities used are non-steroidal anti-inflammatory drugs (NSAIDs), anticonvulsants, cannabis, and several types of cognitive behavioral therapies. These methods are primarily focused on patient populations following surgical procedures and show mixed results (Kumar et al., 2017). Additionally, the efficacy of opioid-sparing has yet to be tested long term and is usually examined in situations where patients are only taking opioids for an acute period of time. However, the exposure time of these acute treatments would be sufficient for the development of adverse events with high doses of opioids. Moreover, several case studies examining opioid sparing are focused primarily on monitoring adverse effects associated with opioid use. In reality, the use of other analgesics that function on other systems have the potential to induce adverse effects of their own (e.g. liver damage, kidney damage). It is also well characterized that opioids in combination with other drugs, such as anticonvulsants and antidepressants, can synergize to enhance specific side effects, including respiratory depression (Kane et al., 2017; Wong, 2022).

Currently, clinical opioid-sparing efforts focus on utilizing non-opioid therapies, but allosteric modulators of MOR have the potential to play a role in this method as well. We do not yet have an understanding of the extent to which PAMs influence exogenous opioid-induced effects *in vivo*. Previous data reports that one PAM, BMS-986122,

significantly enhances morphine- and methadone-induced antinociception in mouse models, but no studies have been done to examine how this compound alters the adverse effects of opioid drugs (Kandasamy et al., 2021). When in the presence of endogenous opioids alone, BMS-986122 also shows enhanced antinociception with very limited development of tolerance, constipation, reward, and respiratory depression. In this regard, positive allosteric modulators have the potential to enhance the antinociceptive effects of low-dose exogenous opioids without potentiating the adverse effects.

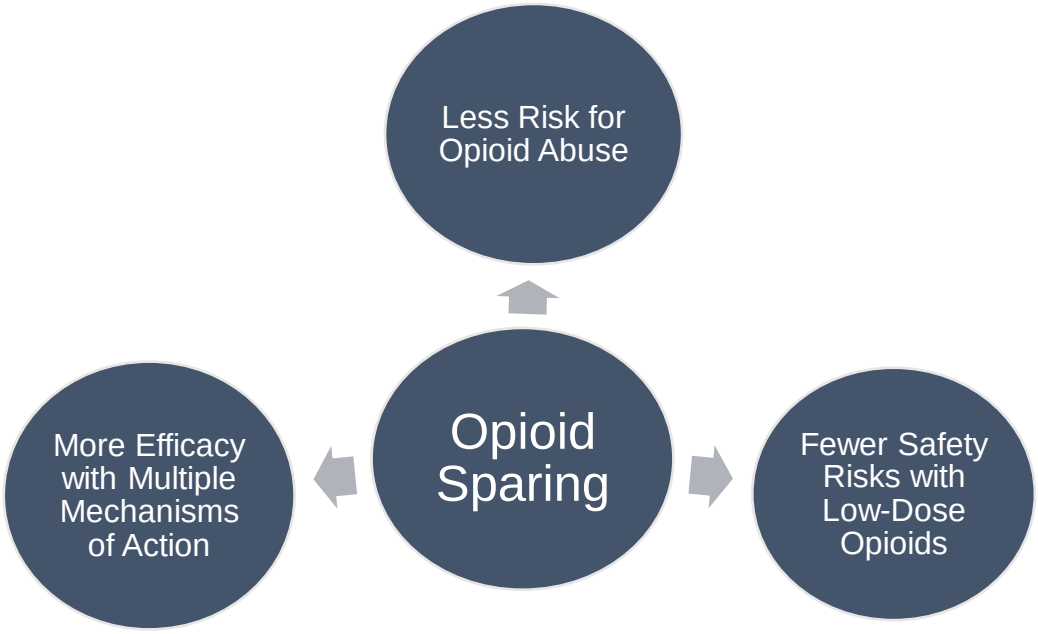

Figure 1.7. The benefits of opioid-sparing multimodal therapy.

Aims

The purpose of the work described in this thesis is to further characterize the function of positive allosteric modulators of the mu opioid receptor and the role that they

may have in replacing traditional opioid therapies and contributing to combatting the opioid epidemic. These compounds have the potential to act as significant tools for furthering our understanding of opioid signaling and the connection between *in vitro* and *in vivo* outputs. Studies to date have explored the ability of PAMs to enhance opioid affinity, efficacy, and/or potency at opioid receptors. However, the influence these alterations have on signaling and behavioral effects are not well understood. This body of work explores a deeper understanding of the impact PAMs have on orthosteric ligands to inform future development of these compounds by examining the influence of positive allosteric modulation on orthosteric agonist signaling bias *in vitro* and understanding the specificity of potentiation induced by PAMs on opioid-mediated effects *in vivo* as follows:

Chapter 2: The Influence of Two Structurally Diverse Positive Allosteric Modulators on Mu-Opioid Receptor Signaling

Chapter two investigates the potential of two positive allosteric modulators of MOR, BMS-986187 and BMS-986122, to promote orthosteric ligand signaling bias. Here we examined how these PAMs impact the bias factors of six orthosteric ligands (DAMGO, fentanyl, methadone, morphine, met-enkephalin, and SR17018) utilizing several different quantifiable comparisons. Furthermore, we surveyed the ability of these orthosteric ligands to alter MOR internalization in the presence and absence of both PAMs.

Chapter 3: Opioid Sparing by a Mu-Opioid Receptor Positive Allosteric Modulator

Evidence from previously published work suggests that MOR PAM BMS-986122 promotes antinociception with reduced adverse effects in vivo by enhancing the action of

endogenous opioid peptides. Chapter three explores the pharmacology resulting from potentiation of exogenous opioid drugs by BMS-986122 across several opioid-induced effects. The purpose of this chapter is to determine whether PAM enhancement is selective for a particular action or actions of opioids and investigate positive allosteric modulation as a potential method for opioid sparing.

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Chapter 2 : The Influence of Two Structurally Diverse Positive Allosteric Modulators on Mu-Opioid Receptor Signaling

Abstract:

The mu-opioid receptor (MOR) is responsible for the beneficial analgesic actions of opioid drugs as well as their unwanted actions, including respiratory depression and addiction liability. A controversial theory suggests MOR agonists that preferentially signal to certain intracellular pathways (specifically a bias towards G-protein over β-arrestin), might show an improved therapeutic index. Evidence from studies of G-protein-coupled receptors (GPCRs) suggests that allosteric modulators can influence ligand signaling profiles. However, no studies have examined this phenomenon at MOR. In this study, we compare the ability of various orthosteric MOR agonists to activate G-protein, as measured by the binding of GTPγ^{35}S, or recruit β-arrestin in the absence or presence of two structurally distinct MOR positive allosteric modulators, BMS-986187 (xanthene-dione) or BMS-986122 (thiazolidine). The orthosteric agonists studied have been previously reported as neutral (morphine, methadone, and Met-Enkephalin), G-protein biased (SR17018) or potentially β-arrestin 2 biased (fentanyl). In our study, fentanyl is biased towards recruitment of β-arrestin 2, and that SR17018, along with Met-Enkephalin, is G-protein biased. Both modulators shifted the GTPγ^{35}S concentration-response curve to the left similarly across the MOR-agonists, although SR17018 was more responsive to

BMS-986187. In contrast, BMS-986187 enhanced the potency for β-arrestin 2 recruitment in the following order; morphine (10-fold) < SR17018 < DAMGO < Met-Enkephalin < methadone < fentanyl (178-fold), whereas BMS-986122 elicited much smaller shifts (1-7 fold). Both PAMs increased receptor internalization induced by DAMGO, morphine, and methadone, but not fentanyl. Overall, these studies provide evidence that PAMs influence the degree and direction by which orthosteric agonists signal downstream of MOR.

Introduction:

Opioids acting at the mu opioid receptor (MOR) are the gold standard for managing pain. Unfortunately, along with pain relief, many patients experience adverse effects, including constipation, addiction liability, and life-threatening respiratory depression (Jones, 2018). The latter two properties have driven the opioid epidemic, resulting in over 564,000 opioid-overdose deaths in the United States between 1999-2020, with 3 million people suffering from opioid use disorder (OUD) (CDC, 2022). Roughly 21-29% of patients prescribed opioids for chronic pain misuse them, with 8-12% developing OUD (Oelhaf *et al.*, 2023). Opioid misuse has broader economic implications, where healthcare, treatment, and criminal justice expenses cost the United States $78.5 billion a year (NIDA, 2019, Florence *et al.*, 2013). Due to the risks associated with opioid use, it is evident that better therapeutic options for pain management are necessary. However, given the effectiveness of the opioid system, it is important to study and manipulate this system to reduce adverse effects while maintaining beneficial effects of MOR activation.

MOR is a seven-transmembrane G-protein-coupled receptor that, when activated, interacts with downstream signaling pathways responsible for both the beneficial and adverse effects of opioid use. Biased agonists are compounds modulating specific signaling pathways over others, most often G-protein activation over β-arrestin 2 recruitment. In the case of MOR, evidence suggests that G-protein-biased agonists may result in fewer or less severe adverse effects, including respiratory depression and constipation (Bohn *et al.*, 1999, 2000; Raehal, Walker & Bohn, 2005; Schmid *et al.*, 2017; Bohn 2022), though this remains heavily debated (Foster & Conn, 2017, Gillis *et al.*, 2020). Nonetheless, there have been efforts to design compounds that preferentially engage G-proteins rather than β-arrestins (DeWire *et al.*, 2013; Manglik *et al.*, 2016; Schmid *et al.*, 2017), and one biased agonist, oliceridine®, is FDA approved for use in hospitals for the management of postoperative pain.

Another potential approach is the use of positive allosteric modulators of MOR (Burford et al., 2015). Compared to traditional orthosteric and biased agonists, allosteric modulators bind to receptors at a site spatially distinct from the orthosteric site to modify ligand-receptor interactions, leading to changes in orthosteric ligand affinity and/or efficacy. Allosteric modulators at MOR can be positive (PAMs), negative (NAMs), or silent (SAMs). MOR-PAMs enhance the activity of endogenous opioid peptides (β-endorphin, enkephalins) released during pain states providing endogenous pain relief. Unlike morphine and exogenous opioids that activate MOR globally throughout the body, MOR-PAM enhancement maintains the spatial and temporal release patterns of the opioid

peptides (Livingston & Traynor, 2018); and as such, shows reduced side effects (Kandasamy *et al.,* 2021).

There is some evidence that allosteric modulators may influence the signaling of other G-protein-coupled receptors, such as calcium-sensing, cannabinoid, and muscarinic receptors (Davey *et al.*, 2012; Wootten, Christopoulos & Sexton, 2013; Cook *et al.*, 2015; Khajehali *et al.*, 2015). Given the implications of allosteric modulation and the potential of differential activation of downstream pathways of MOR in designing safer pain therapeutics, we sought to examine how MOR-PAMs might influence downstream signaling and signaling bias of orthosteric MOR agonists.

We compared the effects of two structurally diverse MOR-PAMs, BMS-986122 and BMS-986187 (fig. 1), that have similar activity at MOR (μM range) *in vitro* and potentially act through a similar or overlapping allosteric site (Livingston *et al.*, 2018). Comparisons were made between the activation of G-protein (as determined by the binding of GTPγ^{35}S) the ability to recruit β-arrestin2, and effects on agonist-mediated receptor internalization. PAMs are known to show probe dependence such that their observed actions are contingent on the agonist occupying the orthosteric site (Bartuzi et al., 2017, Livingston & Traynor, 2014). Therefore, we employed several MOR agonists: the standard peptidic full agonist DAMGO, Met-Enkephalin as a representative endogenous ligand, fentanyl and morphine for their clinical relevance and different potencies, methadone because it has been reported as the most sensitive to MOR-PAMs (Livingston & Traynor, 2014) and

SR17018, a partial MOR agonist and G-protein-biased ligand (Schmid *et al.*, 2017, Grim *et al.*, 2017, Stahl & Bohn, 2022).

Our findings show that BMS-986122 and BMS-986187 enhance signaling to both G-protein and β-arrestin 2, although the degree of the shift varies depending on the PAM and agonist. In general, BMS-986187 promoted larger shifts in the β-arrestin assay, with fentanyl and methadone being especially sensitive, whereas BMS-886122 promoted larger shifts in the GTPγ^{35}S assay, with methadone being the most sensitive. The differential enhancement of signaling to β-arrestin or G-protein highlights the role allosteric modulation can play in altering signaling bias.

Methods:

Materials

Guanosine-5'-O-(3-[^{35}S]thio) triphosphate (GTPγ^{35}S), and reagents were from PerkinElmer. BMS-986122 and BMS-986187 were synthesized by the University of Michigan Vahlteich Medicinal Chemistry Core and characterized as previously described (Burford et al., 2015). β-arrestin 2 Pathhunter cell line and reagents were from Eurofins DiscoverX. All MOR ligands (DAMGO, fentanyl, R-methadone, met-enkephalin, morphine, and β-funaltrexamine (β-FNA)) were obtained by the National Institutes for Drug Abuse drug supply or Tocris Bioscience.

Cell Lines

For GTPγ^{35}S assays, CHO cells stably expressing wild-type human-MOR (CHO-MOR) were grown in DMEM containing 10% FBS and 1% penicillin/streptomycin and maintained in 0.8 mg/ml G418 (geneticin) as previously described (Burford et al., 2015). For β-arrestin 2 recruitment assays, CHO OPRM1 Pathhunter cells (Pathhunter CHO-MOR) from Eurofins DiscoverX were maintained in 0.8 mg/ml G418 and 0.3 mg/ml Hygromycin B per manufacturer instructions. For β-FNA experiments, cells were incubated at 37 ^{o}C with 100 nM β-FNA for 1 hr before prepping membrane homogenates. Stable nonclonal HEK293 cells (American Type Culture Collection CRL-1573) expressing superecliptic phluorin (SpH)-MOR were selected in Geneticin (Invitrogen) and grown in Dulbecco's modified Eagle's medium (Hyclone) + 10% fetal bovine serum (Gibco). The SpH-MOR-S363A point mutant is as described previously (Soohoo and Puthenveedu, 2013).

Membrane Preparations

Cells were harvested, and membrane homogenates were 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). Detached cells were then pelleted by centrifugation at 1600rpm 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 20,000rpm 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 for a final protein

concentration range of 0.5-1.5 mg/mL. Protein concentrations were determined using the bicinchoninic acid quantification method (BCA), with bovine serum albumin (BSA) serving as the standard.

Stimulation of GTPγ^{35}S Binding

Agonist stimulation of GTPγ^{35}S binding was measured using CHO cells expressing wild-type human MOR (10 µg/well). Membrane preparations 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 an orthosteric agonist with BMS 986122, BMS 986187 or Vehicle (DMSO) 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 six times with 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 MOR agonist, DAMGO, at 10 µM to account for variability between different 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 white 384-well plates using Assay Complete Cell Plating Reagent (DiscoverX) 24 hours before drug treatment. The following day, cells were treated with

indicated drug conditions for 60 minutes at 37^0 C. After 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).

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 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, and each field of view in every well on the plate was 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 the drug addition timing matched the experimental conditions. Images were acquired for 20 minutes after drug addition.

For internalization analysis, images were normalized to remove background fluorescence, and each frame's integrated density of fluorescence intensity was

calculated. Intensities were normalized to the mean of the two baseline frames, and 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 the analysis, and fields, where cells left the field of view, were discarded. Each well's remaining fields of view 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.

Data and Statistical Analysis

Bias calculations were performed as previously described (Kenakin et al., 2017). Individual concentration-response curves were used for each ligand to calculate the log(max/EC50). The difference in log(max/EC50) between β-arrestin 2 recruitment and GTPγ^{35}S, (Δlog(max/EC50)), was then calculated for each agonist. Individual results were combined to give means ± SEM. Finally, the differences between the Δlog(max/EC50) values for the reference ligand (DAMGO) and test ligand were calculated to give a ΔΔlog(max/EC50) value, or bias factor. Bias values were calculated using multiple reference ligands, with either DAMGO or individual orthosteric ligands as the reference. Comparisons of bias factor values were done using an unpaired two-tailed t-test to compare each value only to the corresponding reference ligand's value. Significance was measured as *P<0.05 and **P<0.01.

Results:

BMS-986187 and BMS-986122 promote MOR agonist activation of G-proteins.

The effect of BMS-986187 and BMS-986122 on the ability of an orthosteric ligand to activate G-protein signaling or recruit β-arrestin 2 was measured in CHO-hMOR cells. PAMs were used at the maximal solubility limit of 10 μM, although this does give a maximal shift in dose-response curves (Livingston et al., 2018; Stanczyk et al., 2019).

BMS-986187 elicited leftward shifts in the agonist concentration-response curves in the GTPγ^{35}S assay in the order morphine (3.6-fold) = Met-Enkephalin < fentanyl < DAMGO < methadone = SR17018 (18-fold) (Figure 2.1, Table 2.1). BMS-986122 produced similar degrees of shifts but in the order: SR17018 (2.3-fold shift) < morphine = fentanyl = DAMGO < Met-Enkephalin < Methadone. The only notable difference between the two PAMS was with SR17018 which was the most sensitive compound to BMS-986187 but was almost insensitive to BMS-986122.

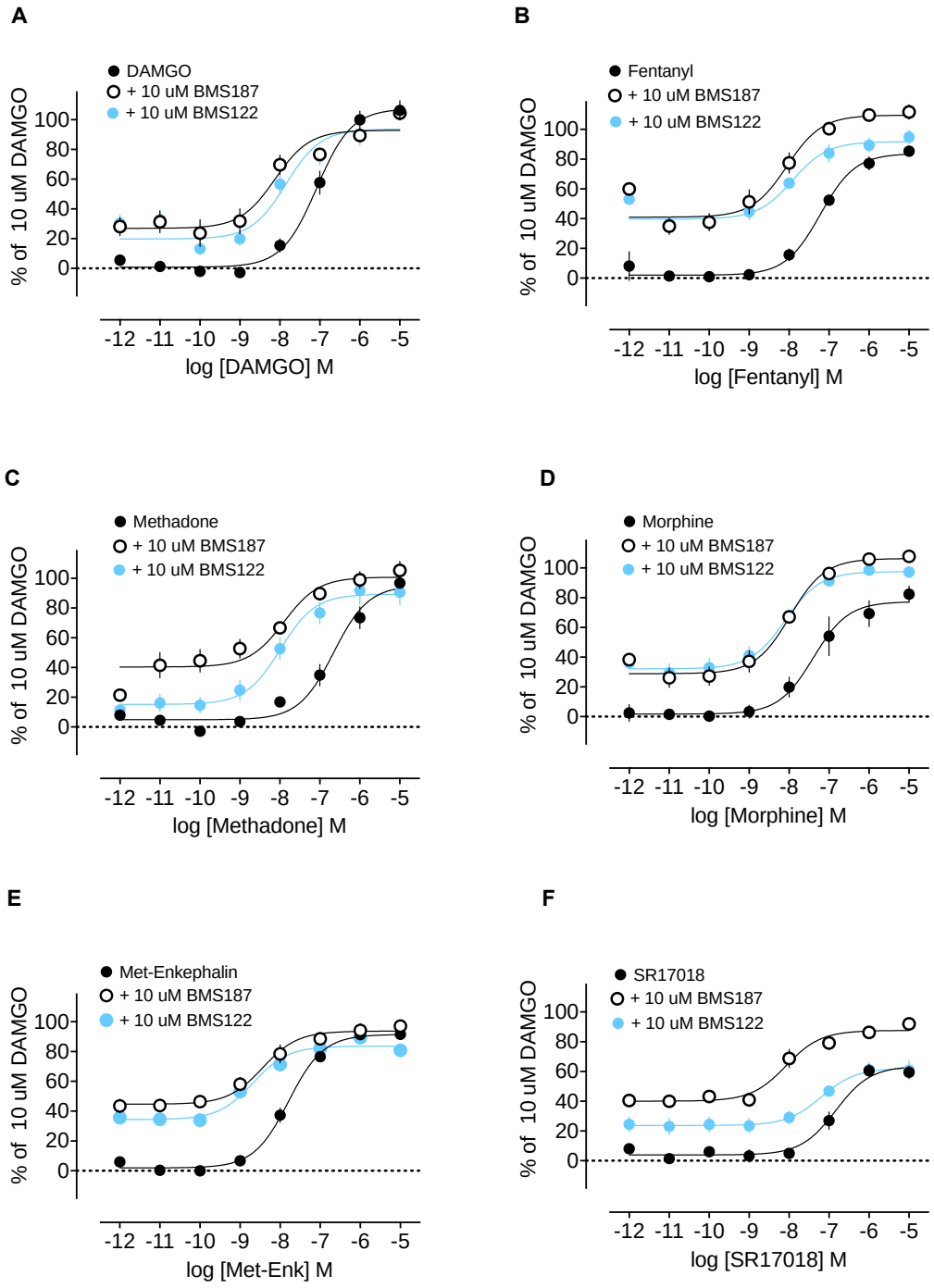

Figure 2.1 The effect of BMS-986187 (BMS187) and BMS-986122 (BMS122) on the activity of the orthosteric MOR agonists in the GTPγS assay in membrane homogenates from CHO cells expressing hMOR. **A.** DAMGO, **B.** Fentanyl, **C.** Methadone, **D.** Morphine, **E.** Met-Enkephalin, and **F.** SR17018. All data are from five separate experiments, each performed in duplicate and expressed as mean ± SEM. See Table 2.1 for the maximal effect and EC50 values.

In the GTPγ^{35}S binding assay, both PAMs produced an elevation of basal activity, indicating they act as “ago-PAMs.” Concentration-response curves show this effect of the PAMs alone occurs at > 1 μM (Figure 2.2). To determine if this is due to high expression level of MOR in the CHO cells we re-examined the ago-PAM activity after treatment of the cells with the MOR irreversible inhibitor, β-FNA. The ago-PAM activity of both modulators was diminished after treatment with 100nM β-FNA and completely absent after treatment with 1μM β-FNA (Figure 2.2).

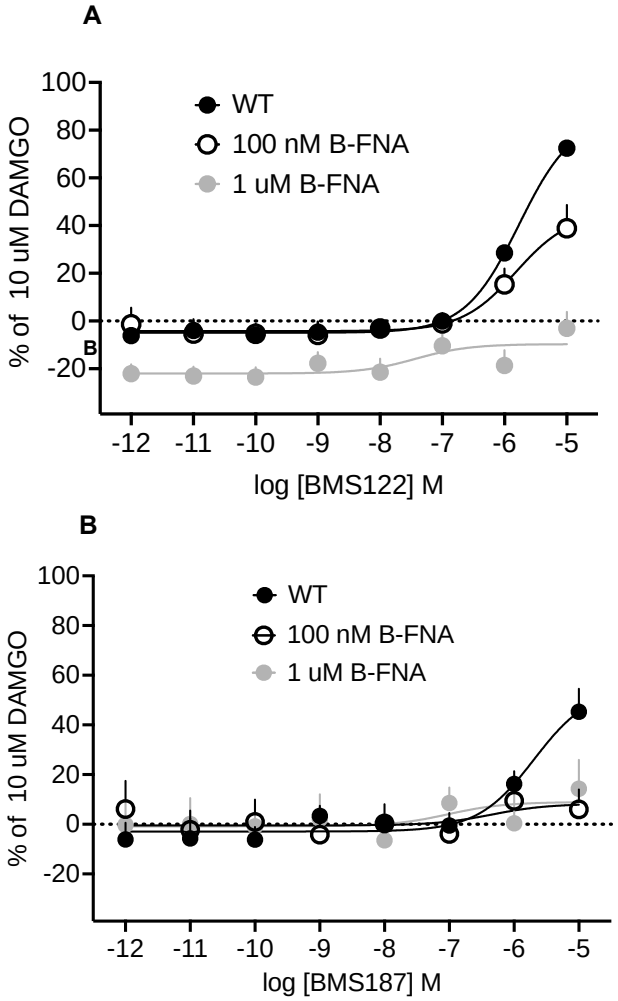

Figure 2.2. The effect of positive allosteric modulators in the GTPγS binding assay in the absence of an orthosteric ligand in membrane homogenates from CHO cells expressing hMOR treated with either 100 nM or 1 μM β-FNA. All data are from three separate experiments repeated in duplicate and expressed as mean ± SEM.

