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SEARCHING FOR NEW ANALGESICS WITHOUT ADDICTION RISKS

by

Mohammad Atiqur Rahman

A Thesis

Submitted to the
Department of Chemistry & Biochemistry
College of Science and Mathematics
In partial fulfillment of the requirement
For the degree of
Master of Science in Pharmaceutical Sciences
at
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Thesis Advisor: Thomas M. Keck, Ph.D.

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Dedication

Dedicated for my younger brother Arifur Rahman Razon for being my side in every situation.

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I would like to express my heartiest gratitude and sincere thanks to my research advisor Dr. Thomas Keck, PhD. for providing me valuable guidance and regular suggestion throughout my study and research at Rowan University. He continually and persuasively expressed a spirit of venture regarding research and development of new ideas. I am also grateful to Dr. Bradford Fischer, Ph.D. for supervising me to finish a project by his valuable directions and help. I would like to convey the deepest appreciation to my thesis committee members for their valuable time. I want to express my gratefulness to my parents (Afruz Khanom & Abdus Satter) and my beloved wife (Shahnaz Rahman) for encouraging me throughout my journey and providing me continuous support for my education. Finally, deepest thanks to all collaborators and my research group.

Abstract

Mohammad Atiqur Rahman
SEARCHING FOR NEW ANALGESICS WITHOUT ADDICTION RISKS
2019-2020
Advisor: Thomas M. Keck, Ph.D.
Master of Science in Pharmaceutical Science

Opioids are widely used to treat acute and chronic pain. But opioid addiction to these compounds can cause social and life-threatening health problems, including the risk of overdose. In this thesis, I evaluated IBNtxA (3-iodobenzoyl naltrexamine), a novel μ opioid receptor (MOR) agonist structurally related to the classical MOR antagonist naltrexone, in drug discrimination studies in order to better understand its subjective effects and more thoroughly its abuse liability. IBNtxA represents an intriguing lead compound for preclinical drug development specifically targeting MOR splice variants, potentially creating effective analgesics with reduced side effects. These results indicate that IBNtxA produces potent antinociception and has low abuse liability, likely driven by substantial κ opioid receptor agonist signaling effects. I also evaluated whether a combination of drugs can produce synergistic antinociceptive effects. Using von Frey testing and hot plate procedures, I measured the antinociceptive effects of morphine, the novel $\alpha 2/\alpha 3$ subunit-containing GABA_A receptor positive allosteric modulator MP-III-024, and their combination. Combinations of morphine and MP-III-024 produced supra-additive effects in both assays, indicating some level of synergy from these compounds. Results from these studies may lead to the development of new analgesic treatments with improved side-effect profiles, including reduced abuse liability.

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Chapter 1

Introduction

1.1. History of Opioids

Opium is an ancient drug derived from the milky sap of the opium poppy and used for medicinal purposes. (Booth, 1986) The use of raw opium in the modern era has been supplanted by more specific preparations of opiates—the naturally occurring compounds in opium—and by semisynthetic and synthetic opioids. There are several clinical effects of opioids, but their most significant effects involve relieving pain. (National Institute of Diabetes and Digestive and Kidney Diseases; 2012)

Around 3400 B.C. in lower Mesopotamia, the opium poppy was first cultivated. This poppy juice was known to produce euphoric effects, so Sumerians called it “Hul Gil” or “the Joy Plant”. (Schiff, 2002) After that, opium was cultivated in ancient Egypt around 1300 B.C. An Egyptian medical document called the Ebers Papyrus describes that poppy grains used to stop a crying child from crying at once. (Brownstein, 1993) At that time, opium was widely cultivated, traded, smoked, and used medically throughout the ancient world to every major civilization in Europe and Asia. It was used to treat pain and many other ailments successfully. (Schiff, 2002; Askitopoulou et.al., 2002; Booth, 1986; Dikötter et al., 2004). An ancient Greek physician, Hippocrates, described that for treating pain, internal diseases and epidemics, opium is an effective choice of drug. He also mentioned that the mixture of white poppy juice and the seed of nettle work as a narcotic, hypnotic and cathartic drug. (Kleisaris, et. al., 2014)