BMS-986187 and BMS-986122 differentially promote MOR agonist recruitment of β-arrestin 2.

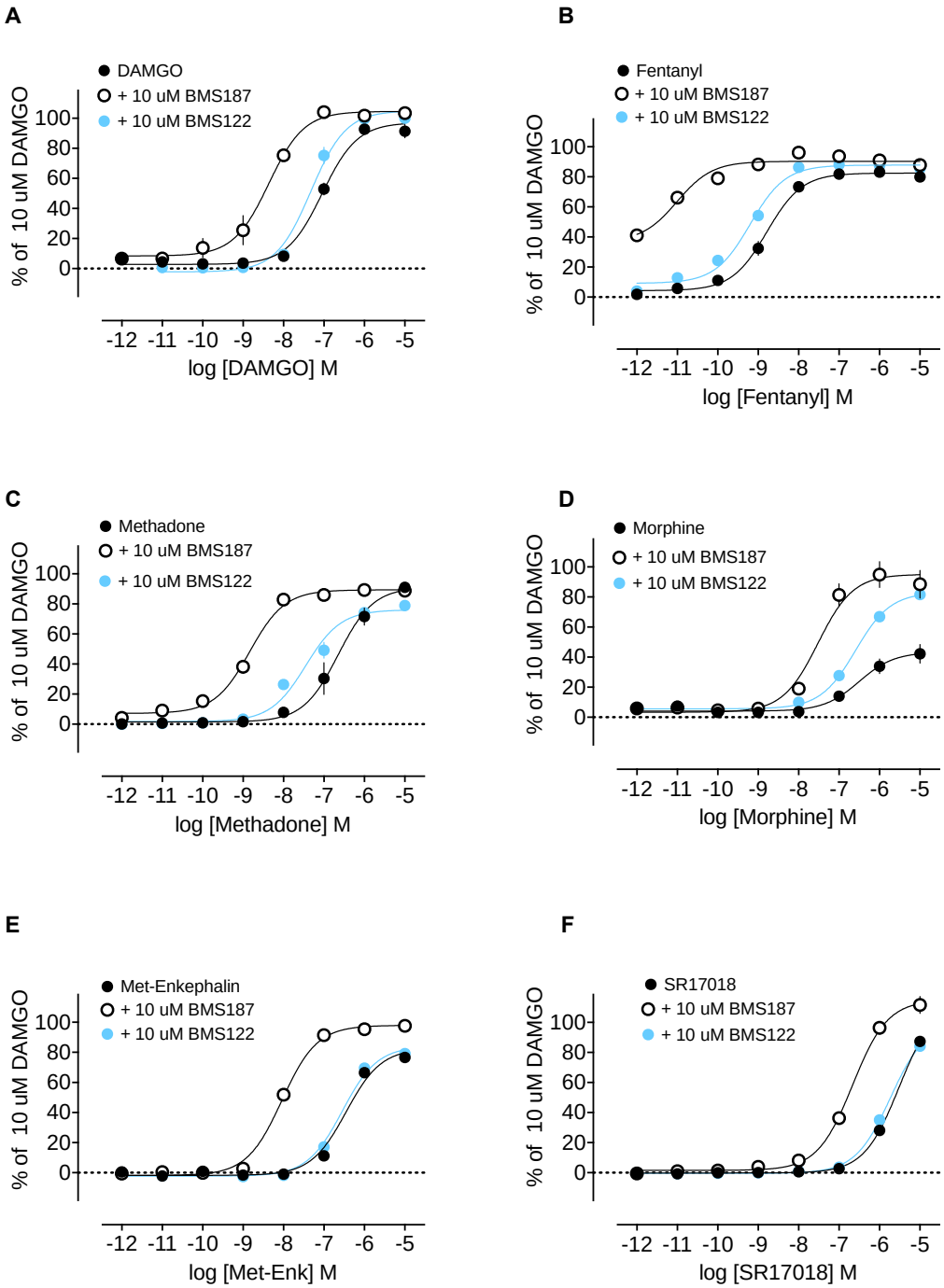

Figure 2.3. Effect of BMS-986187 (BMS187) and BMS-986122 (BMS122) on the orthosteric agonist-mediated recruitment of β-arrestin 2 using Pathhunter CHO cells expressing hMOR. **A.** DAMGO, **B.** Fentanyl, **C.** Methadone, **D.** Morphine, **E.** Met-Enkephalin, and **F.** SR17018. All data

are from five separate experiments, each performed in triplicate and expressed as mean ± SEM. See Table 2.1 for the maximal effect and EC50 values.

BMS-986187 promoted sizeable differences between the agonists by shifting concentration-response curves ranging from morphine (10-fold) < SR17018 < DAMGO < Met-Enkephalin (39-fold) >> methadone (151-fold) < fentanyl (178-fold) (Figure 2.3, Table 2.1). BMS-986122 promoted much smaller shifts ranging from 6.5-fold for methadone to just 1.2-fold for morphine, although the maximal response to morphine, which was the only compound that showed partial agonism in this assay, was increased by both PAMs. It is also notable that the PAMs alone did not exhibit agonist activity in recruiting β-arrestin 2 (Figure 2.4).

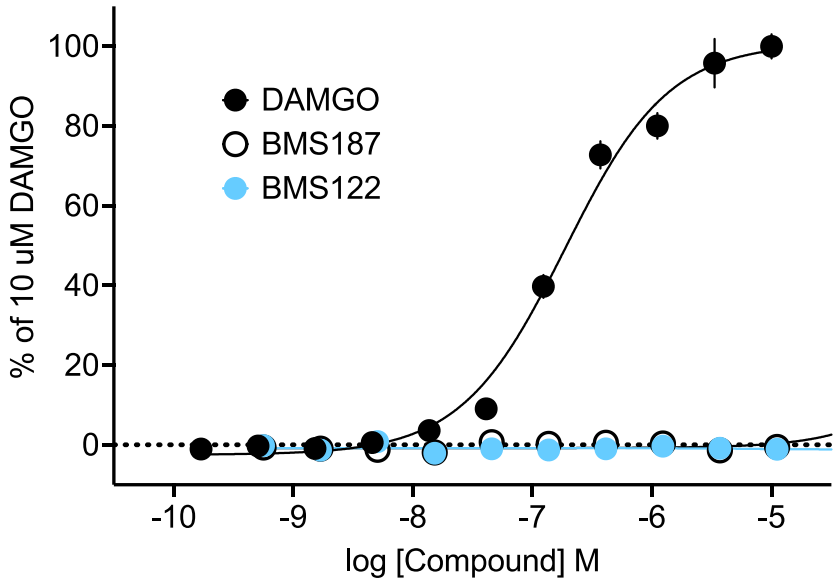

Figure 2.4. BMS-986187 and BMS-986122 do not display agonist activity in β-arrestin 2 recruitment. All data are from three separate experiments, each performed in duplicate using Pathhunter CHO cells expressing hMOR and expressed as mean ± SEM.

Table 2.1. Maximal effect and EC_{50} values for G protein activity and β-arrestin recruitment. Values are shown as mean (95% CI) for agonists in the presence and absence of BMS-986187 (BMS187) or BMS-986122 (BMS122) for 5 individual experiments, each performed in duplicate.

	GTPγS		**β-Arrestin**	
	% Max (95% CI)	**EC_{50} nM (95% CI)**	**% Max (95% CI)**	**EC_{50} nM (95% CI)**
DAMGO	108 (100-115)	84 (55-125)	97 (92-102)	90 (70-164)
+ *BMS187*	93 (85-101)	8 (3-21)	105 (99-111)	4 (2.7-6.9)
+ *BMS122*	94 (87-102)	14 (6.7-36)	105 (100-110)	47 (37-60)
Fentanyl	84 (79-88)	60 (42-85)	83 (79-86)	1.6 (1.2-2.3)
+ *BMS187*	110 (102-117)	9 (3.9-20)	90 (88-93)	0.009 (0.005-0.02)
+ *BMS122*	92 (86-98)	12 (5-31)	88 (86-90)	0.63 (0.5-0.8)
Methadone	95 (86-105)	221 (125-406)	91 (83-100)	226 (138-380)
+ *BMS187*	101 (93-109)	13 (4.2-38)	89 (87-92)	1.5 (1.2-1.8)
+ *BMS122*	89 (80-99)	10 (4-27)	76 (72-80)	35 (23-54)
Morphine	77 (67-88)	40 (15-100)	43 (37-51)	312 (127-779)
+ *BMS187*	106 (100-113)	11 (5.9-19)	95 (87-103)	30 (18-50)
+ *BMS122*	98 (92-103)	8 (4.3-15)	83 (80-87)	254 (201-322)
Met-Enkephalin	91 (87-96)	16 (12-23)	83 (80-86)	352 (299-415)
+ *BMS187*	94 (88-99)	4 (1.4-9.3)	98 (95-101)	9 (7.7-11)
+ *BMS122*	84 (79-88)	2 (1.0-5)	84 (81-88)	282 (235-341)
SR17018	64 (57-71)	147 (81-266)	114 (109-120)	3055 (2646-2564)
+ *BMS187*	88 (83-92)	8 (4.3-17)	115 (110-120)	215 (172-269)
+ *BMS122*	62 (55-70)	65 (19-198)	100 (96-104)	1848 (1598-2146)

To compare the relative degrees of PAM-mediated leftward shifts in the concentration-response curves for the orthosteric agonists we determined the ratio between the shift in the GTPγ^{35}S assay/shift in the β-arrestin assay (Table 2.2). For all

agonists, with the exception of SR17018, the ratio for BMS-986187 was < 1, indicating preferential shifts in the β-arrestin recruitment response curves. Conversely, for BMS-986122, a ratio of > 1 for all agonists, suggesting a bias to greater shifts in the GTPγ^{35}S concentration-response curves. Although the effects of BMS-986187 were much greater than those of BMS-986122. For SR17018, the fold shift ratio in the presence of either PAM was the same and favored G-protein signaling (1.30 for BMS-986187 and 1.35 for BMS-986122).

Table 2.2. Fold shift values and ratios for (GTPγS/β-arr) EC_{50} values. Values calculated as mean EC_{50} of agonist + vehicle/mean EC_{50} of agonist + 10 μM modulator (values from Table 2.1). Fold shift ratios are calculated as GTPγS/β-arrestin for both BMS-986187 (BMS187) and BMS-986122 (BMS122). Ratio values larger than 1 indicate a larger shift in the GTPγS EC_{50}, whereas values smaller than 1 demonstrate a larger shift in the β-arrestin EC_{50}.

	BMS-986187		**BMS-986122**	
	Fold Shift	**Ratio (GTPγS/β-arr)**	**Fold Shift**	**Ratio (GTPγS/β-arr)**
DAMGO GTPγS	10.5	0.47	6.0	3.16
DAMGO β-arrestin	22.5		1.9	
Fentanyl GTPyS	6.7	0.04	5.0	2.0
Fentanyl β-arrestin	177.8		2.5	
Methadone GTPγS	17.0	0.11	22.1	3.4
Methadone β-arrestin	151.0		6.5	
Morphine GTPγS	3.6	0.35	5.0	4.17
Morphine β-arrestin	10.4		1.2	
Met-Enk GTPγS	4.0	0.10	8.0	6.15
Met-Enk β-arrestin	39.1		1.3	
SR17018 GTPγS	18.4	1.30	2.3	1.35
SR17018 β-arrestin	14.2		1.7	

Determination of ligand bias in the presence and absence of BMS-986187 and BMS-986122.

To compare the ability of agonists to signal to G-protein versus recruit β-arrestin we determined the bias of each ligand by comparing responses normalized to the standard full MOR agonist, DAMGO using the method of Kenakin (2017) as follows: for each orthosteric ligand and respective signaling response, individual experimental curves were used to calculate log(max/EC50). The difference in log(max/EC50) between β-arrestin recruitment and GTPγ35S, Δlog(max/EC50), was then calculated. The difference between these values for each orthosteric ligand were then compared to a neutral ligand to calculate ΔΔlog(max/EC50) values. Individual results were combined to give means ± SEM values. DAMGO was chosen as a reference ligand as it has been reported to exhibit no bias between the two assays (McPherson *et al.*, 2010, Manglik, 2016, Conibear & Kelly, 2019). Relative to DAMGO (Figure 2.5, Table 2.4), fentanyl presented with a bias factor value of -1.65, representing a bias of 45-fold for β-arrestin 2 over G protein. Methadone shows a neutral bias with a value of -0.05, whereas Met-Enkephalin (1.31 or 20-fold), and SR17018 (0.99 or 10-fold) display significant G protein bias relative to DAMGO.

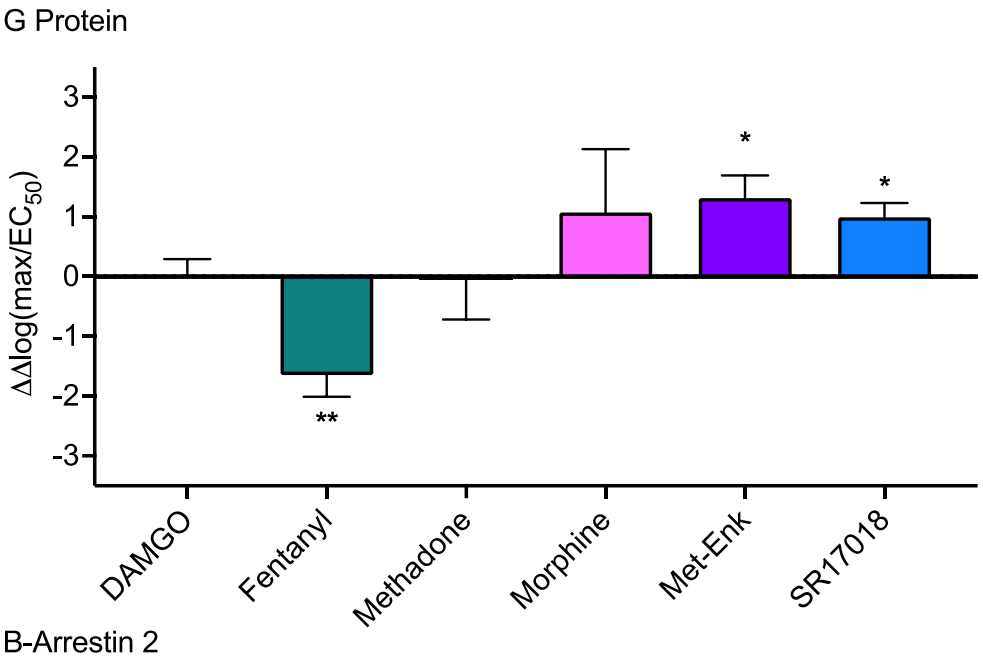

Figure 2.5. Orthosteric agonist bias factors compared to DAMGO. Bias factors were determined using ΔΔlog $(\frac{max}{EC50})$ from each individual experiment. The max and EC50 values used in the calculations were determined in the previously shown concentration-response curves in the GTPγS and β-arrestin assays. Positive values signify a GTPγS bias, whereas negative values signify a β-arrestin bias. Bias factors shown are follows; (DAMGO: 0, Fentanyl: -1.65, Methadone: -0.05, Morphine: 1.07, Met-Enk: 1.31, SR17018: 0.99). Data are represented as mean ± SEM (n =5). *P<0.05, **P<0.01 as determined by one-way ANOVA with Tukey's post-hoc test.

To determine the effect of the PAMs on the degree of bias we compared the compounds to DAMGO in the presence of the PAMs since the PAMs induce a shift in the DAMGO concentration-response curves for both assays (Figure 2.6A, Table 2.3). Under these conditions, in the presence of BMS-986187, the β-arrestin bias factor for fentanyl increased to -2.56 or 363-fold. The only other compound to show a significant bias was SR17108 which showed an increase in G-protein bias to 1.6 or 40-fold. This suggests a probe dependence for BMS-986187 toward fentanyl and SR17018. In contrast, there was no change in the bias of compounds relative to DAMGO in the presence of BMS-986122

(Figure 2.6B, Table 2.3). Fentanyl retained its β-arrestin bias (-1.74 or 55-fold) and Met-Enkephalin maintained a slightly enhanced G-protein bias (1.67 or 47-fold).

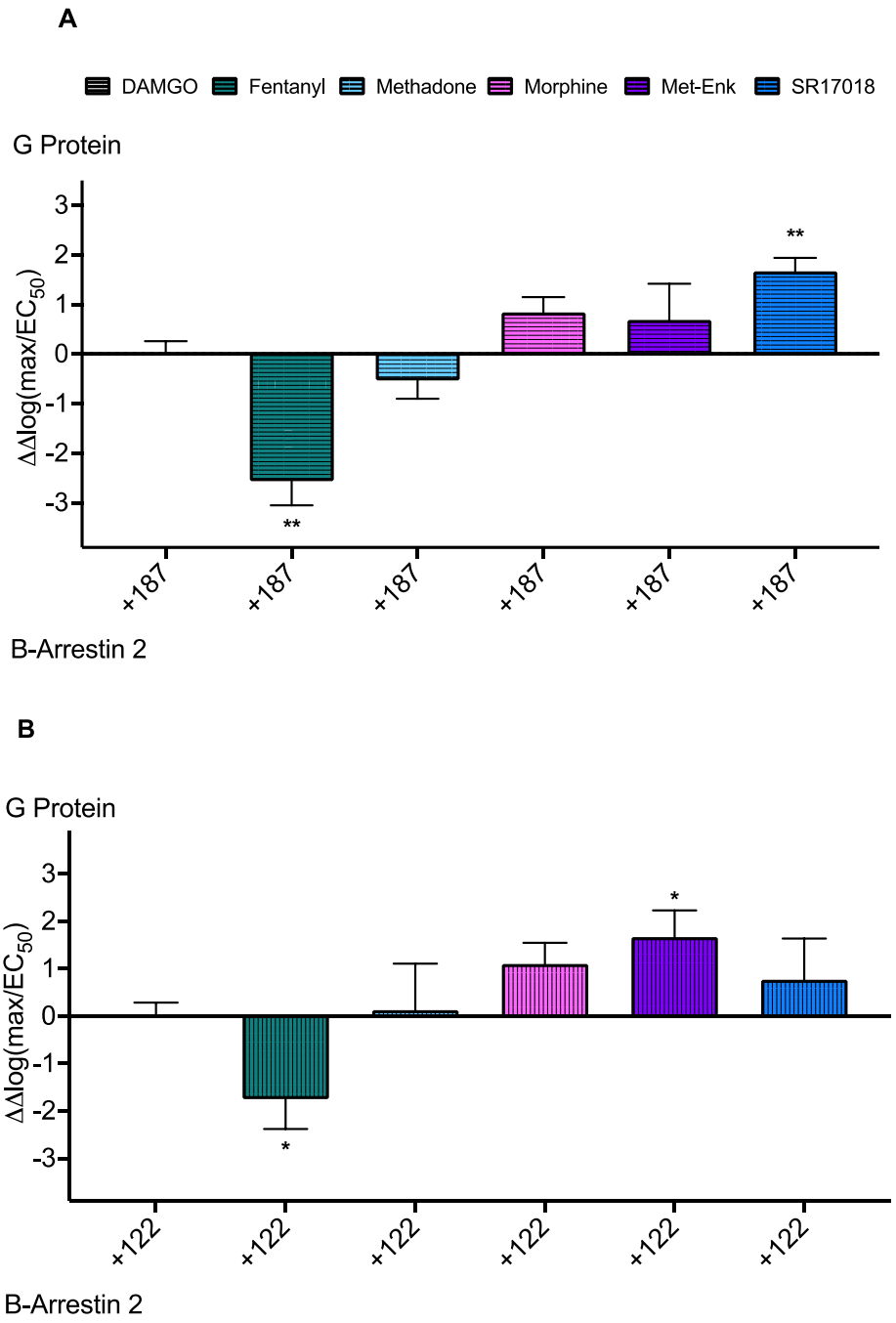

Figure 2.6. The effect of signaling bias relative to DAMGO in the presence of positive allosteric modulators. Bias factor calculations are formulated using either **A.** DAMGO + BMS-986187 (187) or **B.** DAMGO + BMS-986122 (122) as the reference ligand. Positive values indicate a GTPγS bias, whereas negative values a β-arrestin bias. Data are represented as mean ± SEM (n=5). *P<0.05 and **P<0.01 as determined by one-way ANOVA with Tukey's post-hoc test.

Table 2.3. Compiled bias factors using DAMGO + PAM as the reference ligand. Bias factors were determined using $\Delta\Delta\log\left(\frac{max}{EC50}\right)$ and SEM values were propagated from the mean values of each experiment. The max and EC50 values from the GTPγS and β-arrestin 2 recruitment assays used were determined in Figs. 2.1 and 2.2. Positive values represent a GTPγS bias, whereas negative values a β-arrestin bias. Data are represented as mean ± SEM.

	Bias Factors ΔΔlog(Max/EC50)	
	DAMGO + 187 Reference (± SEM)	**DAMGO + 122 Reference (± SEM)**
Fentanyl	-1.22 (± 0.35)	-2.05 (± 0.37)
+ *BMS187*	-2.56 (± 0.49)	
+ *BMS122*		-1.74 (± 0.63)
Methadone	0.38 (± 0.67)	-0.45 (± 0.67)
+ *BMS187*	-0.53 (± 0.37)	
+ *BMS122*		0.13 (± 0.98)
Morphine	1.50 (± 1.06)	0.67 (± 0.66)
+ *BMS187*	0.84 (± 0.31)	
+ *BMS122*		1.10 (± 0.45)
Met-Enkephalin	1.74 (± 0.37)	0.90 (± 0.38)
+ *BMS187*	0.69 (± 0.73)	
+ *BMS122*		1.67 (± 0.56)
SR17018	1.42 (± 0.23)	0.59 (± 0.25)
+ *BMS187*	1.67 (± 0.27)	
+ *BMS122*		0.77 (± 0.87)

To directly observe any changes in the relative abilities of orthosteric agonists to activate G-protein or β-arrestin in the presence of either PAM, we determined bias factors

by using each opioid agonist as its own reference ligand. Under these conditions (Figure 2.7, Table 2.4), BMS-986187 slightly shifted the signaling bias for all orthosteric ligands except SR17018 towards β-arrestin 2 such that fentanyl maintained its β-arrestin bias and Met-Enkephalin and SR17018 lost their original G-protein bias. In contrast, BMS-986122 shifted the bias of all ligands except SR17108 away from β-arrestin and towards G-protein, such that fentanyl lost its β-arrestin bias.