In 1806, the German scientist Friedrich Wilhelm Adam Sertürner dissolved opium into acid and then neutralized it with ammonia. This allowed him to identify the primary active ingredient, a weak base or alkaloid called *Principium somniferum* or morphine. (Brownstein, 1993; Krishnamurti & Rao, 2016) This is the first time they got a safe and effective way to treat pain and that is why Sir William Osler called this God's own medicine. (Young, 2007; Batmanabane, (2014) Morphine was taken orally until the invention of the hypodermic needle by the Scottish physician Charles Wood allowed the use of morphine injections to relieve neuralgia-induced pain. (Rosenblum et. al., 2009) German physician Edward Livenstein described addiction, withdrawal syndrome, relapse and explained that craving for Morphine was basically a physiological response. (Rosenblum et. al., 2009) In 1874, heroin (diacetylmorphine) was first synthesized by the English researcher C.R. Wright. (Merry 1975) Heroin is less addictive than morphine with higher efficacy. (Rosenblum et. al., 2009)

The term "opioid" originated in 1950, proposed by George Acheson, and means opiate-like—a combination of the word opium and the suffix -oid, meaning "like" or "resembling. (Eades et al., 1963) Opioid drugs have structural similarities with morphine but are either synthetic or semisynthetic. (Martin, 1983) The endogenous (i.e., naturally occurring) opioid peptides, endorphins, were first discovered in 1974 by two independent group of investigators—John Hughes and Hans Kosterlitz of Scotland, and Rabi Simantov and Solomon H. Snyder of the United States. (McLaughlin & Zagon, 2013)

1.2. Classifications of Opioid Drugs

Opioid drugs have a broad spectrum of activity. According to their procedure of synthesis, clinical opioids can be classified into three groups:

1. Natural Opioids: Extracted directly from poppy seeds, such as morphine, papaverine, and codeine.
2. Semi-synthetic Opioids: Obtained by the modifications of natural compounds, including morphine esters such as heroin, oxycodone, and oxymorphone.
3. Fully synthetic compounds, such as pethidine, fentanyl, and tramadol. (Jamison & Mao, 2015; Pathan & Williams, 2012)

Based on their binding affinity and effects on the four major opioid receptors, Opioids can also be classified into four types:

1. The δ -opioid receptor (DOR);
2. The κ -opioid receptor (KOR);
3. The μ -opioid receptor (MOR);
4. The nociception/orphanin opioid receptor (NOP).

Opioids can also be classified based on their signaling properties:

1. Full agonists: These activate the opioid receptors in the brain fully getting the full opioid effect (e.g., morphine, etorphine, methadone, meperidine, codeine, hydromorphone, codeine, fentanyl, heroin, hydrocodone, oxycodone, oxymorphone)
2. Partial agonists: These partially activate opioid receptors (e.g., buprenorphine, butorphanol, tramadol, pentazocine, nalbuphine)

3. Antagonists: These block the activity of agonists and partial agonists (e.g., naloxone, naltrexone). (Jamison & Mao, 2015; Waldhoer et al., 2004)

There are some opioid peptides which produced by the body itself called endogenous opioids or endogenous ligands. These are not like the regular clinical opioids. For producing pharmacological actions, these endogenous opioids need to bind to the opioid receptors. (Li et al., 2012; Waldhoer et al., 2004). Though there are numerous known endogenous opioid peptides, they can be classified into three different groups of ligands—enkephalins, endorphins, and dynorphins—which usually signal through the three major receptors, DOR, MOR, and KOR, respectively. (Li et al., 2012)

Table 1

Classifications of Opioids

| | Origin | Function |
|--|---|--|
| <u>Strong:</u> Morphine Pethidine Fentanyl Alfentanil Remifentanil Sufentanil | <u>Naturally Occurring:</u> Morphine Codeine Papavarine Thebaine | <u>Pure Agonists:</u> Morphine Fentanyl Alfentanil Remifentanil Sufentanil |
| <u>Intermediate:</u> Buprenorphine Pentazocine Butorphanol Nalbuphine | <u>Semisynthetic:</u> Diamorphine Dihydrocodeine Buprenorphine | <u>Partial Agonist:</u> Buprenorphine |
| <u>Weak:</u> Codeine | <u>Synthetic:</u> <u>Phenylpyperidines:</u> pethidine, fentanyl, alfentanil, sufentanil <u>Diphenylpropylamines:</u> methadone, dextropropoxyphene <u>Morphinans:</u> butorphanol, levorphanol Benzomorphans: pentazocine | <u>Agonist-Antagonists:</u> Pentazocine Nalbuphine Nalorphine <u>Pure Antagonists:</u> Naloxone Naltrexone |