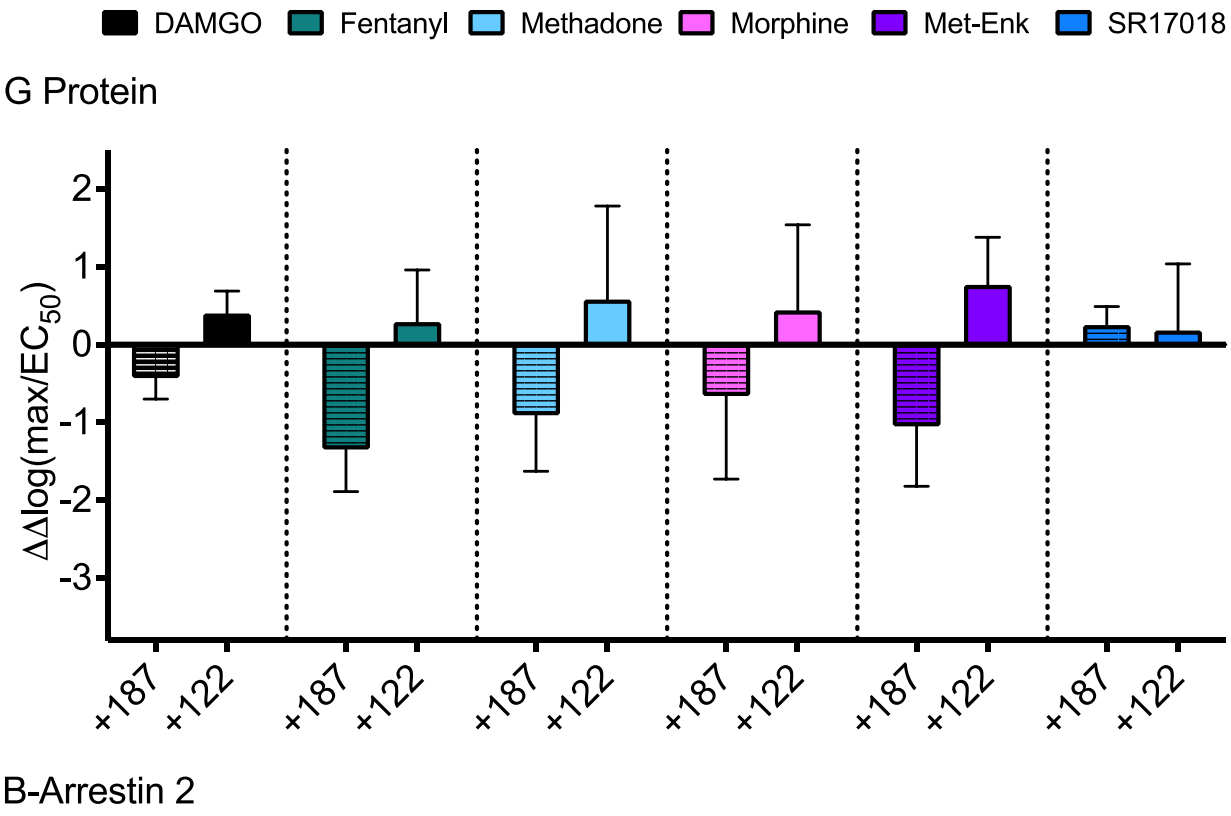

Figure 2.7. The effect of BMS-986187 and BMS-986122 on orthosteric ligand bias using each agonist in the presence of vehicle as its own reference. Bias factor calculations were determined using $\Delta\Delta\log(\frac{max}{EC50})$ for each individual experiment. The max and EC50 values used in the calculations were determined in the previously shown concentration-response curves in the GTPγS and β-arrestin assays. Positive values represent a G protein bias, whereas negative values a β-arrestin bias. Data are represented as mean ± SEM (n =5).

Table 2.4. Bias factors using either DAMGO or orthosteric ligand as the reference for calculations. Bias factors were determined using $\Delta\Delta\log\left(\frac{max}{EC50}\right)$ and SEM values were propagated from the mean values of each experiment. The max and EC_{50} values from the GTPγS and β-arrestin 2 recruitment assays used were determined in Figs. 2.1 and 2.2. Positive values represent a G protein bias, whereas negative values a β-arrestin bias. Data are represented as mean ± SEM.

	Bias Factors ΔΔlog(Max/EC50)	
	DAMGO + veh Reference (± SEM)	**Ligand + veh Reference (± SEM)**
DAMGO	0 (± 0.3)	0 (± 0.29)
+ *BMS187*		-0.43 (± 0.27)
+ *BMS122*		0.40 (± 0.29)
Fentanyl	-1.65 (± 0.36)	0 (± 0.43)
+ *BMS187*		-1.34 (± 0.54)
+ *BMS122*		0.31 (± 0.67)
Methadone	-0.05 (±0.67)	0 (± 0.91)
+ *BMS187*		-0.91 (± 0.72)
+ *BMS122*		0.58 (± 1.20)
Morphine	1.07 (± 1.06)	0 (± 1.47)
+ *BMS187*		-0.66 (± 1.07)
+ *BMS122*		0.43 (± 1.12)
Met-Enkephalin	1.31 (± 0.38)	0 (± 0.46)
+ *BMS187*		-1.05 (± 0.77)
+ *BMS122*		0.77 (± 0.61)
SR17018	0.99 (± 0.24)	0 (± 0.19)
+ *BMS187*		0.25 (± 0.24)
+ *BMS122*		0.18 (± 0.85)

MOR internalization in the presence of BMS-986187 or BMS-986122.

Since BMS-986187 promotes β-arrestin recruitment we hypothesized that this PAM would enhance MOR internalization. We measured this as a loss of SpH-MOR

(superecliptic phluorin-MOR) surface expression in HEK cells (Fig 2.8). DAMGO, methadone, fentanyl, but not morphine afforded a concentration-dependent reduction in MOR surface expression. BMS-986187 co-treatment significantly decreased expression at 100nM DAMGO from 58 ± 2 % to 33% ± 4, which represented a plateau. BMS-986122 had no effect. Methadone (1 μM) decreased surface expression to 56% ± 10, whereas BMS-986187 or BMS-986122 co-treatment reduced surface expression to 35% ± 3 and 37% ± 4, respectively. In the presence of either BMS-986187 and BMS-986122, morphine at 1μM, reduced surface expression to 47% ± 5 and 52% ± 6, respectively. Finally, fentanyl treatment displayed an efficient concentration-dependent reduction in surface expression that was not enhanced by either BMS-986187 or BMS-986122.

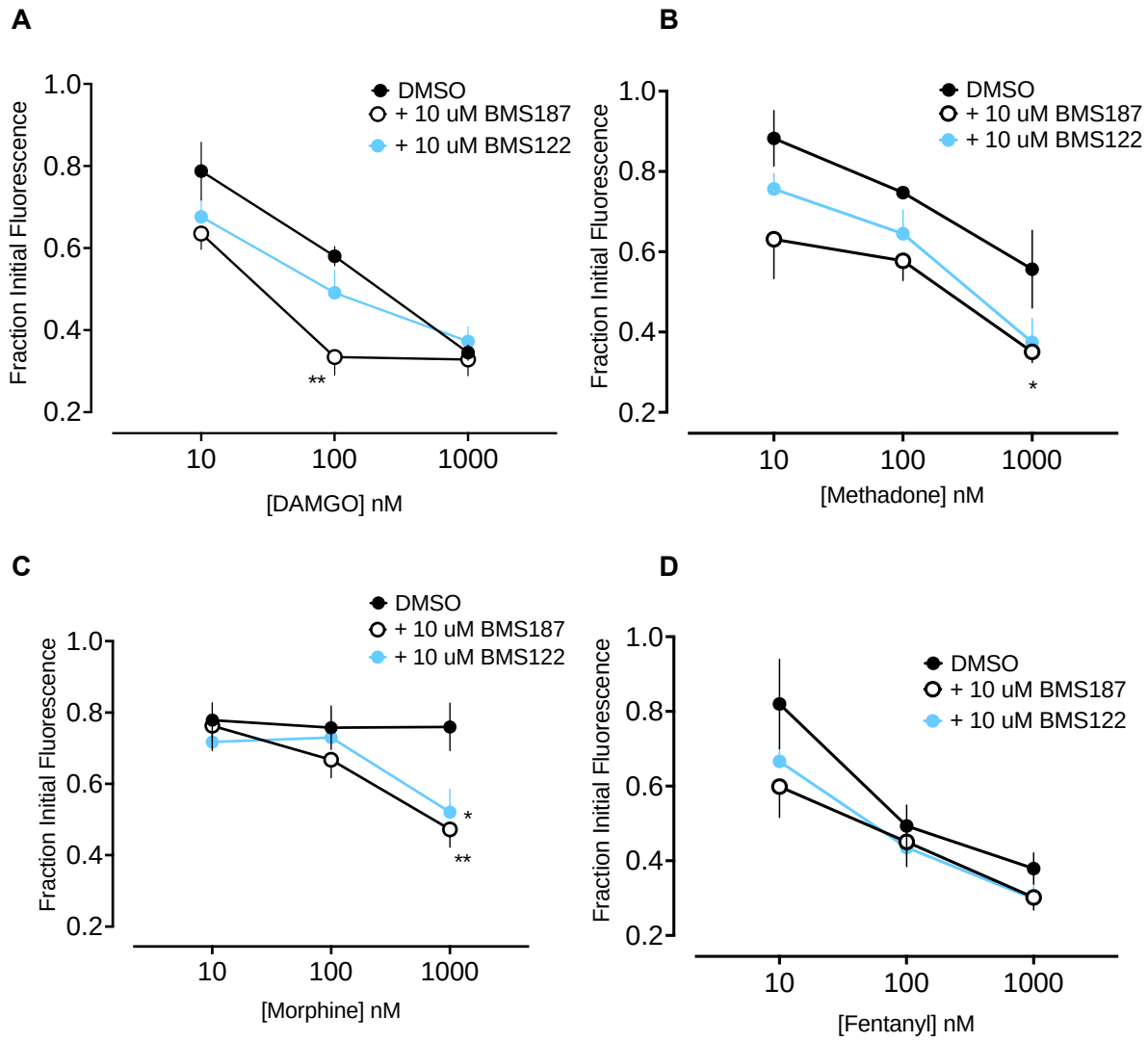

Figure 2.8. The effect of positive allosteric modulation on MOR agonist-induced internalization in HEK293 cells. BMS-986187 (BMS187) enhances **A.** DAMGO, **B.** Methadone, and **C.** Morphine-induced internalization of MOR. BMS-986122 (BMS122) potentiates **B.** Methadone and **C.** Morphine-induced internalization of MOR. Neither modulator had an impact on **D.** Fentanyl-induced internalization of the receptor. Data are represented as means ± SEM from three separate experiments in triplicate. *P<0.05 and **P<0.01 as determined by two-way ANOVA with Dunnett's post-hoc test.

Discussion:

This study demonstrates that the opioid PAM BMS-986187, in general, promotes the ability of agonists acting at MOR to recruit β-arrestin 2 to a greater extent than G

protein activation. Conversely, the PAM BMS-986122 consistently promotes G-protein activation over β-arrestin recruitment. However, there are differences in the degree of shift in potency (EC_{50} values) induced by the modulators, depending on the agonist occupying the orthosteric site, indicating probe-dependent effects. This is especially true for BMS-986187 where the shifts in the β-arrestin assay are between 10- and 170-fold, although much smaller shifts are seen in the G-protein assay (4- to 22-fold). Moreover, the order of sensitivity of orthosteric agonists to BMS-986187 is different across the two assays. With BMS-986122, the shifts observed are much smaller, and probe dependence is much less evident in both the β-arrestin and G-protein assays. Several of the orthosteric agonists show bias toward either G-protein or β-arrestin when compared to DAMGO. The degrees of bias show a trend to move toward β-arrestin in the presence of BMS-986187 and toward G-protein in the presence of BMS-986122. Despite the differences induced by the two modulators, the enhancement of agonist-mediated internalization of MOR is similar. Both modulators behaved as ago-PAMs in the G-protein assay but were silent in the β-arrestin assay.

In the GTPγ^{35}S assay the orthosteric agonists were effective with EC_{50} values between 16 nM for Met-enkephalin to 220 nM for methadone. Compared to DAMGO, morphine, fentanyl, and SR17018 behaved as partial agonists in this system. In the β-arrestin assay, the compounds were either equipotent with the GTPγ^{35}S assay (DAMGO, methadone), more potent (fentanyl), or less potent (morphine, Met-Enkephalin, SR17018). These results translate to significant G protein bias for Met-Enkephalin and SR17018. Previous literature has presented morphine as neutral, methadone as β-

arrestin biased, and Met-enkephalin and SR17018 as G-protein biased (Schmid et al., 2017, Doi et al., 2016, Gomes et al., 2020). In contrast to the other opioids, fentanyl showed a significant β-arrestin bias in support of previous findings comparing the GTPγ^{35}S assay to the β-arrestin recruitment assay (Schmid et al., 2017). However, this finding depends on the assays employed, the receptor and cell type, and the method of calculation (Burgueño et al., 2017; Schmid et al., 2017; Conibear and Kelly, 2019; Ramos-Gonzalez et al., 2023; Rivero et al., 2012; Winpenny et al., 2016; Vasudevan et al., 2020). Moreover, choosing to evaluate bias relative to DAMGO was based on a previous study suggesting DAMGO has a neutral signaling profile (Piekielna-Ciesielska *et al.,* 2021), although Rivero et al., 2012 show that compared to the endogenous ligand Leu-enkephalin, DAMGO trends towards G-protein bias. Furthermore, if we had used the endogenous Met-enkephalin as the neutral reference ligand this would have resulted in no observed bias for SR17018 and an increased β-arrestin bias factor for fentanyl (-1.65 to -2.95).

BMS-986187 and BMS-986122 enhanced the potency and efficacy of MOR orthosteric agonists as determined in both the GTPγ^{35}S and β-arrestin assays. However, the degree of shift in agonist dose-response curves observed with the allosteric modulators depended on the agonist occupying the orthosteric site. This probe dependence is not qualitatively or quantitatively consistent across the two assays and differs between the modulators. Large shifts in the β-arrestin assay in the presence of BMS-986187 are seen with fentanyl and methadone compared to other opioids. In contrast, in the G-protein assay, compounds show much smaller shifts and generally

similar shifts across the compounds examined. In the presence of BMS-986122 shifts in the GTPγ^{35}S assay are larger than in the β-arrestin assay, but overall shifts are much smaller than observed with BMS-986187 suggesting BMS-986122 is a less efficacious modulator. There is also much less of a probe dependence with BMS-986122 with the exception of methadone which is more sensitive than the other opioids in accordance with previous reports (Livingston et al, 2014; 2018). Compared to the other opioids, the structurally different benzimidazole derivative, SR17018, shows similar shifts in the GTPg^{35}S and b-arrestin assays in response to either BMS-986187 or BMS-986122. This different response compared to the more traditional opioid drugs and peptides may be because this compound has been suggested to be a non-competitive agonist which binds to a site separate from the orthosteric site. Nonetheless, our data shows this site is sensitive to allosteric modulation and SR17018 has previously been shown to be displaced by naloxone (Stahl et al., 2021; Fritzwanker et al., 2021).

In line with its ability to increase MOR-agonist-mediated β-arrestin recruitment to a greater extent than G-protein activation, BMS-986187 significantly enhanced the β-arrestin bias for fentanyl and caused methadone to shift to β-arrestin biased and also tended to reduce the G-protein bias of morphine and Met-enkephalin, but it did not change the bias of SR17108. In contrast, BMS-986122 did not cause any significant change in the degree of bias across the different opioids, although the bias for every ligand did tend to move towards G protein.

We have previously shown BMS-986187 to be a G-protein-biased ago-PAM at the delta-opioid receptor (DOR) (Stanczyk *et al.*, 2019) which appears to contradict its effects to significantly enhance β-arrestin signaling of MOR agonists. BMS-986187 alone stimulated GTPγ^{35}S binding at MOR but did not stimulate β-arrestin recruitment, suggesting BMS-986187 on its own is unlikely to be β-arrestin biased at MOR. It is possible the larger effect on β-arrestin recruitment with orthosteric agonists could be explained by the higher efficacy requirements of the assay, suggesting that increased MOR receptor reserve would have a greater impact on this pathway than on G-protein activation. However, it is unclear why the same would not apply to BMS-986122 since previous pharmacological work has suggested these two structurally distinct PAMs bind to a geographically similar allosteric site on MOR (Livingston *et al.*, 2018). Computational studies supported by mutagenesis also predict a similar site for both compounds on the extracellular side of the receptor (Bartuzi et al., 2016; Shang et al., 2016); although the studies with BMS-986187 were modeled at DOR, not MOR. Common amino-acids interacting with the two modulators are in transmembrane (TM) domains 2 and 7 as follows (Ballesteros & Weinstein, 1995): Trp7.35, His 7.36, Ile 7.39, Tyr 2.64, and the residue at 2.63, although this is Asn in MOR and Lys in DOR. This difference, plus the more extensive allosteric site for the larger BMS-986187 involving residues in TM 1 and some interactions with TM6 (Shang et al., 2016), could explain the receptor selectivity of the two molecules. This explanation suggests the PAMs may not necessarily transmit their effects at MOR in the same way, allowing for variation in how orthosteric ligands respond to potential conformational changes in the binding pocket. However, there are several other predicted allosteric sites on MOR (Chan et al., 2023) that are available to

BMS-986122 and BMS-986187. Additionally, recent NMR evidence indicates a site for BMS-986122 that is close to the cytosolic side of the receptor, specifically a cleft close to T162 on TM3. The binding of BMS-986122 to this region would change the interactions between TM3 and TM6, leading to stabilization of TM6 in an active position that favors G protein binding (Kaneko et al., 2022).

Consistent with the observed enhancement of β-arrestin 2 recruitment, BMS-986187 produced a greater loss in cell surface MOR expression than BMS-986122 for DAMGO and methadone. In addition, both BMS-986122 and BMS-986187 increased surface expression loss when paired with morphine. This is consistent with the β-arrestin 2 recruitment data, where both PAMs increased the potency and efficacy of morphine, though BMS-986187 to a larger extent. However, the fentanyl-induced internalization does not support the β-arrestin 2 recruitment data. Neither BMS-986187 nor BMS-986122 promoted fentanyl-mediated loss in cell surface MOR expression despite BMS-986187 showing a 178-fold shift in the β-arrestin 2 recruitment assay. It is possible that fentanyl alone recruits sufficient β-arrestin 2 for efficient internalization of MOR.

Overall, the findings show that allosteric modulators can differentially affect G-protein activation and β-arrestin recruitment downstream of MOR and therefore alter the preference of a ligand for these two responses. However, there is a probe dependence such that the direction and strength of the effect depend on the modulator and the orthosteric agonist. The hypothesis that biased signaling at MOR might alter the pharmacological profile of opioid drugs remains controversial. It will be of interest to

determine if PAMs appear to have a marked effect on compounds *in vivo* (Kandasamy et al., 2021). Future studies may examine if PAMs, in particular BMS-986187, potentially alter the magnitude of orthosteric responses or the pharmacological profile of orthosteric agonists *in vivo*.

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Chapter 3 : Opioid Sparing by a Mu Opioid Receptor Positive Allosteric Modulator

Abstract:

Opioids that act at the mu-opioid receptor (MOR) are the gold standard for the clinical management of pain but can induce serious side effects such as constipation, addiction liability, and the fatal overdose effect of respiratory depression. Positive allosteric modulators (PAMs) of MOR enhance the actions of both small molecule opioids and peptidic opioids in vitro. Moreover in vivo these compounds enhance endogenous opioid peptide antinociception, but do not to cause constipation, reward or respiratory depression. Thus, they act as potentially safer analgesics than traditional opioid therapeutics. However, the *in vivo* effects of PAMs on exogenous opioids have not been studied. We hypothesize that MOR PAMs could be used to selectively enhance the beneficial effects induced by opioids, but not the adverse effects. This theory would be clinically applicable for opioid sparing, that is combining opioid drugs with other compounds to reduce the total dosage of opioids used and help to combat the current opioid epidemic. In this study, we show the influence the MOR PAM, BMS-986122, has on effects induced by three clinically relevant opioids; morphine, methadone, and fentanyl. Here, we show that BMS-986122 enhances MOR opioid agonist-induced antinociception, but not the adverse effects of constipation, respiratory depression or

reward as measured by conditioned place preference. Overall, these data provide a rationale for further development of MOR PAMs to be used in opioid sparing.

Introduction:

Opioids remain the gold standard for clinical management of moderate to severe pain. Traditional opioid therapeutics like morphine act as agonists of the mu opioid receptor (MOR). Although highly effective in relieving pain their use is associated with severe adverse effects, such as constipation, addiction liability, and fatal respiratory depression. Over 106,000 Americans died from an opioid overdose in 2021, including illicit and prescription opioids (NIDA, 2022). The rate of overdose deaths involving synthetic opioids has steadily increased since 2019, and fentanyl-induced overdoses continue to be a growing concern in the United States (NIDA, 2022). As such, it is imperative to study potential alternatives for pain management.

Current clinical efforts to maintain effective pain relief while taking lower doses of opioids involves “multimodal analgesia” or employing multiple pain relief methods to minimize side effects and reduced the potential for development of addiction (Ghai et al., 2022). This “opioid-sparing” technique involves utilizing both non-pharmacological and pharmacological techniques in combination with opioid drugs. Non-pharmacological methods include cognitive behavioral therapy, music therapy, acupuncture, and several neuromodulation therapies such as “electro analgesia”. (Tsivian et. al, 2012; Hole et al., 2015; Ntritsou et al., 2014; Colquhoun and Novella, 2013; Geziry et al., 2018; Tsegaye et al., 2023). Common pharmacological methods of opioid sparing include the use of

acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs) cannabinoids, N-methyl-D-aspartate (NMDA) receptor antagonists such as ketamine, anticonvulsants, steroids, alpha-2 adrenergic receptor agonists, such as clonidine (Cao et. al, 2016; Oliveira et al., 2011; White et al., 2012; Martinez et al., 2019). For example, the NMDA receptor antagonist ketamine has been shown to reduce narcotic use by 40% and delay the use of the first dose of opioids following surgical procedures (Laskowski et al., 2011, Kim et al., 2013, Kanupriya et al. 2017, Cohen et al., 2022). While pre-clinical studies have suggested that cannabinoids may be effective in opioid sparing, several randomized controlled trials show they have little to no effect in reducing opioid dosing but increase nausea in chronic cancer pain patients (Nielsen et al., 2022, Mun et al., 2022, Noori et al., 2021).

While current opioid-sparing methods have the potential for a positive impact to reduce opioid use, the level of effectiveness for chronic pain patients is heavily variable and the risk for rebound pain is high (Kumar et al., 2017). Furthermore, using additional pharmacological approaches increases the risk of exposure to adverse events induced by both the opioid system and adjuvant therapy. One potential alternative to reduce the opioid dose is to enhance the analgesic effects of existing drugs. This would be especially useful if analgesia could be selectively enhanced, at the expense of unwanted effects.

Positive allosteric modulators (PAMs) of MOR work by interacting with a site on MOR separate from the orthosteric site where the traditional opioid agonists bind. The MOR- PAM, BMS-986122, has been previously shown to enhance opioid drug and

endogenous opioid antinociception (Livingston & Traynor, 2018, Kandasamy *et al.,* 2021). However, it is not known if this PAM also enhances the unwanted actions of opioid drugs. In this work, we examined the effect of BMS-986122 on three clinically relevant opioids, morphine, methadone, and fentanyl, and their antinociceptive, constipation, respiratory, and rewarding actions. These opioids were chosen for study because of their clinical relevance, as well as their structural and pharmacological differences. Morphine is a traditional clinical standard for pain management, fentanyl is potent *in vivo* and prevalent in opioid overdose deaths, and *in vitro* methadone responds most robustly to the presence of BMS-986122 (Livingston & Traynor, 2014). Our findings demonstrate that BMS-986122 potentiates opioid-induced antinociception with no obvious effects to enhance opioid-mediated constipation or respiration, or reward. These data highlight a potential role for the development of MOR-PAMs as adjuvant therapies for opioid pain management.

Methods:

Drugs:

For behavioral experiments, drugs were dissolved as follows; BMS-986122 was dissolved in 10% dimethyl sulfoxide, 10% ethoxylated castor oil, and 80% sterile H2O; morphine sulfate, methadone, and fentanyl HCl were dissolved in sterile saline (0.9% NaCl); and buprenorphine was dissolved in sterile H2O. Drugs were obtained from the NIDA drug supply program (opioid agonists) and synthesized by the University of Michigan Vahlteich Medicinal Chemistry Core (BMS-986122).

Animals:

Behavioral experiments were conducted using male and female wildtype ICR: CD-1 mice between 8 and 16 weeks of age, bred in-house or purchased from Envigo Laboratories. All animals were group-housed by sex on a 12 h light/dark cycle (lights on 0700 hours). All testing was performed during the light phase. Mice and rats had free access to food and water in their home cage. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of the University of Michigan.

Warm Water Tail Withdrawal:

Using a 50°C water bath, mouse tail withdrawal latencies were measured following i.p. administration of BMS-986122 or vehicle (30 min pretreatment) and opioid agonist or saline. For cumulative dose-response curves, time points were collected, and injections were administered in either 30 or 15 min intervals depending on the agonist present. For time course experiments, data were collected at various time points following a single bolus dose of BMS-986122 or vehicle and opioid agonist. A latency of 20 sec was set as the cutoff time point to prevent tissue damage.

Hot Plate Assay:

Using a 52°C hot plate, mouse latencies (forepaw licking or jumping) were measured following i.p. administration of BMS-986122 or vehicle (30 min pretreatment) and opioid agonist or saline. For cumulative dose-response curves, time points were

collected, and injections were administered in 30 min intervals. A latency of 60 sec was set as the cutoff time point to prevent tissue damage.

Constipation Assay:

Single-housed mice were food-deprived for 16 h and then exposed to blue-colored food (21g chow, 40 mL sterile H2O, 5-10 drops of blue food dye) for 1 h. Following food exposure, mice were given injections of BMS-986122 or vehicle (i.p.) and opioid agonist or saline (s.c.) and the time to first blue fecal bolus and the total number of fecal boli were measured for 6 h thereafter.

Respiratory Depression:

Using the MouseOx pulse oximeter system (Starr Life Sciences Corp.), blood oxygen saturation, breath rate, and heart rate were measured in awake mice following BMS-986122 or vehicle (30 min pretreatment i.p.) and opioid agonist or saline (i.p.). Mice were single-housed and habituated to the collars for at least 2 h prior to the experiment.

Conditioned Place Preference (CPP) Assay:

Mice were conditioned to one side (black or white) of a two-compartment chamber (Med Associates Inc.) with fentanyl, BMS-986122, fentanyl + BMS-986122, or vehicle (i.p.). Chamber side bias was determined before conditioning, and the preferred side was paired with the vehicle. Conditioning consists of a 30 min morning session in the vehicle-paired chamber and a 30 min afternoon session in the drug-paired chamber. After 5 d of

conditioning, mice were given free access to both chambers for 30 min. CPP scores were calculated as the difference between time spent on the drug-paired side on test day compared to bias day. A positive CPP score correlates to increased reward and a negative value indicates aversion.

Data and Statistical Analysis:

Data analysis was performed using GraphPad Prism version 9.5.0 (GraphPad, San Diego, CA, USA). Statistical comparisons were made by two-way ANOVA with Tukey's post-hoc tests to correct for multiple comparisons for significant ANOVAs only. The criterion for significance was as follows; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. For respiration experiments, data were averaged into 5 minute (oxygen saturation and heart rate) or 10 minute (breath rate) bins and "peak effects" were reported. Peak effects were determined as the means of the data points displaying the most significant reduction in oxygen saturation, breath rate, or heart rate for each individual experiment.

Results

BMS-986122 enhances spinal-mediated opioid antinociception

The antinociceptive effects of morphine, methadone, and fentanyl in the presence of BMS-986122 were measured using the 50°C warm-water tail-withdrawal (WWTW) assay in CD-1 mice. The opioid agonists produced dose-dependent antinociception, with agonist potency ranked: fentanyl > methadone > morphine as expected (Meert and

Vermeirsch, 2005). Each opioid reached maximal effect (cutoff of 20 seconds) at the highest dose tested. The presence of 10 mg/kg BMS-986122 potentiated the effects of the opioids (Figure 3.1A-B) such that morphine at 5.6 mg/kg increased tail-withdrawal latency from 7.6 to 11.3 seconds, methadone at 3.2 mg/kg from 12.6 to 17. 4 seconds and 0.1 mg/kg fentanyl from 7.5 to 15.1 seconds. The BMS-986122-mediated potential of the fentanyl dose-response curve was larger (Figure 3.1C).

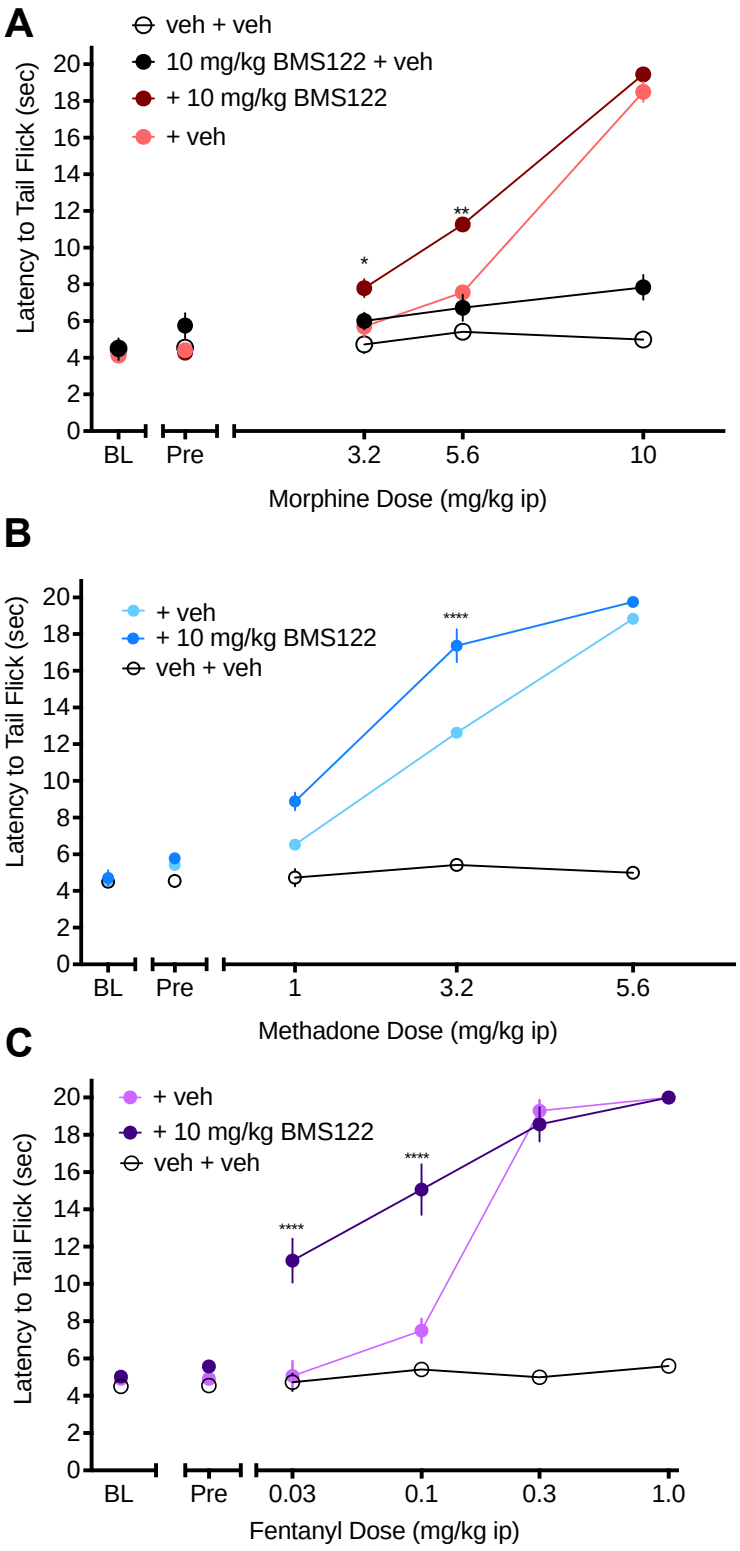


Figure 3.1. BMS-986122 enhances opioid-induced antinociception. Cumulative dose-response curve in the mouse WWTW assay at 50°C for morphine ($F=60.5$, $p<0.0001$) (**A**), methadone ($F=52.1$, $p<0.0001$) (**B**), and fentanyl ($F=57.3$, $p<0.0001$) (**C**) in the presence and absence of 10

mg/kg BMS-986122 (BMS122). A 20-second cutoff was set to prevent tissue damage. Data shown are means ± SEM for all groups (n = 6-12 for each group). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 as determined by two-way ANOVA with Tukey's post-hoc test.

To study the time course of BMS-986122 we administered single doses of agonist in the presence of 10 mg/kg BMS-986122 (Figure 3.2). The effects of BMS-986122 followed the time course pattern of the orthosteric agonist. With the 3.2 mg/kg dose of morphine, enhancement by BMS-986122 was observed from the 15 through 60 min time points (Figure 3.2A). Similarly, with 3.2 mg/kg methadone (Figure 3.2B) the enhanced effect of BMS-986122 was at 15-60 minutes, with the largest enhancement observed at 45 minutes; the 1 mg/kg dose shows only a slight increase in effect in the presence of BMS-986122 at 30 minutes. BMS-986122 potentiated the effect of both 0.03 and 0.1 mg/kg fentanyl for 15-45 minutes (Figure 3.2C). In each experiment, BMS-986122 and vehicle controls did not show any elevation in effect above baseline measurements.

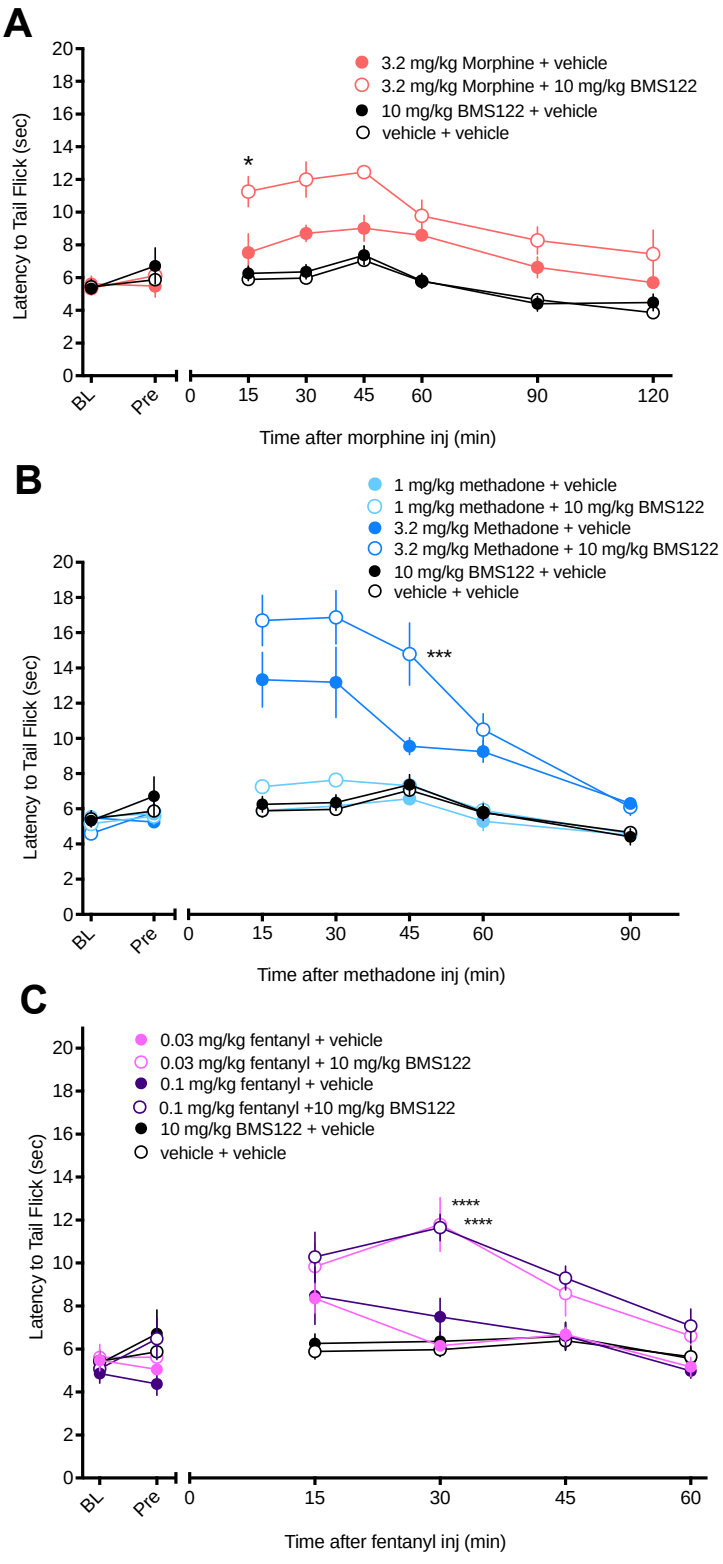


Figure 3.2. BMS-986122 promotes opioid action over time. Time-course in the 50°C mouse WWTW assay following a single injection of morphine (F=3.0, p<0.0001) (**A**), methadone (F=8.7, p<0.0001) (**B**), and fentanyl (F=3.1, p<0.0001) (**C**) in the presence or absence of 10 mg/kg BMS-

986122 (BMS122). A 20-second cutoff was set to prevent tissue damage. Data shown are means ± SEM for all groups (n = 6-10 for each group). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, as determined by two-way ANOVA with Tukey's post-hoc test.

BMS-986122 enhances supraspinal-mediated opioid antinociception

The 52°C Hot Plate assay is a higher efficacy requiring heat nociception assay. The opioid agonists produced dose-dependent antinociception, with potency rankings the same as in the WWTW assay. BMS-986122 administration caused an enhancement of the effect of each dose of morphine, most notably at 32 mg/kg with an increase from 30.6 to 47.6 seconds (Figure 3.3A). Methadone displayed a smaller degree of potentiation in the presence of BMS-986122, with 5.6 mg/kg showing the largest increase from 28.5 to 39.8 seconds (Figure 3.3B). Similarly to the results in the WWTW assay, fentanyl showed the largest potentiation in the presence of BMS-986122, for example at the 1 mg/kg dose, there was an enhancement in latency from 39.8 to 58.7 seconds (Figure 3.3C). The partial agonist buprenorphine was more potent than either morphine or methadone, but less potent than fentanyl, and only reached a latency of 27.6 seconds. BMS-986122 increased the latency time of buprenorphine to 37.5 seconds, though it did not reach maximal efficacy (Figure 3.3D). BMS-986122 showed no effect in the absence of an orthosteric agonist.

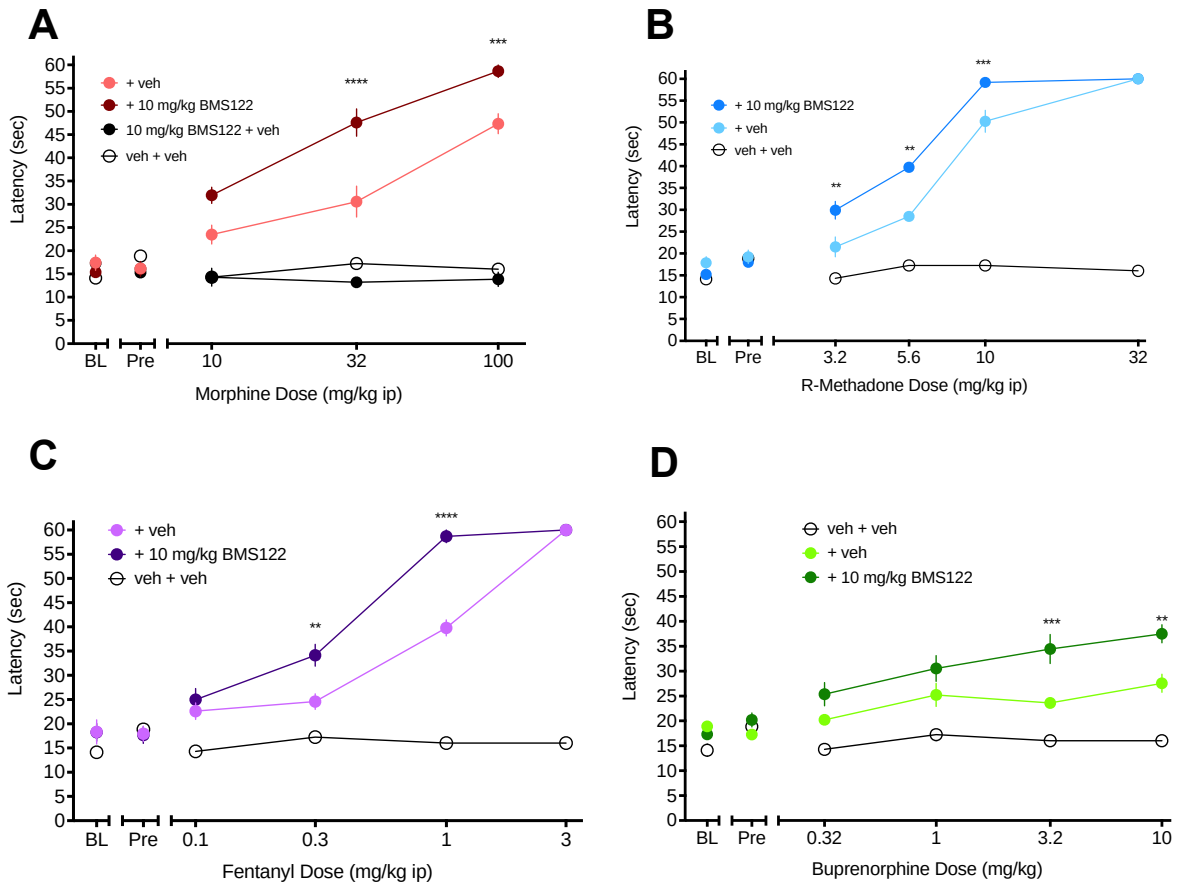
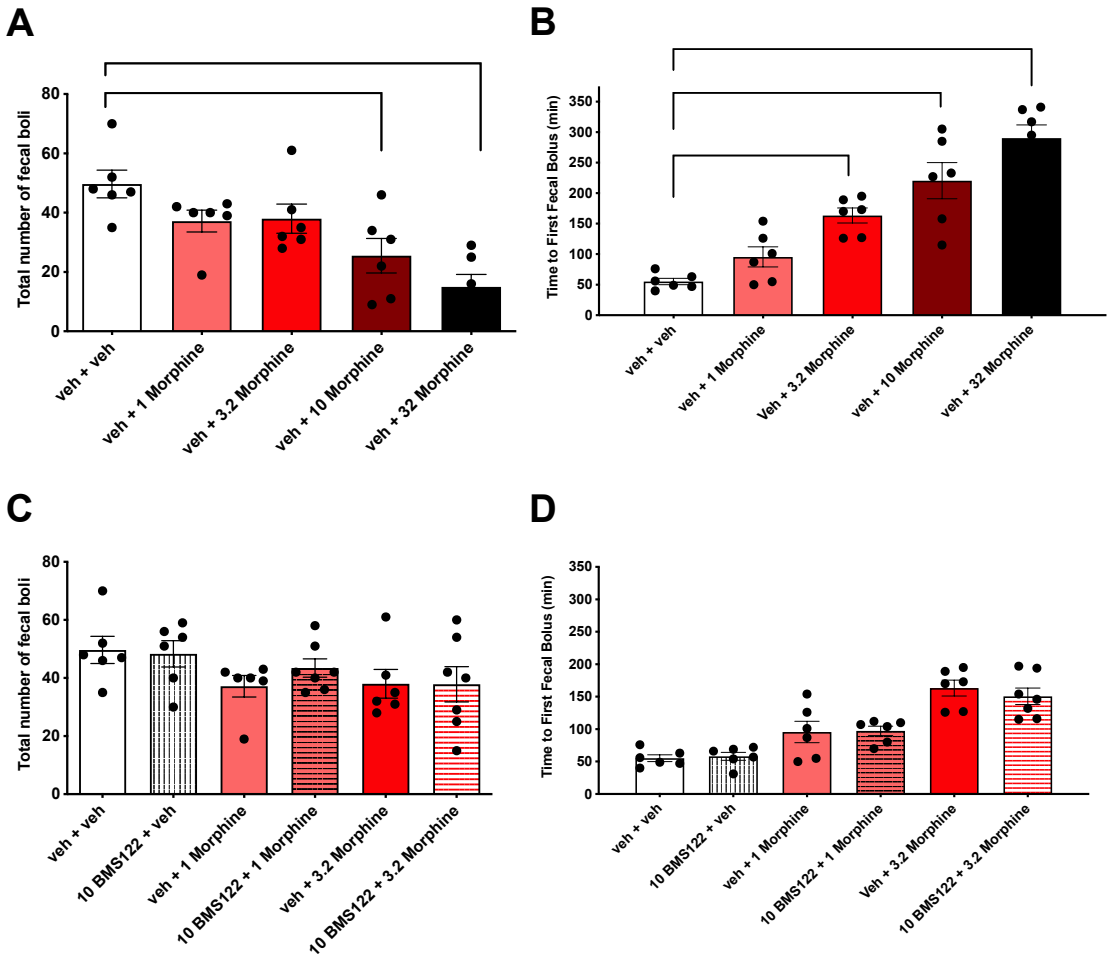


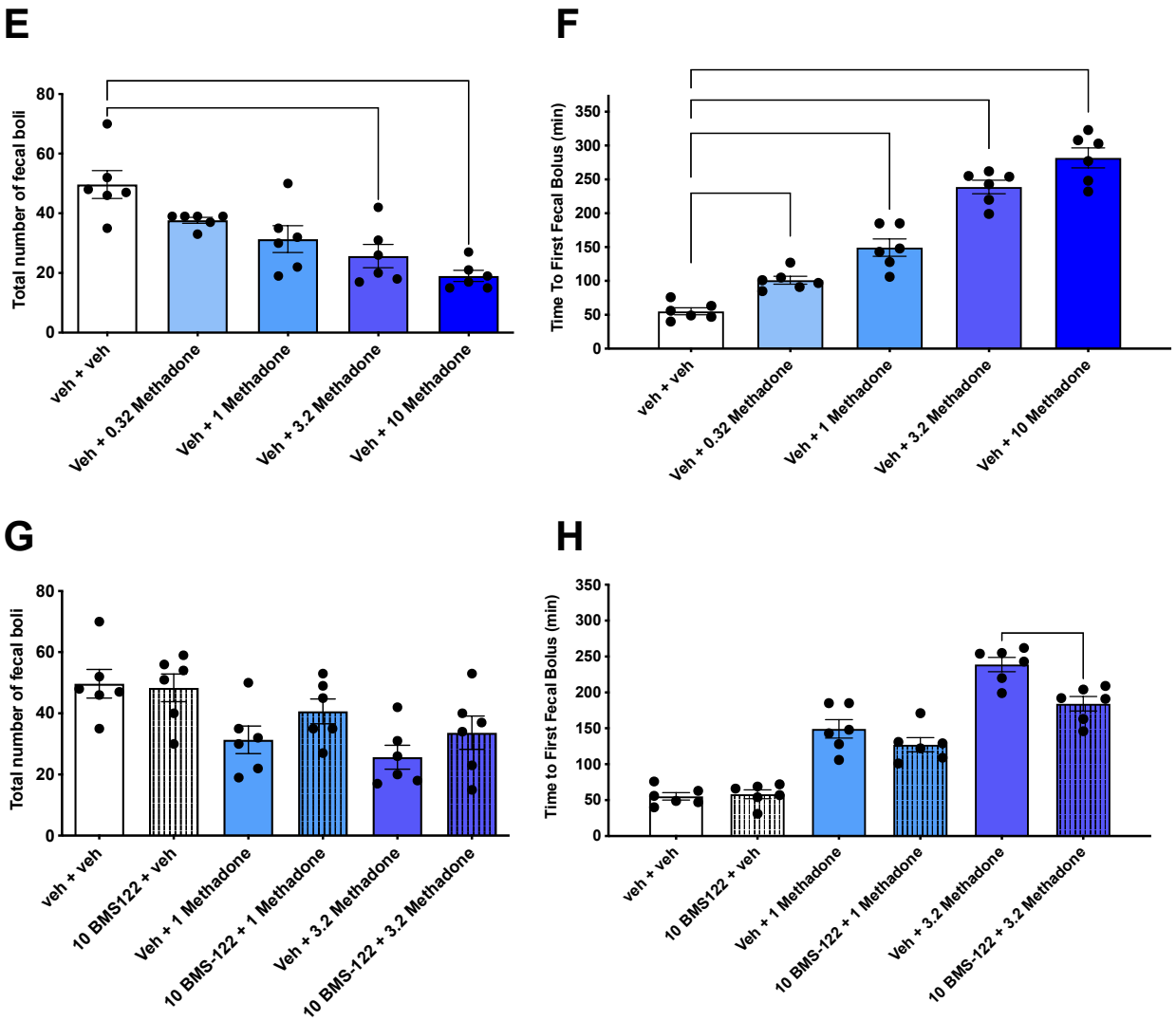
Figure 3.3. BMS-986122 potentiates supraspinally-mediated opioid-induced antinociception. Cumulative dose-response curve in the 52°C mouse hotplate assay for morphine (F=36.3, p<0.0001) (**A**), methadone (F=71.0, p<0.0001) (**B**), fentanyl (F=50.6, p<0.0001) (**C**), and buprenorphine (F=6.8, p<0.0001) (**D**) in the presence or absence of 10 mg/kg BMS-986122 (BMS122). A 60-second cutoff was set to prevent tissue damage. Data shown are means ± SEM for all groups (n = 6 for each group). **P<0.01, ***P<0.001, ****P<0.0001 as determined by two-way ANOVA with Tukey's post-hoc test.