Note. This information is obtained from Roth et al., 2002; Pathan & Williams, 2012; Goldstein, & James, 1984; Yaksh, 1987; Kieffer, 1997

1.3. Molecular Targets of Opioids

Opioid receptors belong to the super-family of G protein-coupled receptors (GPCRs) which are the most abundant class of cell-surface receptors in the central nervous system. (Mansour et al., 1993; Vortherms, & Roth, 2005) The presence of opioid receptors is high in the central nervous system (CNS), but they are found in many peripheral tissues like the tissue of small intestine, large intestine, adrenal, kidney, lung, spleen, testis, ovary and uterus of the mammalian groups of organism. (Wittert et al., 1996) Opioids have their

action at a cellular level, activating opioid receptors distributed throughout the CNS. The concentrations of opioid receptors are high in different areas of CNS, including the nuclei of tractus solitarius, periaqueductal grey, cerebral cortex, thalamus, and the substantia gelatinosa of the spinal cord. (Henriksen, & Willoch, 2008)

Three major subtypes of opioid receptors have been identified: Delta (δ), Mu (μ) and Kappa (κ) opioid receptors. Endogenous peptides like endomorphines, enkephalins, dynorphins, naturally occurring alkaloids, and other semisynthetic and synthetic small molecule ligands activate these receptors. (McCurdy et al., 2003) Another receptor subtype, called the nociception opioid receptor (NOP receptor), is phylogenetically related to other three, but it does not bind the same ligands. (Shang, & Filizola, 2015)

Delta (δ) opioid receptors (DORs) are mainly located in the brain, particularly in neural areas involved with olfaction and motor integration. (Mansour et al., 1988) DOR signaling is responsible for spinal, supraspinal analgesia and reduce gastric motility. (Trescot et al., 2008) Delta agonists and antagonists has anxiolytic activity of the opioid tone facilitated by DOR. (Saitoh et al., 2005; Perrine et al., 2006) DORs are a G protein-coupled receptor that respond to enkephalins as endogenous ligands. (Hart et al., 1985; Quock et al., 1999) Based on receptor binding studies, endogenous opioids have greater selectivity for δ -opioid receptor (DOR) over clinical opioids. DORs are mainly existing in pontine nuclei, amygdala and olfactory bulbs of CNS. Primarily the DOR is responsible for analgesia, physical dependence, euphoria, convulsant, and antidepressant effects. (Chung & Kieffer, 2013; Mao, 1999).

Mu (μ) opioid receptors (MORs) are located mostly presynaptically in the periaqueductal gray region, and in the superficial dorsal horn of the spinal cord. MORs are

also found in the external plexiform layer of the olfactory bulb, the nucleus accumbens, layers of the cerebral cortex, nuclei of the amygdala, intestinal tract, and the nucleus of the solitary tract.

MORs are responsible for different physical conditions related to supraspinal analgesia, respiratory depression, euphoria, sedation, decreased gastrointestinal motility, and physical dependence. (Benyamin et al., 2008) Three different MOR subtypes, μ_1 , μ_2 , and μ_3 , are known. These are not separate genes; they are splice variants of a single gene. (Cadet, 2004) μ_1 is associated with analgesia, euphoria, and serenity. μ_2 is associated with respiratory depression, pruritus, prolactin release, physical dependence, euphoria, reduced gastrointestinal motility, miosis and sedation. (Pasternak et al., 2013) μ_3 is associated with vasodilation. (Mao, 1999; Stein et al., 2003)

κ opioid receptors (KORs) are mainly present in the substantia gelatinosa, hypothalamus, periaqueductal gray, and claustrum in the brain. KOR activation is responsible for producing spinal analgesia, sedation, miosis, dysphoria, neuroprotection, and diuresis. There are three different subtypes of KOR, namely κ_1 , κ_2 and κ_3 . (Lalanne et al., 2014; Stein et al., 2003)

The natural ligand of the nociceptin opioid receptor (NOP) is the 17 amino acid neuropeptides known as nociceptin (N/OFQ) (Malmberget al., 1997). The expression of this receptor mainly in cortex, ventral forebrain, hippocampus, hypothalamus, amygdala, and in the dorsal horn of spinal cord. (Donica et al., 2013; Koob et al., 2014). NOP activation produces physiological responses such as anxiety, food intake, learning, locomotor etc. can be produced. (Donica et al., 2013)

Table 2

Different Opioid Ligands and Receptor Targets

| Opioid Ligands | Mu Opioid Receptor | Kappa Opioid Receptor | Delta Opioid Receptor | Nociceptin Receptor |
|--|--------------------|-----------------------|-----------------------|---------------------|
| Endogenous Ligands | | | | |
| β -endorphin | +++ | +++ | +++ | - |
| Leu-enkephalins | + | - | + | - |
| Dynorphin A & B | ++ | +++ | + | + |
| Nociceptin/orphanin FQ | - | - | - | +++ |
| Clinical and Nonclinical Ligands | | | | |
| Agonists | | | | |
| Morphine | +++ | + | + | - |
| Diamorphine | +++ | + | + | - |
| Fentanyl | +++ | + | - | - |
| Pethidine | +++ | + | + | - |
| Partial Agonists | | | | |
| Buprenorphine | ++ | + | - | - |
| Pentazocine | - | ++ | - | - |
| Antagonists | | | | |
| Naloxone | +++ | ++ | ++ | - |
| Naltrexone | +++ | ++ | ++ | - |
| + = low affinity, ++ = moderate affinity, +++ = high affinity, - = no affinity | | | | |

Note. This information is adapted from Lemberg et al., 2008; Kumar et al., 2019; Lemberg et al., 2008; Nielsen et al., 2007; Ross & Smith, 1997; Leander 1987

1.4. Mechanism of Action of Opioid Agonists

Generally, pain sensations are signaled by primary sensory neurons releasing predominantly substance P and glutamate in the dorsal horn of the spinal cord. Spinothalamic tracts help to transmit nociceptive information to the brain. The activation of descending pathways depends on the ascending information. This ascending information can trigger the descending pathways, from the midbrain periaqueductal grey area, which exercise an inhibitory control over the dorsal horn. (Ossipov et al., 2014)

Opioid activation of opioid receptors produces intracellular signaling effects typical of $G\alpha_{i/o}$ -coupled GPCRs. Initially, guanosine triphosphate (GTP) binds with the $G\alpha$ subunit and GTP converts into the guanosine diphosphate (GDP). GDP generates α -GTP complex to dissociate away from the $\beta\gamma$ complex. (Pathan & Williams, 2012; McDonald & Lambert, 2005; Stein, 2016). The available free α -GTP and $\beta\gamma$ interact with separate target proteins. As a result, inhibition of adenylate cyclase happened as well as cyclic adenosine monophosphate (cAMP) decreases inside the cell. (McDonald & Lambert, 2005; Pathan & Williams, 2012).

With respect to synaptic signaling, opioids can act at two different sites, the presynaptic nerve terminal, and the postsynaptic neuron. The postsynaptic actions of opioids are normally inhibitory whereas the presynaptic action of opioids is to inhibit neurotransmitter release. Inhibition of the neurotransmitter release is their major effect in the nervous system. Neurotransmitter release from neurons is normally preceded by depolarization of the nerve terminal and Ca^{2+} entry through voltage sensitive Ca^{2+} channels are the process to release Neurotransmitter from neurons. Opioids have direct effects on Ca^{2+} channels to reduce Ca^{2+} entry or on increasing the outward K^+ current and thus the inhibition of

neurotransmitter release happened. As a result, repolarization time and the duration of the action potential becomes lower. Opioids has both effects because opioid receptors are ostensibly coupled via G-proteins straight to K^+ channels and voltage-sensitive Ca^{2+} channels. All MOR, DOR, and KOR signaling can also regulate Ca^{2+} channels in both pre- and post-synapse reduces Ca^{2+} inside the cell and impaired the neurons' excitability. (Simons, 1988; Stein, 2016) These intercellular events cause hyperpolarization as well as hinder neuronal firing in key nociceptive circuits. As a result, it eventually reduces pain. (McDonald & Lambert, 2005; Pathan & Williams, 2012; Simons, 1988; Stein, 2016)

1.5. Use of Opioid Drugs

1.5.1. As analgesics. Opioids are very effective drugs for the treatment of pain. The management of acute severe pain and chronic pain is completely depending on the opioid analgesics. A lot of people are suffering from the chronic pain all over the world. In just the United States, more than 100 million peoples are suffering from acute and chronic pain and around 6-8 million undergo long-term treatment by opioid drugs. (Jamison & Mao, 2015; Kalso et al., 2004). Opioid analgesics work effectively against both cancer and non-cancer pain. There is a significant effect of intravenous infusion of opioid analgesics to heal the neuropathic pain like central pain, postherpetic neuralgia and mixed neuropathic pain. Different doses of oral opioids are effective against neuropathic, musculoskeletal, and other non-cancer pain. (Kalso et al., 2004). WHO confirmed the effectiveness of opioid drugs to manage the most challenging cancer pain. Almost 75% of the cancer pain managed by applying opioid analgesics. (Thapa et al., 2011). Morphine is enough alone to manage severe cancer pain of almost 85% of patient. It is like a single pharmacotherapy. Combination therapy with morphine and other analgesic can provide synergistic effects.