The effect of BMS-986122 on opioid-induced constipation

The constipating effects of morphine, methadone, and fentanyl were examined in the presence and absence of 10 mg/kg BMS-986122 in CD-1 mice. The total number of fecal boli over 6 hours and the time to first fecal bolus following drug administration were determined. Vehicle-treated mice had a total number of 50 fecal boli over 6 hours and an average time of 55 minutes to the first fecal bolus. BMS-986122, administered on its own,

showed no significant difference compared to vehicle-treated mice. All three opioids caused dose-dependent constipation. Fentanyl was especially effective and at 10 mg/kg delayed the time to the first fecal bolus to 356 minutes (Figure 3.4I-J). In the presence of 10 mg/kg BMS-986122 the effects of submaximal doses of fentanyl were unchanged, measured either as the number of fecal boli or time to first bolus. A similar lack of an effect of BMS-986122 on constipation was observed for morphine and methadone.




E
Total number of fecal boli
0
20
40
60
80
veh + veh
Veh + 0.32 Methadone
Veh + 1 Methadone
Veh + 3.2 Methadone
Veh + 10 Methadone
F
Time To First Fecal Bolus (min)
0
50
100
150
200
250
300
350
veh + veh
Veh + 0.32 Methadone
Veh + 1 Methadone
Veh + 3.2 Methadone
Veh + 10 Methadone
G
Total number of fecal boli
0
20
40
60
80
veh + veh
10 BMS122 + veh
Veh + 1 Methadone
10 BMS-122 + 1 Methadone
Veh + 3.2 Methadone
10 BMS-122 + 3.2 Methadone
H
Time to First Fecal Bolus (min)
0
50
100
150
200
250
300
350
veh + veh
10 BMS122 + veh
Veh + 1 Methadone
10 BMS-122 + 1 Methadone
Veh + 3.2 Methadone
10 BMS-122 + 3.2 Methadone

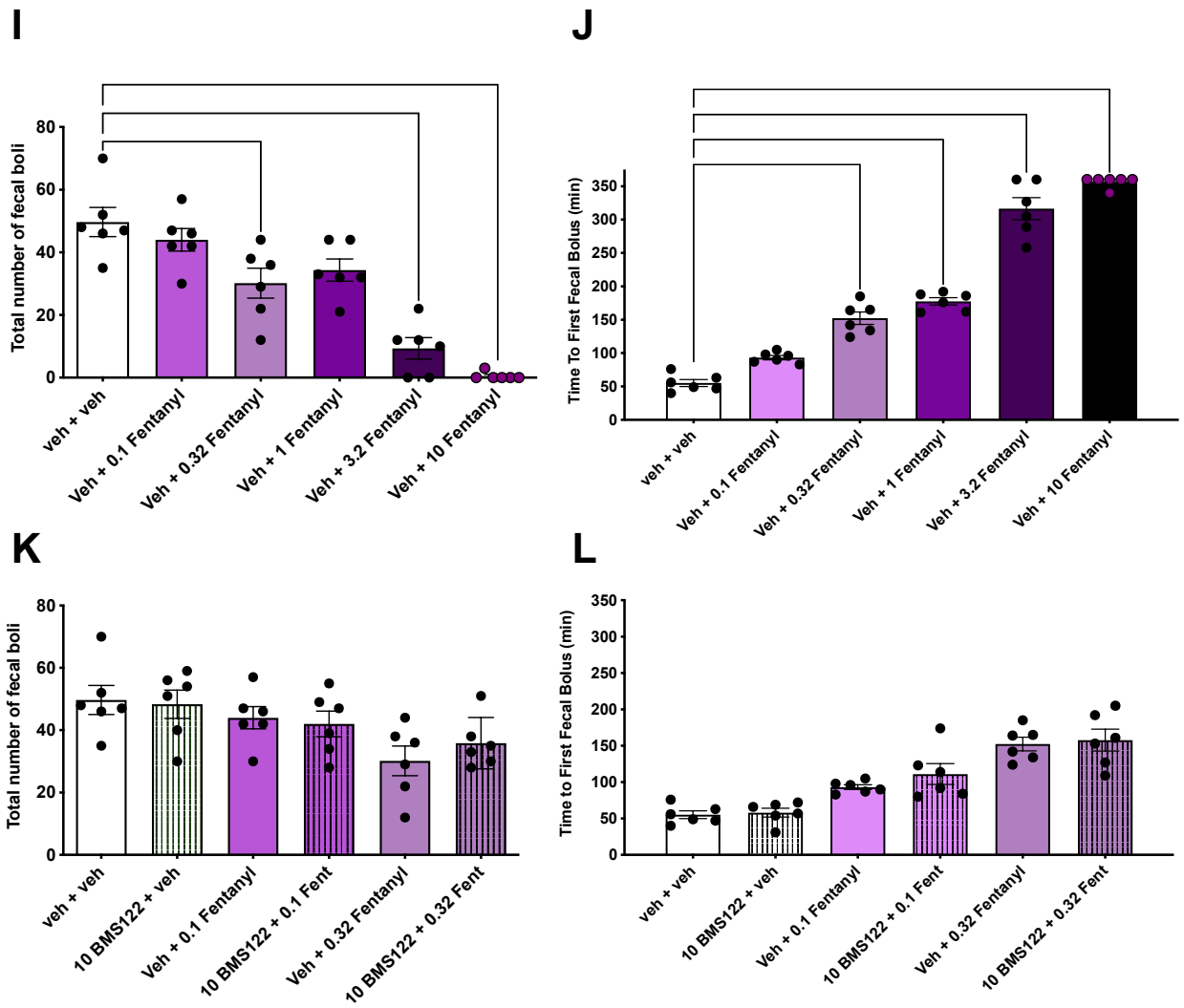


Figure 3.4. BMS-986122 does not enhance opioid-induced constipation. Constipation dose-response curves following administration of morphine ($F=6.5$, $p<0.0001$) (**A-D**), methadone ($F=7.3$, $p<0.0001$) (**E-H**), and fentanyl ($F=22.1$, $p<0.0001$) (**I-L**). Opioid administration alone produces dose-dependent decreases in the total number of fecal boli (**A, E, I**) and an increase in delay in time to the first fecal bolus (**B, F, J**). Opioid + BMS-986122 treatment does not potentiate these effects (**C-D, G-H, K-L**). Data shown are means ± SEM for all groups (n = 6 for each group). *P<0.05, **P<0.01 ***P<0.001, ****P<0.0001 as determined by two-way ANOVA with Tukey's post-hoc test.

The effect of BMS-986122 on opioid-induced respiratory depression and heart rate

Opioid-induced respiratory depression (reduced breath rate and blood oxygen levels) and heart rate were determined using the mouse PulseOx system on awake, free-moving CD-1 mice. Fentanyl displayed a dose-dependent decrease in oxygen levels, reaching 74% saturation following 10 mg/kg fentanyl. The effect was rapid in onset and oxygenation returned to baseline levels 50 min after drug administration. In the presence of BMS-986122, fentanyl-induced reduction in oxygen saturation was not significantly altered at submaximal 1 or 3.2 mg/kg doses (Figure 3.6A-B). In contrast to fentanyl, neither morphine (up to 100mg/kg) or methadone (up to 10mg/kg) caused a reduction in blood oxygen percent in either the absence or presence of BMS-986122 (Figure 3.5A-B).

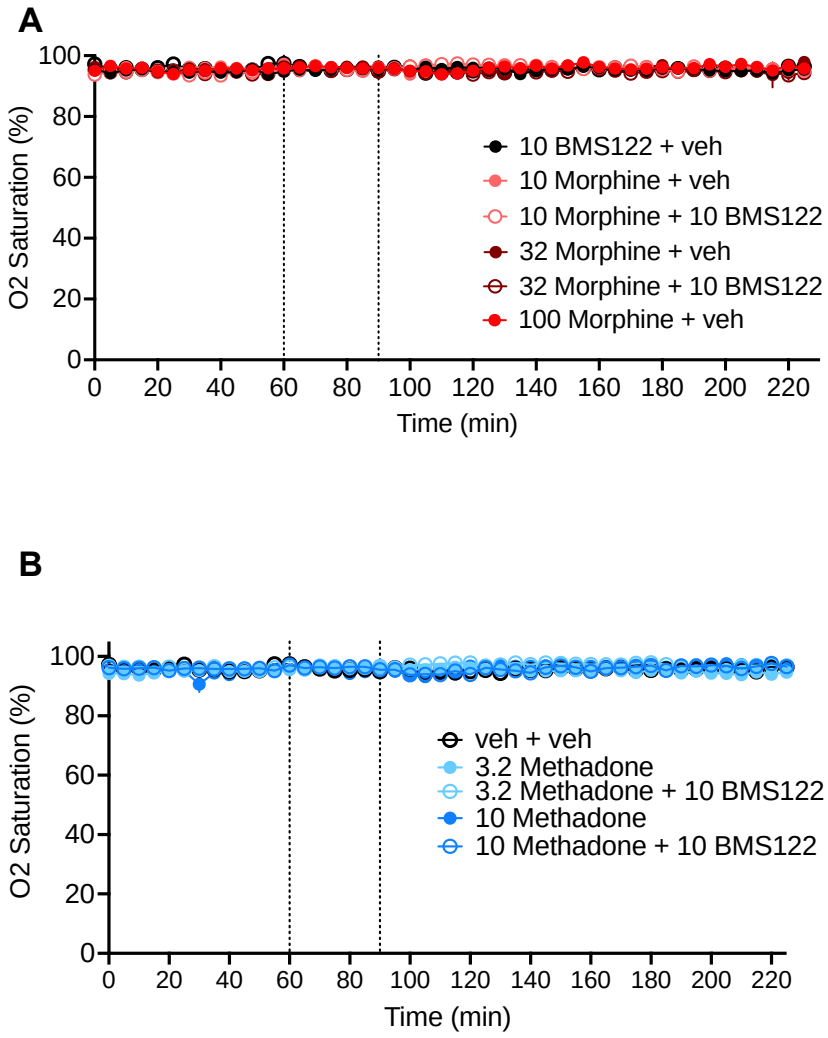


Figure 3.5. Morphine and methadone do not produce decreases in mouse blood oxygen saturation. Blood oxygen saturation dose-response for morphine (**A**) and methadone (**B**) with and without 10 mg/kg BMS-986122 (BMS122) over time. Dotted lines indicate saline (BL) and pre-treatment (BMS122) injection times. Data are presented as means ± SEM (n = 6-8 for each group).

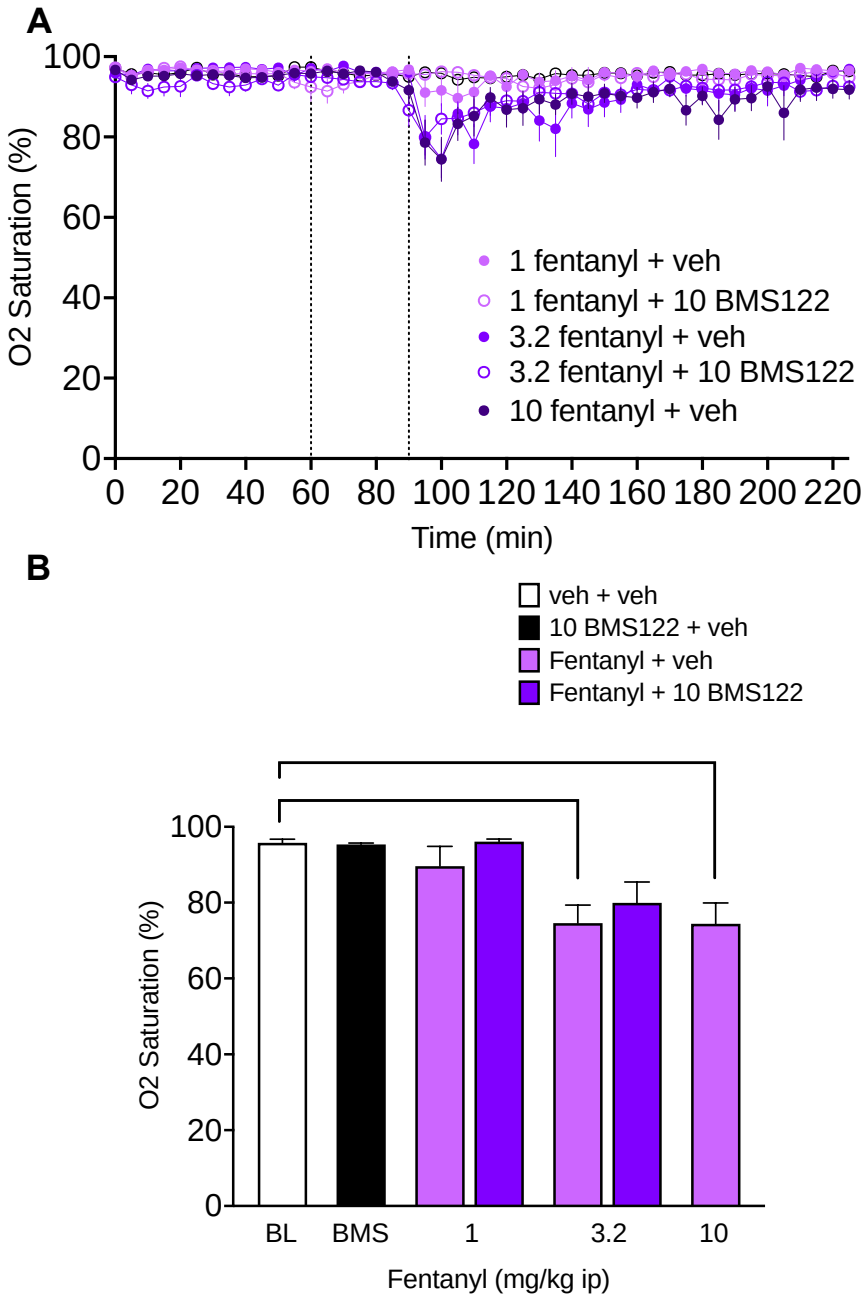
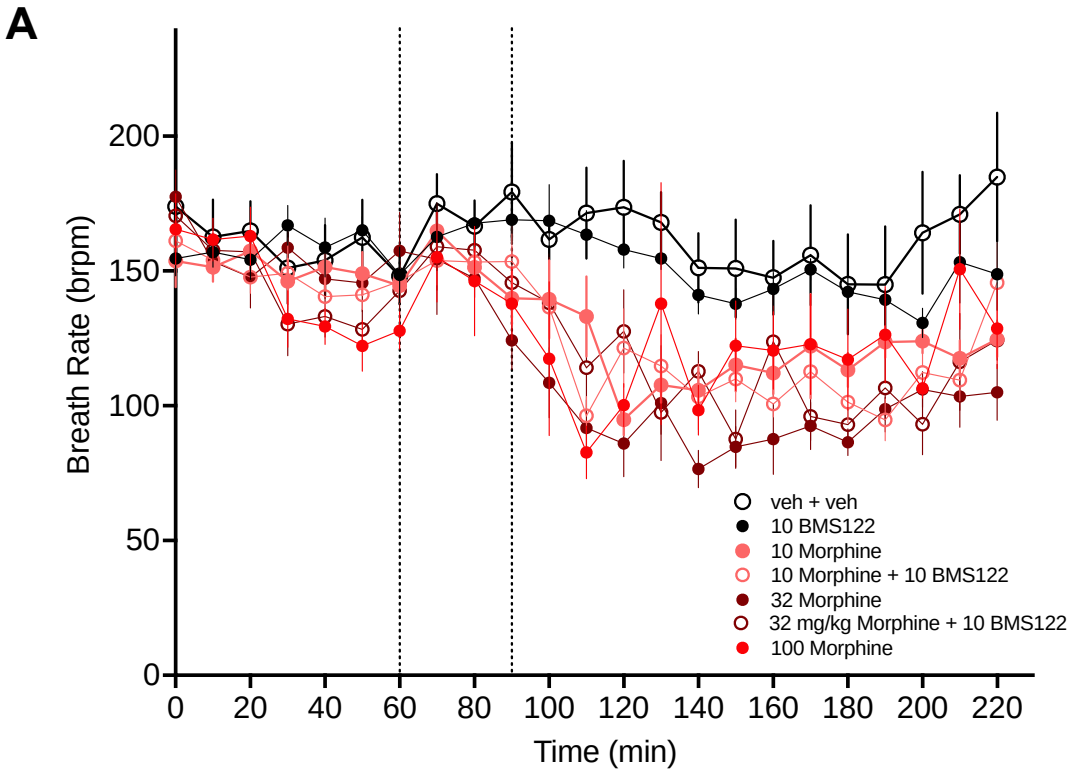
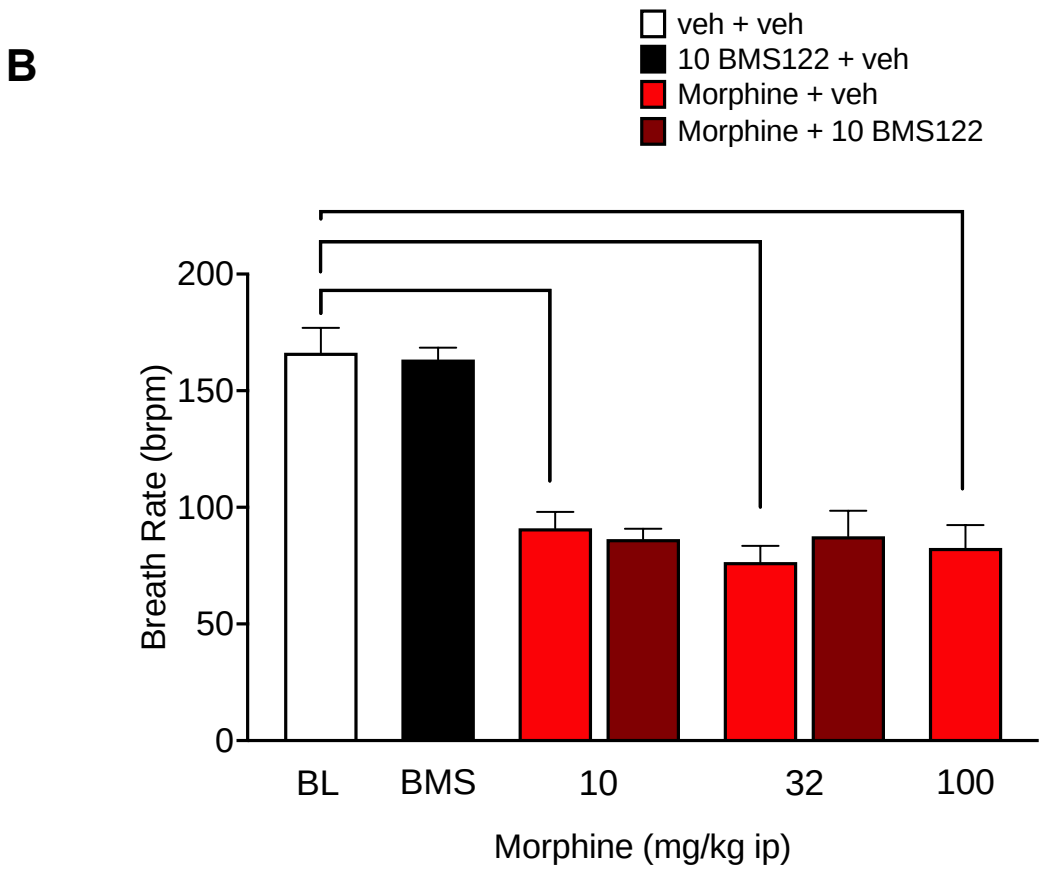
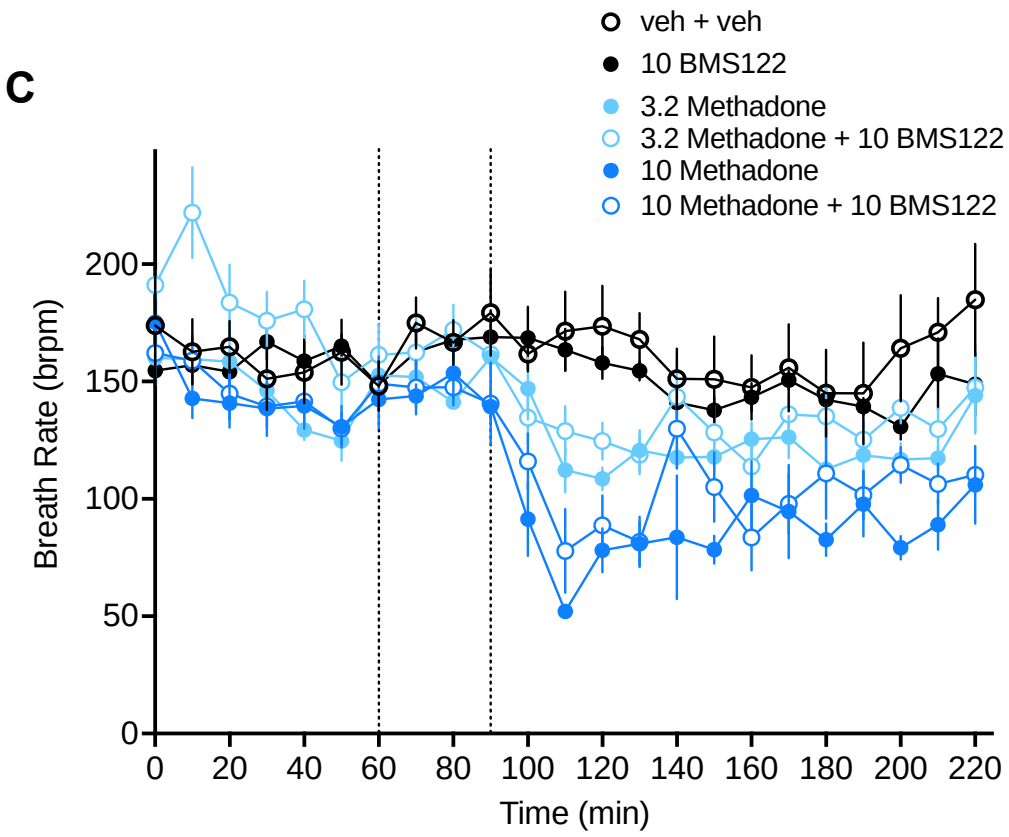