(Gilson et al., 2004). These opioid analgesics are very effective against cancer pain like severe pain but due to some abusive properties which make it intricate. (Thapa et al., 2011)

1.5.2. Treatment options for pulmonary edema. Opioids, especially morphine, have been used for a long time to treat pulmonary edema. In pulmonary edema, a patient's left ventricle fails to properly operate, leading to elevated hydrostatic pressure and increased pulmonary circulation. As a result, extra fluid accumulates in the interstitium and alveoli of the lungs. (Ellingsrud & Agewall, 2016) To treat pulmonary edema, reduction of hydrostatic pressure through lowering preload and afterload is required and can be achieved by using the vasodilatory properties of morphine. (Mattu et al., 2005)

1.5.3. Treatment options for diarrhea. Opioid drugs can be used to treat irritable bowel syndrome with diarrhea (IBS-D). There is no effective treatment method is available to treat IBS-D, so opioids can be a treatment of choice. A Schedule IV drug called eluxadoline was approved by the FDA to manage IBS-D and features a mixed pharmacology: it is a MOR agonist, which has both DOR antagonist activity and KOR agonist activity. Eluxadoline, provides relief of IBS-D-associated symptoms with significantly lower side effects, specifically constipation, by targeting the local opioid receptors in the gut, which reduces the side effects of the central nervous system. (Maltz & Fidler, 2017)

1.5.4. As a cough suppressant. Codeine and hydrocodone have been used in cough medications along with other drugs like chlorpheniramine (an antihistamine), pseudoephedrine (a decongestant), and guaifenesin (an expectorant). Some studies show that codeine does not have any significant effect on chronic obstructive pulmonary disease (COPD) in adults or on acute cough in children. The United States Food & Drug

Administration (FDA) does not recommend cough medication with opioids if the patient is younger than 18 years. (Smith et al., 2006; McCrory et al., 2013)

1.5.5. As an anesthetic. There are some available narcotic analgesic opioids, especially morphine, used as anesthetic agents. In particular, for patients with cardiovascular disorders, opioids are used in different major surgeries to prevent the occurrence of cardiac depression. (Bovill et al., 1984; Hug, 1992)

1.6. Adverse Effects of Opioids

Opioids undoubtedly are effective analgesics, but they have well-known side effects that include respiratory depression, sedation, constipation, bradycardia, tolerance, hyperalgesia, dependence, immunologic effects, hormonal change, sleep disturbances, and abuse and addiction. (Ballantyne & Mao 2003; DeWire et al., 2013)

Opioid analgesia in patients can be difficult to manage because of risks associated with tolerance, hyperalgesia, withdrawals symptoms and dependency, euphoria and drug abuse, and opioid addiction. (Fields & Margolis, 2015; Jamison & Mao, 2015; Volkow & McLellan, 2016)

1.6.1. Respiratory depression. For survival, humans are totally dependent on the cardiorespiratory or ventilatory control system for adequate uptake of oxygen and removal of CO₂ using lungs. (Dahan et al., 2010) Potent opioid analgesics depress ventilation by acting on μ -opioid receptor (MOR) located on respiratory neurons in the brainstem. This potentially life-threatening cause of substantial morbidity and mortality called opioid-induced respiratory depression (OIRD) (Van der Schier et al., 2014; Dahan et al., 2010) OIRD initiates cardiorespiratory arrest with subsequent hypoxia and hypercapnia, resulting

fatalities. (Dahan et al., 2010; Morgan et al., 2006; Oderda et al., 2007; Oderda et al., 2013) Opioid receptors expressed abundantly in the CNS specifically respiratory neurons which is directly related to OIRD. (Pattinson, 2008) Though some cases of opioid-induced respiratory depression acts as a beneficial for pain patients, but ultimately it may increase the mortality if the opioid addicts take similar dose in different condition or relapse after a period of abstinence. (Siegel et al., 1982)