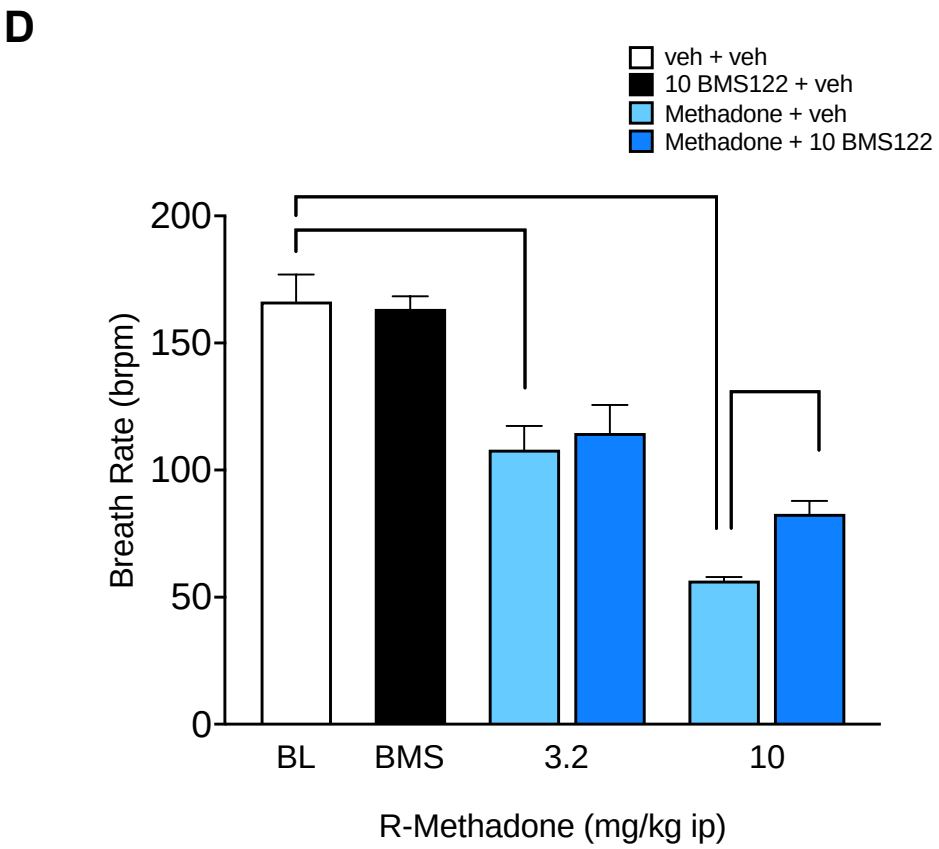
Figure 3.6. BMS-986122 does not potentiate fentanyl-induced decreases in mouse blood oxygen saturation. Dose-response for fentanyl with and without 10 mg/kg BMS-986122 (BMS122). Dotted lines indicate saline (BL) and pre-treatment (BMS122) injection times. Data are presented as means ± SEM over time (**A**) and peak effect time (**B**) (n = 6-7 for each group). ($F=120.2$, $p<0.0001$) ***P<0.0001 as determined by two-way ANOVA with Tukey's post-hoc test.

Opioid-induced depression of breath rate (brpm) was variable using the pulse-oximeter, but there were significant decreases in peak effects for morphine, methadone, and not fentanyl. Mice treated with BMS-986122 showed no change from vehicle-treated animals with an average brpm of 163 over 60 minutes of baseline measurements and 166 brpm over 30 minutes of pretreatment measurements, respectively. Morphine decreased breath rate to 92 brpm which was sustained for 100 minutes. Methadone also dose-dependently reduced breath rate to a maximal effect at 56 at 10 mg/kg. Maximal effects were observed 30 mins after drug administration and were maintained for at least 90 mins. Fentanyl showed less of a decrease in breath rate compared to morphine and methadone, with the largest reduction observed at 100 brpm with both 1 and 3.2 mg/kg. In the presence of BMS-98622, there was no further lowering of breath rate with morphine, methadone, or fentanyl even at submaximal doses of the opioids.


A
Breath Rate (brpm)
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
200
220
Time (min)
veh + veh
10 BMS122
10 Morphine
10 Morphine + 10 BMS122
32 Morphine
32 mg/kg Morphine + 10 BMS122
100 Morphine


B
veh + veh
10 BMS122 + veh
Morphine + veh
Morphine + 10 BMS122
Breath Rate (brpm)
200
150
100
50
0
BL
BMS
10
32
100
Morphine (mg/kg ip)


C
veh + veh
10 BMS122
3.2 Methadone
3.2 Methadone + 10 BMS122
10 Methadone
10 Methadone + 10 BMS122
Breath Rate (brpm)
200
150
100
50
0
0
20
40
60
80
100
120
140
160
180
200
220
Time (min)


D
veh + veh
10 BMS122 + veh
Methadone + veh
Methadone + 10 BMS122
Breath Rate (brpm)
200
150
100
50
0
BL
BMS
3.2
10
R-Methadone (mg/kg ip)

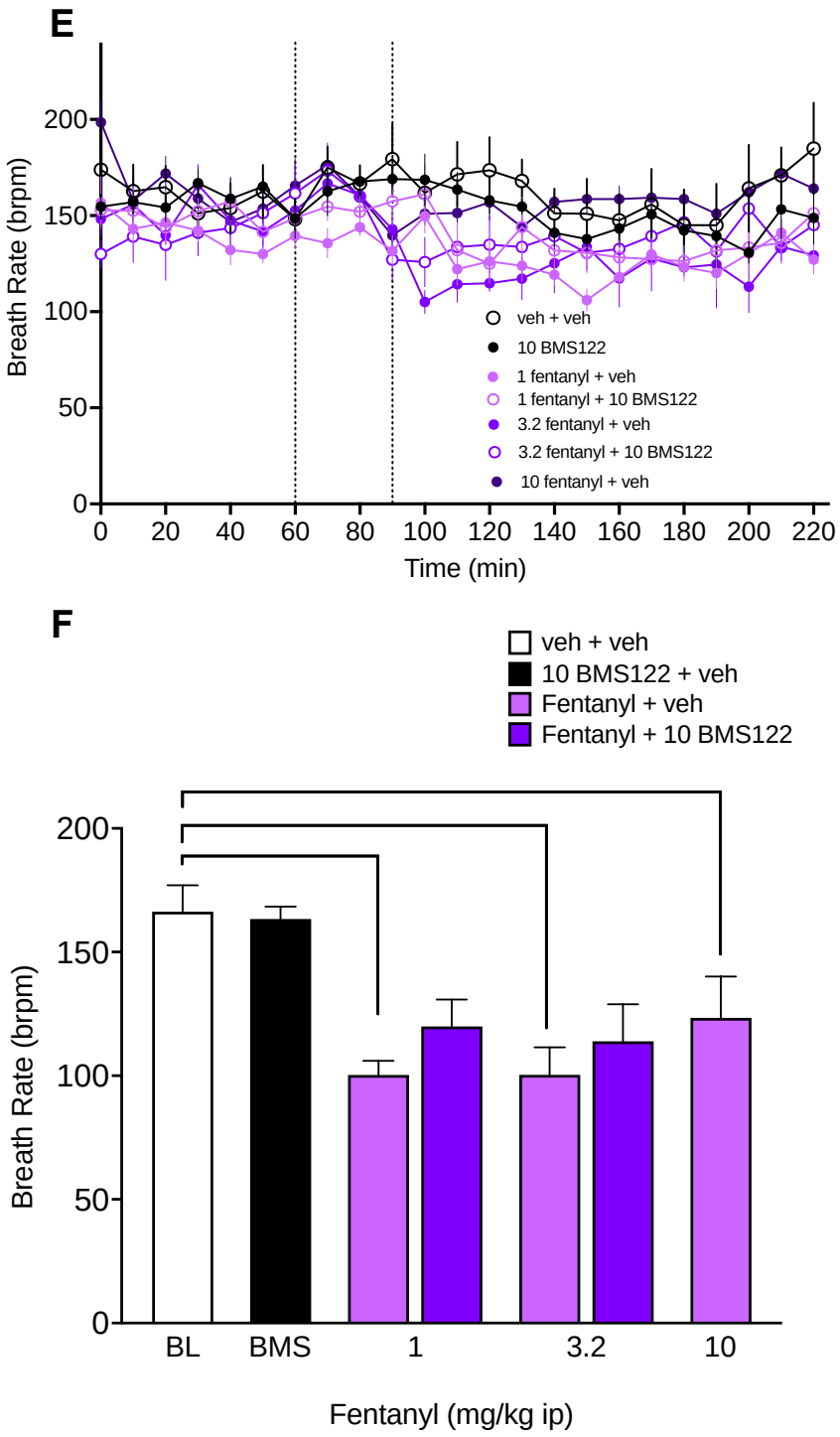
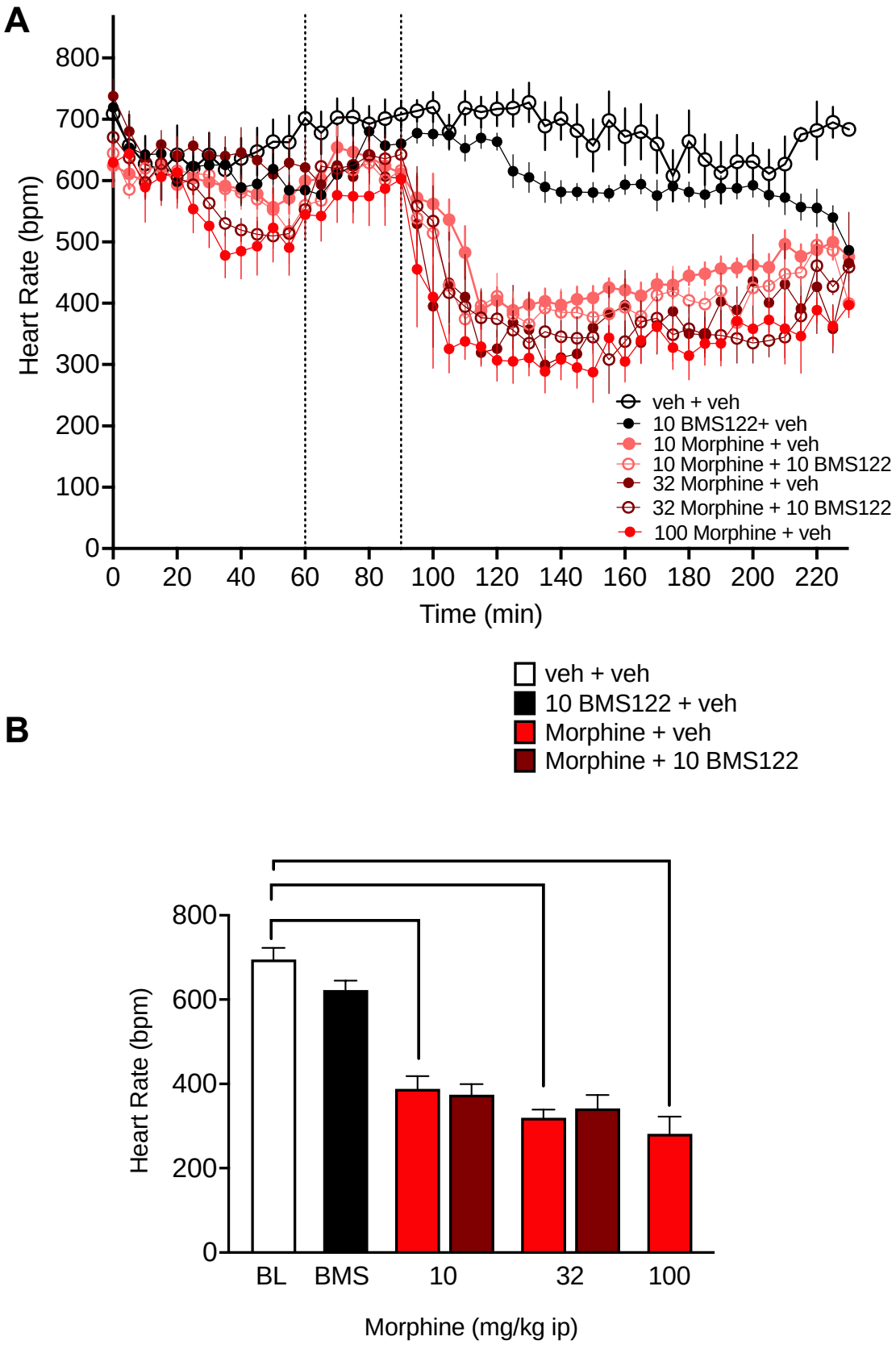
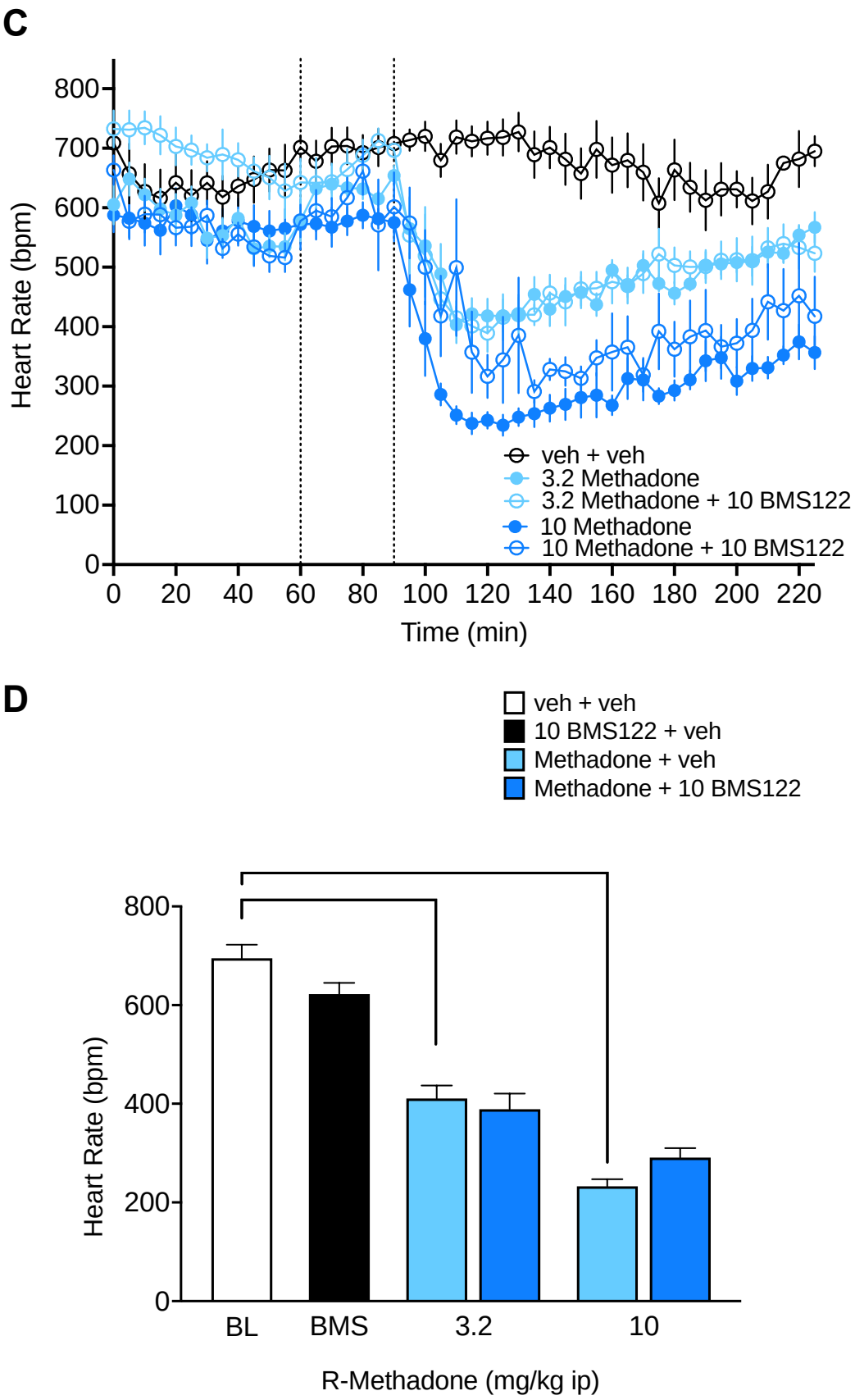


Figure 3.7. Opioid-induced respiratory depression is not worsened by BMS-986122. Dose-response for morphine ($F=13.5$, $p<0.0001$) (**A-B**), methadone ($F=14.1$, $p<0.0001$) (**C-D**), and fentanyl ($F=10.3$, $p<0.0001$) (**E-F**) with and without 10 mg/kg BMS-986122 (BMS122). Dotted lines indicate saline (BL) and pre-treatment (BMS122) injection times. Data are presented as means ± SEM over time (**A, C, E**) and peak effect time (**B, D, F**) (n = 6-7 for each group). *P<0.05, ***P<0.001, ***P<0.0001 as determined by two-way ANOVA with Tukey's post-hoc test.

Mouse heart rate following vehicle treatment was an average of 695 beats per minute (bpm) averaged over 150 mins. The average rate in the presence of 10 mg/kg BMS-986187 was 622 bpm during pretreatment measurements. Morphine afforded a dose-dependent decrease in heart rate, reaching 281 bpm at 100 mg/kg morphine, an effect that was sustained for over 120 minutes. Methadone gave a reduction in heart rate to 233 bpm at 10 mg/kg after only 20 minutes post administration and lasted over two hours. Fentanyl had less of an effect on heart rate, reaching a maximal effect of around 381 bpm at both 1 and 3.2 mg/kg that lasted over 2 hours. BMS-986187 had no effect on the reduction of heart rate caused by any of the three agonists.


A
Heart Rate (bpm)
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
120
140
160
180
200
220
Time (min)
veh + veh
10 BMS122+ veh
10 Morphine + veh
10 Morphine + 10 BMS122
32 Morphine + veh
32 Morphine + 10 BMS122
100 Morphine + veh
B
veh + veh
10 BMS122 + veh
Morphine + veh
Morphine + 10 BMS122
Heart Rate (bpm)
800
600
400
200
0
BL
BMS
10
32
100
Morphine (mg/kg ip)


C
Heart Rate (bpm)
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
120
140
160
180
200
220
Time (min)
veh + veh
3.2 Methadone
3.2 Methadone + 10 BMS122
10 Methadone
10 Methadone + 10 BMS122
D
veh + veh
10 BMS122 + veh
Methadone + veh
Methadone + 10 BMS122
Heart Rate (bpm)
800
600
400
200
0
BL
BMS
3.2
10
R-Methadone (mg/kg ip)

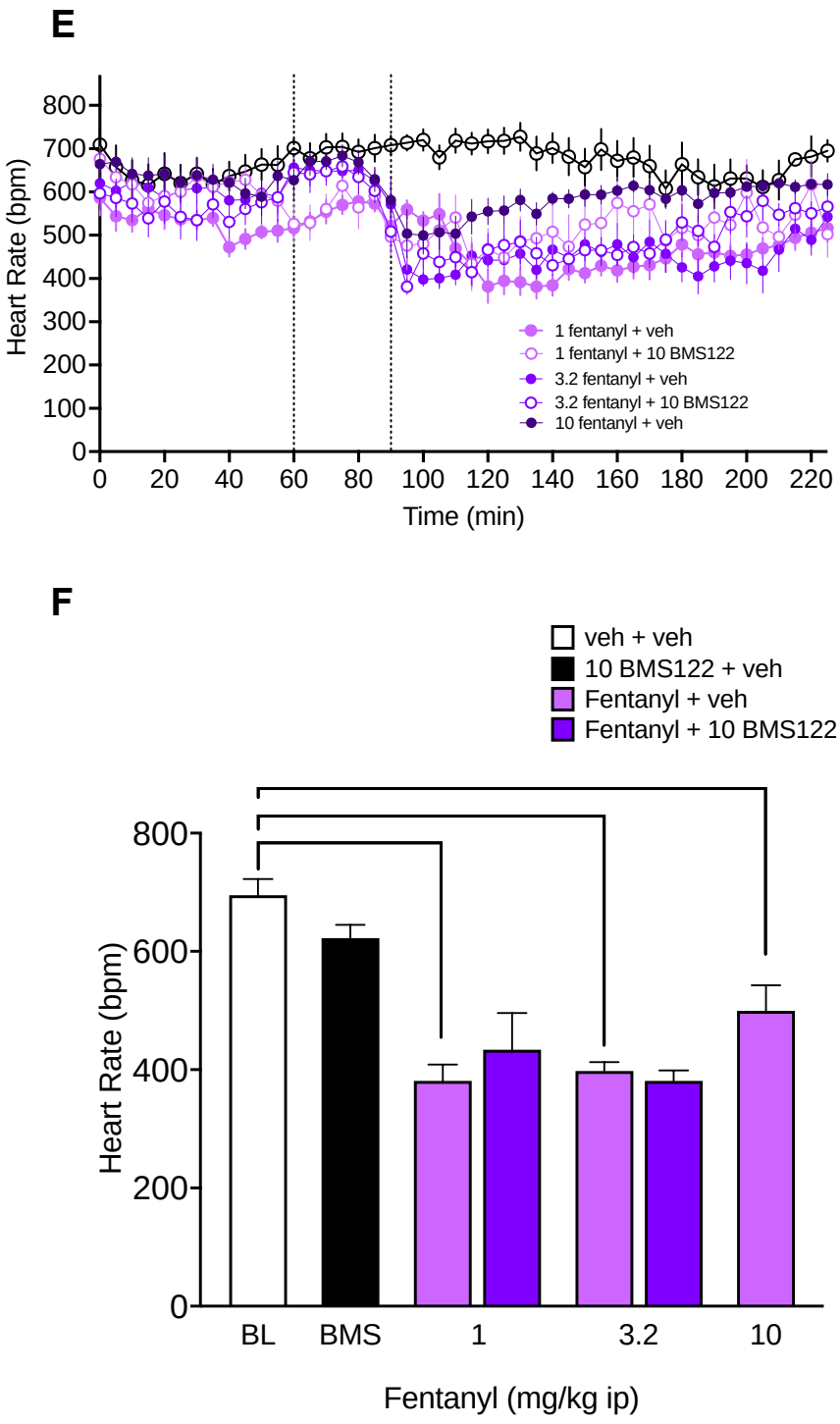


Figure 3.8. BMS-986122 does not worsen opioid-induced decreases in mouse heart rate. Dose-response for morphine (F=13.5, p<0.0001) (**A-B**), methadone (F=17.4, p<0.0001) (**C-D**), and fentanyl (F=10.0, p<0.0001) (**E-F**) with and without 10 mg/kg BMS-986122 (BMS122). Dotted lines indicate saline (BL) and pre-treatment (BMS122) injection times. Data are presented as means ± SEM over time (**A, C, E**) and peak effect time (**B, D, F**) (n = 6-7 for each group). ****P<0.0001 as determined by two-way ANOVA with Tukey's post-hoc test.

Effect of BMS-986122 on the rewarding action of fentanyl

To examine the effect of BMS-986122 on opioid-induced reward, we studied the effects of fentanyl response in the mouse conditioned place preference (CPP) assay in CD-1 mice. The vehicle control (DMSO: castor oil: sterile water, 10:10:80) produced an aversive effect on its own. As such, we normalized the CPP data to this condition and set this as the “baseline” value. Mice showed a significant preference for the fentanyl-paired chamber at 0.1 mg/kg (485 secs $\pm$ 97.7), but not 1 mg/kg (218 secs $\pm$ 56.4) when compared to the vehicle condition (Figure 9A). In the presence of BMS-986122 (Figure 9B), the CPP score for 0.1 mg/kg fentanyl was reduced to 317 $\pm$ 107 secs, whereas the score for 1 mg/kg fentanyl was enhanced to 439 $\pm$ 90.2 secs. Therefore, in the presence of BMS-986122, the effect of 0.1 mg/kg fentanyl compared to vehicle was no longer significant, whereas the effect of 1 mg/kg became significant. These opposite effects at 0.1 and 1.0 mg/kg fentanyl compared to the vehicle control suggests that BMS-986122 shifts the peak effect observed with fentanyl to the right. Additionally, the PAM did not produce a significant preference when administered alone. Additional studies were conducted with fentanyl in the absence of the vehicle and are discussed in Chapter 4.

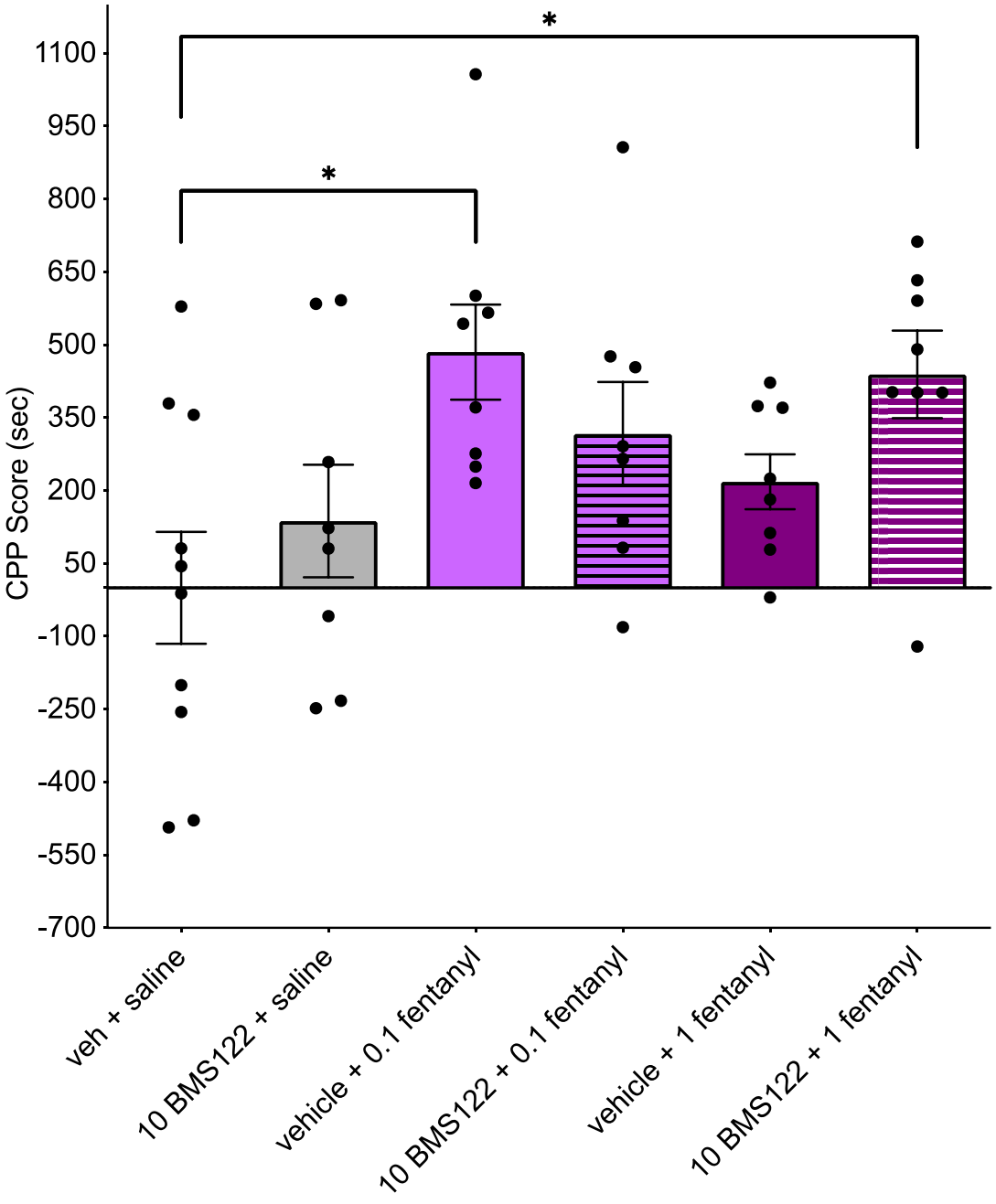


Figure 3.9. BMS-986122 attenuates fentanyl-induced reward. CPP scores following conditioning to fentanyl in the absence or presence of BMS-986122. CPP scores are the difference in time spent on the drug-paired side on bias and test day. Data are normalized to vehicle + saline condition and presented as mean ± SEM for all groups (n = 8-10 for each group). ($F=1.6$, $p<0.05$) *$P<0.05$ as determined by two-way ANOVA with Tukey's post-hoc test.

Discussion:

In this study, we demonstrate that the positive allosteric modulator (PAM) BMS-986122 preferentially enhances MOR agonist-induced antinociception in response to thermal noxious stimuli without an enhancement of opioid-mediated respiratory depression, constipation, or reward. These data are consistent with previous findings that BMS-986122 potentiates endogenous and exogenous opioid-mediated antinociception, using other mouse models and methods (Kandasamy *et al.*, 2021). One caveat to the study is that for solubility reasons, we could not use doses of BMS-986122 higher than 10 mg/kg. Nevertheless, the findings suggest further study of MOR-PAMs as opioid-sparing agents is warranted.

In CD-1 mice, BMS-986122 shows no antinociceptive activity on its own in the warm-water tail withdrawal or hot-plate assays in the absence of exogenous opioids. This is contrary to data previously reported in 129S1/SvlmJ (129) mice (Kandasamy *et al.*, 2021) but aligns with findings that peripheral administration of BMS-986122 in C57/BL6 mice does not produce antinociception. We have previously suggested that this strain difference is due to differences in stress response and level of endogenous opioid release, which is reportedly higher in 129 strains of mice (Chan et al., 2017; Schlussman et al., 2011). For the current studies, it was important that BMS-986122 has no behavioral actions on its own to ensure a clear examination of the interaction between the MOR-PAM and exogenous opioid drugs.

In the mouse WWTW assay, the potency of the antinociceptive effects of morphine and methadone was enhanced to a small but significant degree with maximal effects seen in the middle of the dose response curves. It was unexpected to see the largest potentiation of antinociceptive effects for fentanyl, as we have previously reported that BMS-986122 shows a probe-dependence *in vitro* and that methadone is the most sensitive orthosteric agonist (Livingston & Traynor, 2014). However, PAM potentiation of effect did depend on the time after administration of each agonist. Moreover, the effect of BMS-986122 followed the time course of the orthosteric agonists suggesting the PAM was active throughout the whole period of time the opioids were effective.

The effects of BMS-986122 on morphine- and methadone-mediated antinociception in the WWTW assay were similar. Yet with the partial agonist morphine, BMS-986122 enhances its maximal response as determined in measures of G-protein activation using the in the GTPγ^{35}S assay (Burford et al., 2013). For methadone, which shows a higher maximal effect, there is an increase in potency. To examine this further we used the higher efficacy-requiring hot-plate assay where higher doses of agonists were required and included the low efficacy partial agonist buprenorphine. BMS-986122 potentiated opioid-mediated antinociception to a similar degree compared to the warm-water tail withdrawal assay. Additionally, the PAM enhanced the maximal response to buprenorphine. These data suggest that although we see probe dependence in the potentiation of agonist-induced antinociception by the PAM BMS-986122, the effect is not dependent on the agonist efficacy or efficacy requirement of the assay.

Opioid-induced constipation significantly impairs patient quality of life, regardless of opioid efficacy in pain relief, and can lead to discontinuation of opioid use (Lang-Illievich & Bornemann-Cimenti, 2019). Morphine, methadone, and most significantly, fentanyl caused dose-related constipation in the CD-1 mice. However, at submaximal doses of the orthosteric agonists, 10 mg/kg BMS-986122 did not potentiate the severity of constipation.

Respiratory depression is the fatal effect associated with opioid overdose. Neuronal control of breathing is vulnerable to opioid action, particularly at MOR-expressing Pre-Bötzinger and Kölliker-Fuse neurons, although the exact mechanism of action remains controversial (Varga et al., 2020; Bachmutsky et al., 2020; Baetsch et al., 2021; Montandon & Horner, 2014; Lalley et al., 2014; Saunders & Levitt, 2020). We measured breath rate, blood oxygen saturation, and heart rate in the mouse using pulse oximeter collars that record data from the carotid artery. We did not observe a reduction in blood oxygen levels with morphine or methadone, even at doses of 100 mg/kg and 10 mg/kg respectively, although there was a significant reduction in breath rate. This suggests the preservation of oxygen levels due to compensatory mechanisms maintaining the oxygen supply to continue function and avoid damage (Hoiland et al., 2016). Example compensatory mechanisms reported in clinical studies include increased tidal volume and conserving minute ventilation, where gas exchange remains stable when the respiratory rate is decreased (Boland et al., 2013; Bouillon et al., 2003; Barbour et al., 2004).

In contrast, we did observe a reduction of blood oxygen following fentanyl administration, possibly because compensatory mechanisms cannot fully overcome the actions of this potent opioid. Even so, it is notable that there was only a 25% reduction in blood oxygen levels even at 10 mg/kg fentanyl which is 30-times the dose required to give a maximal response in the WWTW assay and 10-times the dose needed to give a maximal response in the hot-plate assay. BMS-986122 did not cause a reduction in blood oxygen levels nor did it cause morphine or methadone to show a decrease or amplify the decrease caused by a submaximal dose of fentanyl. We previously reported BMS-986122 slightly decreased respiratory rate in the 129S1/SvlmJ strain of mice using the Comprehensive Lab Animal Monitoring System (Kandasamy et al., 2021), again perhaps indicating an enhanced opioid peptide release in this strain of mice in response to stress. In our studies, morphine, methadone, and fentanyl produce significant decreases in heart rate, whereas a slight decrease was observed with BMS-986122. Although we see this decrease with BMS-986122, there is not an additive or synergistic effect when in the presence of any of the agonists, and the PAM did not worsen opioid-induced reductions in heart rate.

Fentanyl produces significant place preference in the CPP assay at a dose of 0.1 mg/kg. In line with a previous report, BMS-986122 does not produce a significant place preference. The effect of fentanyl displays a significant peak at 0.1 mg/kg that decreases at the higher dose of 1 mg/kg. With the addition of BMS-986122, we observed an apparent rightward shift in the fentanyl effect, where the lower dose was attenuated, and the higher dose was potentiated. It is important to note that we face a limitation in that the vehicle

(10:10:80- DMSO: Castor Oil: Sterile Water) had a significant aversive effect on its own following the 5-day conditioning period (discussed in Chapter 4). It is possible that the vehicle effect, along with the genetic variation of the outbred CD-1 mouse strain, may be responsible for the significant variability observed in this assay. These data suggest that BMS-986122 may attenuate, rather than enhance, the action of fentanyl in the CPP assay, though further studies are required to confirm.

Overall, the present study shows that the MOR-PAM, BMS-986122, has the potential to selectively enhance the antinociceptive effects of opioids without potentiating the adverse effects such as constipation, respiratory depression, and reward. It is unclear why the enhancing actions of BMS-986122 should be restricted to antinociception, although there is the caveat that we were restricted by solubility issues to a maximal dose of 10 mg/kg so it is possible effects may be observed at higher doses. The agonist requirements of the assays do not explain the apparently selective action since the doses needed to be effective in the measured behaviors are in the order, lowest first: WWTW assay = conditioned place preference, < constipation < hot-plate assay < respiratory depression. In previous studies with the 129S1/SvlmJ strain of mice, we also showed a selective effect of BMS-986122 on antinociception in the absence of an exogenous agonist, so it is feasible that BMS-986122 is acting on aspects of an endogenous pain system that does not involve opioids, in spite of *in vitro* evidence that the compound is a MOR-PAM. Nonetheless, this study provides further justification for continuing the development of positive allosteric modulators of MOR as opioid sparing agents.

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Chapter 4 : Discussion

Summary

Significance

The studies in this dissertation characterize positive allosteric modulators (PAMs) of the mu-opioid receptor (MOR), from examining cellular downstream signaling to behavioral effects in mice. These data are the first to investigate the role of PAMs in biased signaling and opioid sparing. These studies provide significant support for the further development of PAMs to reduce opioid usage. Collectively, this work adds to the wealth of knowledge necessary for the discovery and development of alternative opioid therapies.

Chapter 2

Studies examining the enhancement of mu-opioid effects *in vitro* by BMS-986187 and BMS-986122 revealed differential probe dependence between these two chemically unique PAMs. Despite acting at a supposed conserved site on MOR (Livingston et al., 2018), BMS-986187 promotes orthosteric agonist-induced β-arrestin recruitment whereas BMS-986122 promotes G protein activation. The only exception to this phenomenon was observed with SR17018, where both PAMs promote similar shifts in

the concentration-response curves for activation of either signaling pathway. These data suggest that while these PAMs may interact with the receptor at a similar geographic location, they facilitate potentiation of orthosteric agonists differently. In addition, this work shows that although these PAMs can produce large shifts in orthosteric agonist potency, these shifts are not enough to significantly change the direction of signaling bias for an orthosteric ligand. Furthermore, similar to the effect of these PAMs in promoting differential potency shifts in β-arrestin recruitment, they exhibit probe dependence in altering agonist-induced loss in cell surface MOR. This finding further suggests that PAMs can differentially affect both G protein and β-arrestin signaling downstream of MOR.

This work adds to the breadth of literature examining biased agonism of MOR but with the additional intersection of positive allosteric modulation that will help to further tease apart the mechanisms underlying signaling bias and its relevance. Additionally, this work provides a novel foundation for comparing bias altered by allosteric modulators, providing a pathway for future studies to build on. Overall, it is clear that activation of MOR by different orthosteric and allosteric combinations can lead to diverse signaling profiles.

Chapter 3

Previous work laid the foundation for the benefit of administering PAMs to enhance endogenous opioid effects *in vivo*, but this study is the first to explore the effects of PAMs on exogenous opioids in animal models. This chapter describes an *in vivo* analysis of the ability of MOR PAM, BMS-986122, to enhance exogenous opioid-induced effects. This work is a study of the effects induced by three clinically relevant opioids, morphine,

methadone, and fentanyl, in the presence of BMS-986122. These data reveal that BMS-986122 enhances the antinociceptive effects of these opioids, exhibiting probe dependence in the degree of potentiation. While antinociception was enhanced, constipation, respiratory depression, and reward was not. This finding highlights that PAMs may selectively enhance certain effects induced by opioids, specifically the beneficial effects over the adverse effects. Selective potentiation of antinociception would be useful in opioid sparing to lower the total amount of opioids used.

While these data suggest that BMS-986122 does not worsen exogenous opioid-induced adverse effects, they also suggest the PAM attenuates the rewarding effects of fentanyl, though more data are necessary to support this conclusion. While this would be an ideal result, we faced several challenges when studying opioid-induced reward. To start, the conditioned placed preference (CPP) assay is a method sensitive to variability, particularly when using the outbred CD-1 mouse strain, which has not been previously characterized in such assays. Initially, we designed this study using a 3-day conditioning protocol but were unable to visualize any effect regardless of the drug treatment, due to variability. To combat this, we increased conditioning to 5 days and were able to produce the fentanyl dose-response curve shown in Figure 4.1. In this figure, we see that 1 mg/kg fentanyl produces the maximum response with a CPP preference of 298 $\pm$ 65 secs, whereas 0.1 and 0.3 mg/kg fentanyl show slightly smaller effects of 226 $\pm$ 48 secs. and 80 $\pm$ 54 secs, respectively.

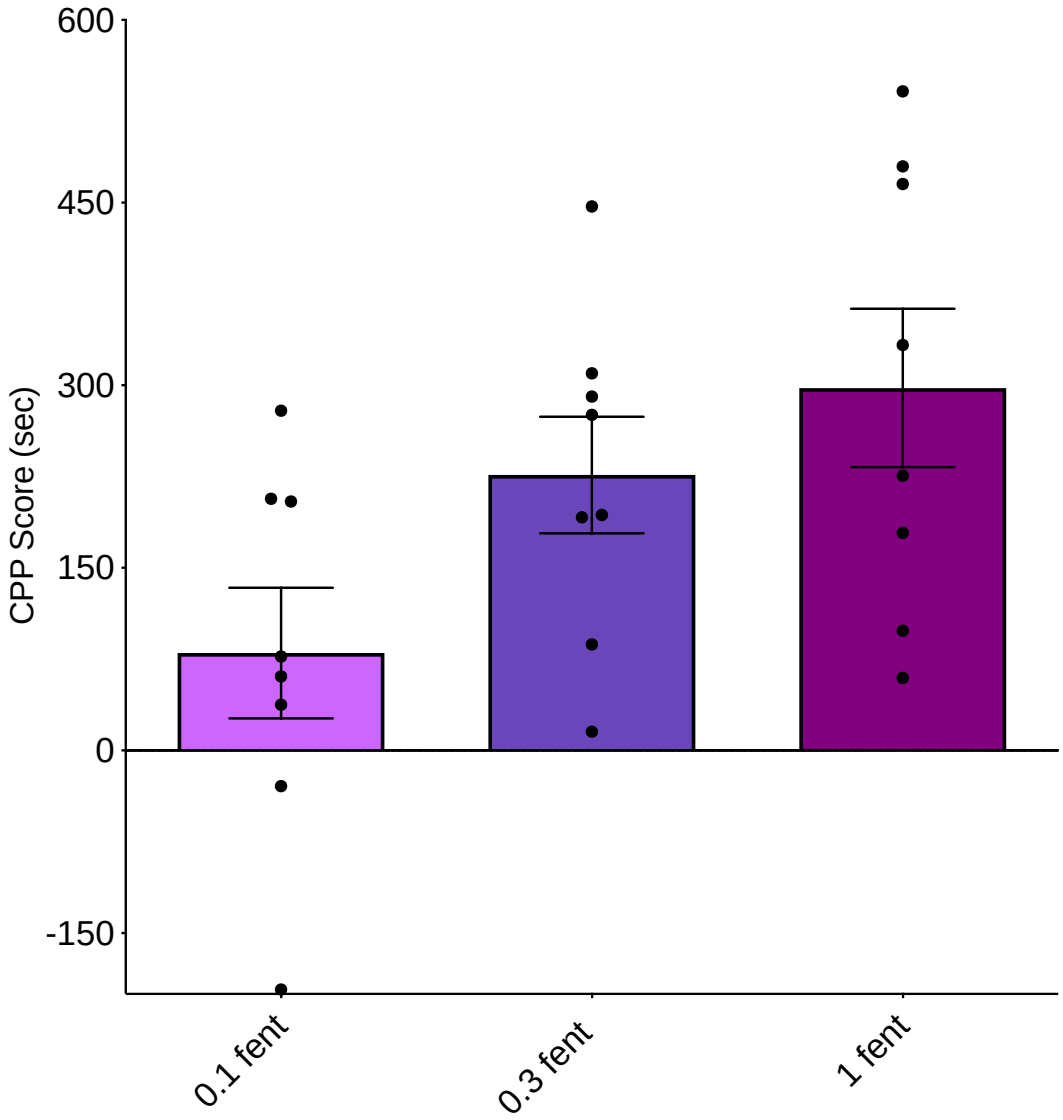

Figure 4.1. Fentanyl produces dose-dependent CPP scores. CPP scores are the difference in time spent on the drug-paired side on bias and test day. Data are presented as mean ± SEM for all groups (n = 8 for each group).

Furthermore, we faced a challenge when introducing pretreatment conditions, especially with the addition of the BMS-986122 vehicle control. The vehicle is comprised of DMSO, castor oil, and sterile water (10:10:80 ratio), and the maximum solubility we can achieve with BMS-986122 is a concentration of 1 mg/mL. When tested in the CPP

assay, this vehicle displayed an aversive response of -157± 115 secs. (Figure 4.2). We hypothesize that the vehicle may cause discomfort for some, but not all, animals, leading to the high level of variance observed. The vehicle pretreatment may also cause irritation o the point where endogenous opioids are released, leading to the rewarding response observed with three of the ten animals, though this has not been examined. Additionally, 10 mg/kg BMS-986122 was able to overcome the aversion caused by the vehicle from a score of -157± 115 secs to -19. ± 116 secs, although the variability is just as high as observed with the vehicle condition. This would support the idea that vehicle-induced discomfort may promote endogenous opioid release that BMS-986122 can then potentiate to reduce the aversion displayed with the vehicle condition on its own.

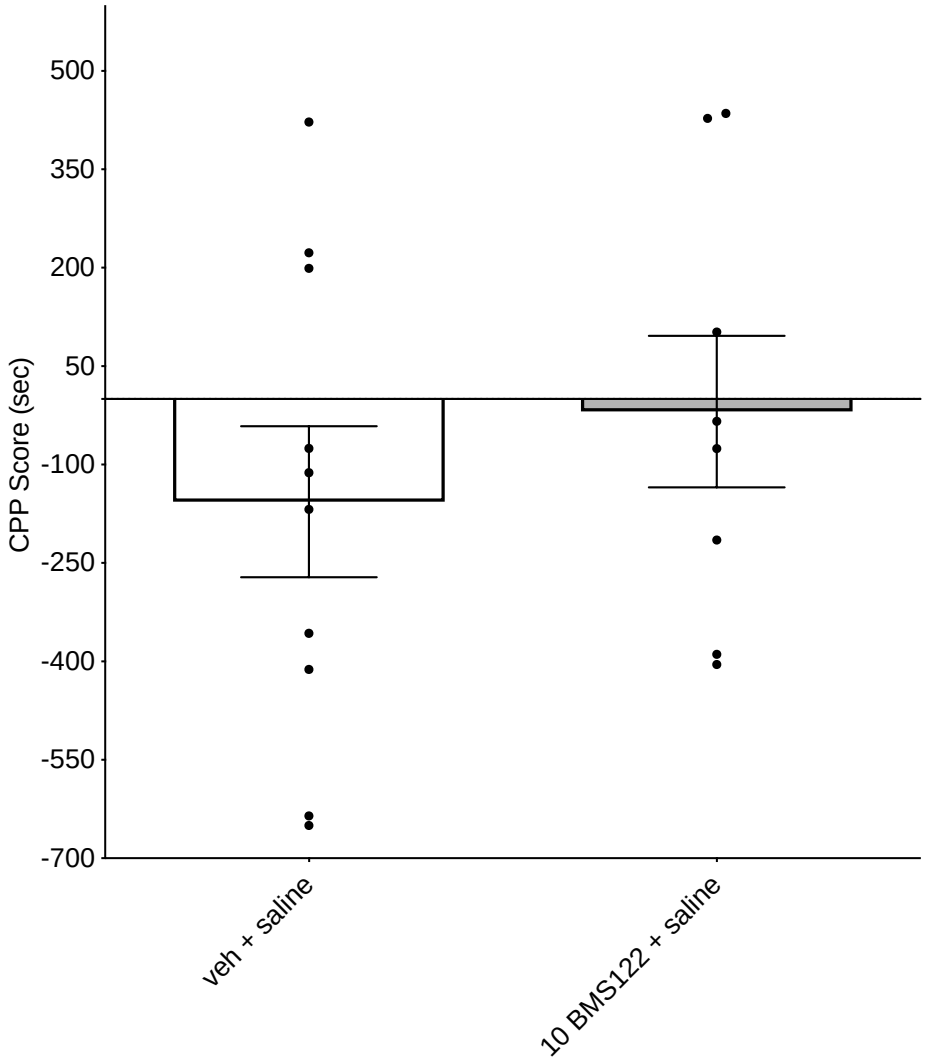

Figure 4.2. The BMS-986122 vehicle displays an aversive effect in CPP that BMS-986122 (BMS122) is able to overcome. CPP scores are the difference in time spent on the drug-paired side on bias and test day. Data are presented as mean ± SEM for all groups (n = 8-10 for each group).

Once we established a vehicle response, we needed to examine what effect the vehicle had on the fentanyl dose-response curve shown in Figure 4.1. To do this, we chose to test the two doses with the smallest and largest effect in the initial experiment, 0.1 and 1 mg/kg fentanyl. This study revealed that the vehicle pretreatment shifted the

fentanyl dose-response curve to the left, such that the magnitude of the effect has switched suggesting a biphasic response to fentanyl. Thus, 0.1 mg/kg fentanyl + vehicle pretreatment gave the largest response of 329 ± 98, and the 1 mg/kg fentanyl + vehicle pretreatment was reduced to 61 ± 56.

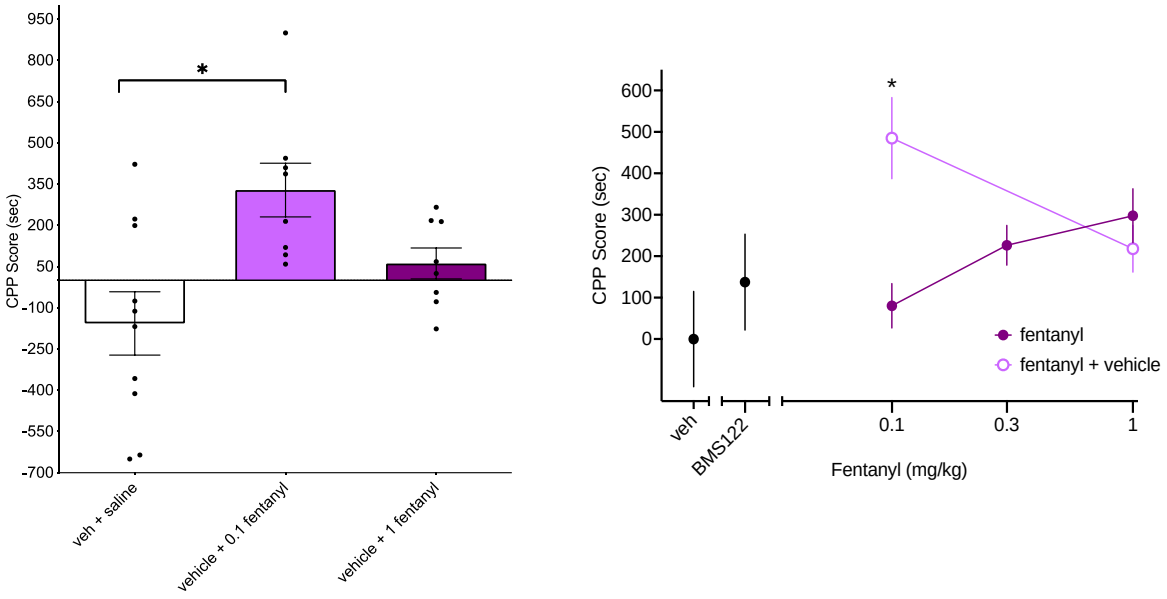

Figure 4.3. The BMS-986122 vehicle alters the fentanyl dose response in the CPP assay. CPP scores are the difference in time spent on the drug-paired side on bias and test day. Data are presented as mean ± SEM for all groups (n = 8-10 for each group). *P<0.05 as determined by two-way ANOVA with Tukey's post-hoc test.

Going forward, we chose to use the fentanyl in the presence of the vehicle as a reference for our BMS-986122 pretreatment + fentanyl experiments. As such, we normalized these data using the vehicle + saline condition as baseline, as every test condition included either the vehicle, or BMS-986122 in the vehicle. Interestingly, when we administer 10 mg/kg BMS-98122, the vehicle + 0.1 mg/kg fentanyl effect is *reduced* to 159.9 ± 107, whereas the vehicle + 1 mg/kg fentanyl effect is *increased* to 282.4 ± 90.2

(Figure 4.4). These data suggest that while the vehicle pretreatment potentiates the effect of fentanyl doses, BMS-986122 blocks this potentiation. Furthermore, both BMS-986122 + fentanyl doses look comparable to the fentanyl dose response without the presence of the vehicle. Thus, we can conclude that the vehicle confounds these results, and future studies should aim to troubleshoot potential methods for reducing vehicle interference as well as overall experimental variability.

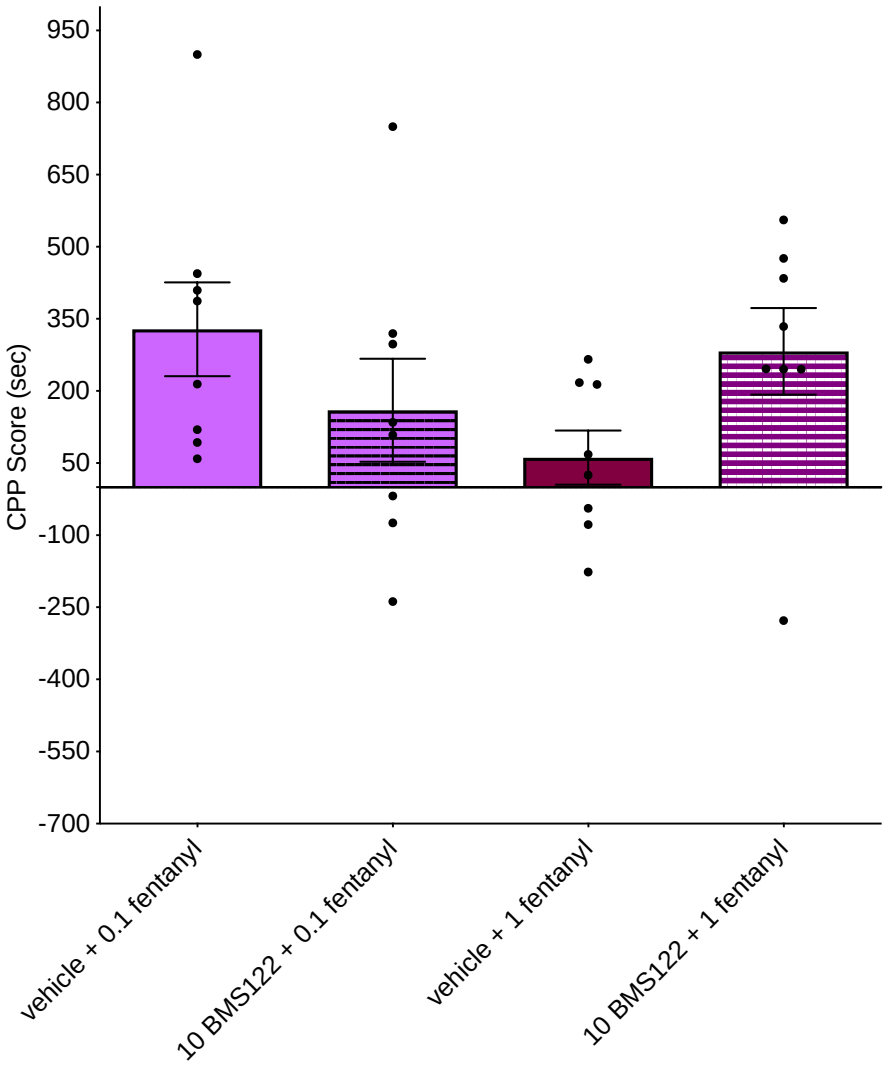

Figure 4.4. BMS-986122 blocks vehicle-induced potentiation of the fentanyl dose response curve in the CPP assay. CPP scores are the difference in time spent on the drug-paired side on bias and test day. Data are presented as mean ± SEM for all groups (n = 8 for each group).

While the work in this chapter faced several challenges due to solubility concerns of the PAM BMS-986122, the data obtained are useful for informing future studies on examining the behavioral effects of exogenous opioids. Further examination of how PAMs may alter both the beneficial and adverse effects induced by several structurally distinct and clinically relevant opioids may reveal methods for manipulating opioid therapies to develop safer alternatives. Overall, this study provides significant support for further development of positive allosteric modulators of the mu opioid receptor.

Future Directions

The studies included in this thesis provide significant insight into the ways PAMs can alter MOR signaling once the receptor is activated by orthosteric agonists. This work provides a significant framework for future studies that further characterize positive allosteric modulation of the mu-opioid receptor, as well as at other G protein-coupled receptors. Thus, there are several areas the field can continue to explore to further our understanding of elucidating PAM function.

Structure-activity relationships to improve compound characteristics:

As mentioned in the chapter summaries, we faced challenges with studying BMS-986122 and BMS-986187 due to poor solubility. As such, we were limited to examining 1 effective dose of BMS-986122 *in vivo* and were unable to fully dissolve BMS-986187 into a vehicle safe for animal use. Previous work using BMS-986187 in animals reports a

water-based vehicle that did not dissolve BMS-986187 in our studies (DiCello et al., 2021). In addition, BMS-986187 is well characterized as a PAM at the delta opioid receptor (DOR), and both PAMs exhibit interaction at DOR and BMS-986187 interacts with KOR (kappa opioid receptor). (Livingston et al., 2018; Stanczyk et al., 2019). In the future, isolating function to one receptor would be ideal for limiting possible off-target effects such as convulsions (DOR) or dysphoria (KOR). Work in collaboration with the Vahlteich Medicinal Chemistry Core and Center for Chemical Genomics at the University of Michigan will allow for the continued development of molecules with improved solubility, efficacy, and selectivity. Furthering our understanding of how changes to the chemical structure alters PAM interaction, function, and receptor selectivity would broaden the ability to develop future compounds with improved and very specific characteristics.

Determine where allosteric modulators bind on MOR:

While several studies have aimed to discover the binding site for MOR PAMs, much is still unknown. Remaining questions include where the site is located on the receptor, which residues are important for binding, and how those interactions influence PAM signaling remains unknown. Previous literature showing that BMS-986122 interacts with MOR and DOR and BMS-986187 at MOR, DOR, and KOR, although not with equivalent activities, but not the nociceptin opioid receptor (Livingston et al., 2018). Thus, we can hypothesize that the binding site for PAMs may be somewhat conserved across the three traditional opioid receptors. In addition, in silico studies have proposed potential binding sites for PAMs on MOR and DOR, located near the extracellular loops of the receptor, engaging transmembrane domains one, two, and/or seven (Bartuzi et al., 2016;

Shang et al., 2016). These studies show some similarities to the reported PAM binding sites in other GPCRs, such as the muscarinic M2 receptor (Kruse et al., 2013). Additionally, recent work utilizing NMR identified a potential site for interaction closer to the cytosolic side of the receptor (Kaneko et al., 2022). Furthermore, molecular dynamics simulations have revealed there are several other predicted allosteric sites on MOR, suggesting that PAMs could potentially interact at multiple locations on the receptor (Chan et al., 2023). Taken together with the body of work in this thesis showing how different PAMs may have differential functions at MOR, it is likely that they may interact with MOR differently.

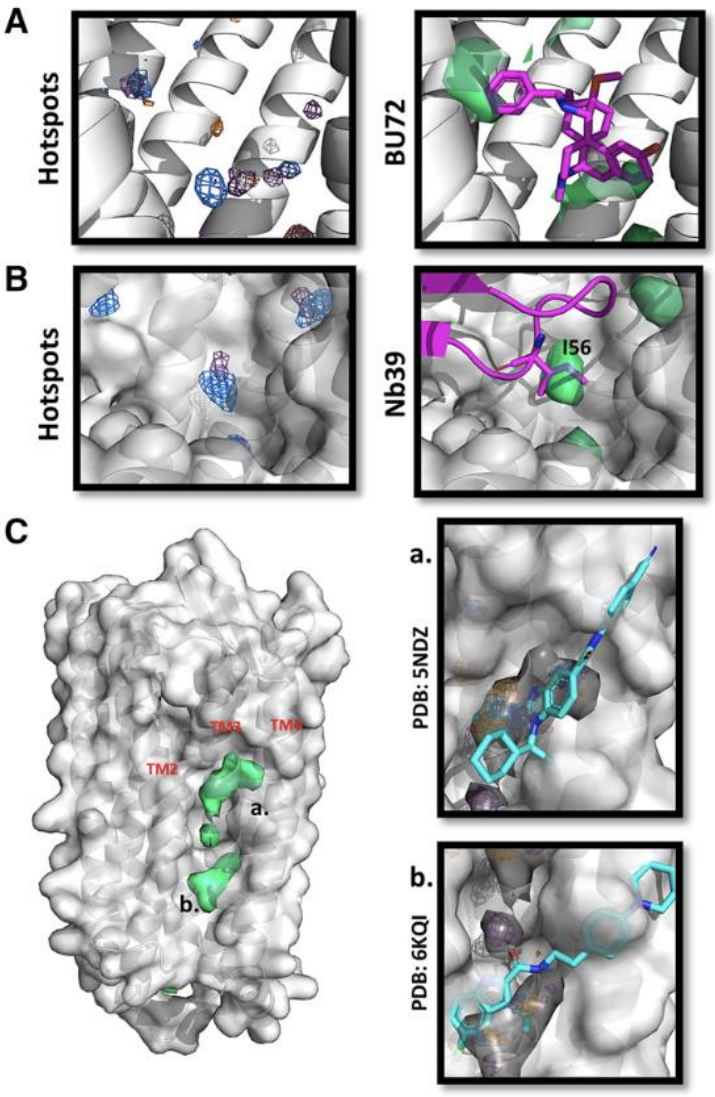

Figure 4.5. Predicted allosteric site for active-state MOR using mixed methods molecular dynamics simulations. BU72 is an orthosteric agonist used to activate MOR and Nb39 is an antibody fragment used to stabilize this active state. This method reveals multiple allosteric binding sites formed near TM2, TM3, and TM4. Image courtesy of Dr. Wallace Chan.

Several modeling studies have aimed to determine the PAM binding site on MOR, yet there has been no experimental validation at MOR of these predictions. Although methods like X-ray crystallography or cryo-EM are more definitive, PAMs exhibit low affinity (μM) in the absence of orthosteric ligands. This would require the presence of an orthosteric ligand which exhibits the highest cooperativity to increase PAM affinity for

MOR. Future studies to determine allosteric binding site(s) would employ a combination of molecular dynamics simulations (MDS), structure-activity relationships (SAR), and mutagenesis. MDS predictions of the allosteric site can inform SAR on what changes to PAM chemical structures are vital for maintaining binding and function at MOR. Additionally, altering MOR itself at the key residues that are predicted for allosteric binding would allow us to test the BMS compounds for loss in affinity, efficacy, etc. Overall, discovering the allosteric binding site of MOR would be useful for informing future drug development of positive allosteric modulators.

Examine the selectivity of PAM action at other GPCRs:

One of the major questions stemming from the work in this thesis is identifying the mechanism for allosteric potentiation of exogenous opioid effects *in vivo*. In particular, why BMS-986122 would only enhance opioid-induced antinociception, but not the other effects. In an effort to identify the specificity of the effect induced by BMS-986122 in mouse antinociception, we employed a non-opioid analgesic, clonidine. Clonidine produces dose dependent antinociception in the mouse warm water tail withdrawal by direct activation of the alpha-2-adrenergic receptor (Skingle et al., 1982; Capasso & Loizzo, 2001). Furthermore, clonidine has been used as a co-treatment with naloxone to shorten withdrawal periods and ameliorate the symptoms of withdrawal (Gold, 1980; Kleber, 2007). In this experiment, we examined the ability of BMS-986122 to enhance clonidine antinociception in the mouse warm water tail withdrawal assay.

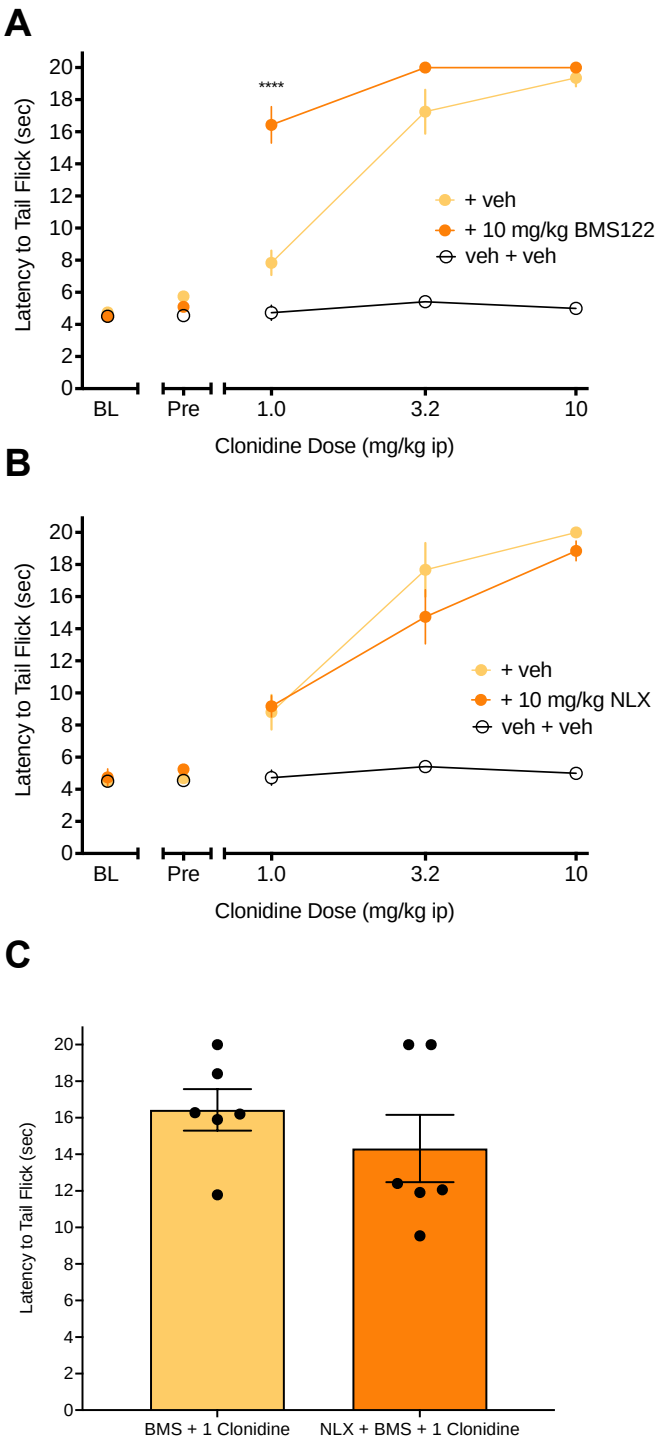

Figure 4.6. BMS-986122 potentiates clonidine antinociception. Cumulative dose-response curve in the mouse WWTW assay at 50°C for clonidine in the presence and absence of 10 mg/kg BMS-986122 (BMS122) (**A**), in the presence and absence of 10 mg/kg naloxone (NLX) (**B**), and in the presence of both BMS122 and NLC (**C**). Effect of 1mg/kg clonidine. A 20-second cutoff was set to prevent tissue damage. Data shown are means ± SEM for all groups (n = 6 for each group). ($F=32.4$, $p<0.0001$) ****$P<0.0001$ as determined by two-way ANOVA with Tukey's post-hoc test.

As shown in Figure 4.6, BMS-986122 potentiates the antinociceptive action of 1 mg/kg clonidine from 7.8 seconds to 16.4 second and shows a slight increase at 3.2 mg/kg clonidine (17.2 to 20 seconds). While this indicates that BMS-986122 may not selectively enhance opioid-induced antinociception, previous studies report that clonidine produces reward in the conditioned place preference assay, an atypical effect for adrenergic agonists (Asin & Wirtshafter, 1985). This effect was hypothesized to be produced by stress induced endogenous opioid release, and was subsequently blocked by naloxone (Navratilova et al., 2015). It has also been proposed that clonidine stimulates the release of dynorphin in rats, suggesting that the kappa opioid receptor may be a possible mechanism of action (Xie et al., 1986). To test if the enhancement of clonidine-induced antinociception was due to endogenous opioid action, we used 10 mg/kg of naloxone, an opioid antagonist, to block this effect. In Figure 4.6 (B), it is shown that we observed no significant decrease of clonidine antinociception on its own. To examine if naloxone would inhibit the enhancement of clonidine by BMS-986122, we gave 10 mg/kg naloxone as a pretreatment to BMS-986122 + 1 mg/g clonidine. In Figure 4.6 (C), we observed no significant decrease of the potentiation induced by the PAM. As such, future studies will need to examine the selectivity of BMS-986122 at other GPCRs. Furthermore, it will be imperative to study other non-opioid antinociceptive drugs with a variety of mechanisms of action, such as gabapentin, in this assay to determine if this effect induced by BMS-986122 is pain specific rather than receptor specific.

Overall Conclusions

Opioid drugs remain the most effective therapies for the management of moderate to severe pain. However, due to the various adverse effects associated with opioid receptor activation (constipation, dependence, euphoria, etc.), drug development efforts have been severely limited. Novel strategies that manipulate opioid receptor function, such as biased agonism and allosteric modulation could be useful for bypassing these limitations. As such, this work highlights how these novel strategies can combine with existing strategies, such as opioid sparing, to alter opioid function in a way that is beneficial. These studies advanced our knowledge surrounding allosteric modulation and the connection between signaling and behavioral outputs, providing support for development of a safer pain therapy. Future work investigating these connections will continue to build our understanding of opioid receptor function and how PAMs can play a role as a safer alternative to traditional opioid therapies.

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