1.6.2. Opioid-induced sedation. Opioids produce sedation and drowsiness, primarily via anticholinergic and other multiple inhibitory effects on cerebral activity. (Ahmedzai, 1997; Slatkin & Rhiner, 2004) Available treatments for opioid-induced sedation include methylphenidate. For cancer patients, administrating 10-15 mg doses of methylphenidate reduced drowsiness significantly. Concurrently, reduction of opioid doses without increasing pain may be possible. (Wilwerding et al., 1995) While other available treatment options for treating sedation include dextroamphetamine, donepezil, modafinil and caffeine, methylphenidate is considered the first-line therapy because of its low side effects and abuse potential. (Reissig & Rybarczyk, 2005) Opioids cause central nervous system depression, which can diminish a patient's ability to operate heavy equipment and drive vehicles. A patient should be able to operate a vehicle after the opioid analgesic regimen reaches a stable condition and patient doesn't have any significant cognitive impairments. (Trescot et al., 2008) One study showed that a group of patients receiving opioid analgesic for chronic pain, they are capable to operate vehicles during daytime. (Cotsonis, 2005) Another study recommended that with stable doses of opioids, patients don't show any impairment of psychomotor abilities observed after opioid administration. (Rosomoff, 2003)

1.6.3. Opioid-induced constipation. Opioid-induced constipation (OIC) is a common problem during opioid administration, even with the single dose. The main cause of constipation is interaction of a plethora of underlying pathophysiologies, lifestyle factors, and medications which leads to opioid-induced bowel dysfunction. (McMillan, 2004) Chronic constipation may be caused of haemorrhoid formation, rectal pain and burning, bowel obstruction, bowel rupture, upper gut dysfunctions, including gastroesophageal reflux disease and death. (Ricardo Buenaventura et al., 2008; Holzer, 2004) 40-95% patients are facing this problem and resulting a significant increase of morbidity and mortality after long-term consequences of constipations. (Datta et al., 2008; Sizar, Gupta, 2019) In the GI tract, opioid drugs prevent gastric emptying and peristalsis. As a result, delayed absorption of medications and increased absorption of fluid happened. The lack of fluid in the intestine is the cause to hardening of stool and constipation. (Sizar, Gupta, 2019) In severe condition of constipation, reduction of opioid dose required resulting in reduced activity of analgesia. In chronic condition hemorrhoid, rectal pain and burning sensation, bowel obstruction, potential bowel rupture and death can be happened. (Datta et al., 2008) This is not clear that this type of constipation in human is centrally or peripherally mediated. Morphine-induced constipation mediated within the CNS and alter autonomic outflow to the gut. (Yuan, Foss, 2000) Also, it affects intestinal motility peripherally by a direct stimulation of opioid receptors in the enteric nervous system. (Sternini 2001) The management of opioid induced constipation is not an easy task. Opioids can be administrating after carefully considering the risk -benefit ratio or taking some alternative options such as Lifestyle modification, alteration of aggravating factors and/or the use of simple laxatives. (Bharucha et al. 2016)

1.6.4. Opioid-induced bradycardia. Opioids mainly binds to the opioid-specific receptors specially in central nervous system (CNS), but opioid specific receptors also found in other different organs like cardiovascular tissue. (Chen, & Ashburn, 2015; Warltier et al., 2000) When opioid administered as an anesthetic agent alone, it produces some effect on heart but do not depress cardiac contractility except the high doses of meperidine. (Chen, & Ashburn, 2015; Warltier et al., 2000) If opioids are combined with other medications, there are significant changes in cardiac function: it impacts the cardiovascular system including vagus nerve-mediated bradycardia. (McIntosh et al., 1992; Lessa & Tibiriçá, 2006; Chen, & Ashburn, 2015) Patients may face vasodilation and decreased sympathetic tone after acute administration of opioids. If given concurrently with the benzodiazepines, leads to decrease cardiac output significantly. Opioids like morphine, hydromorphone, hydrocodone, and meperidine can cause significant decreases in systemic vascular resistance and blood pressure by releasing histamine. But there are no effects on intraoperative ischemia, postoperative myocardial infarction or causing death of opioid-based anesthetics use. (Chen, & Ashburn, 2015; Fareed et al., 2013)

1.6.5. Opioid tolerance. Opioids are well-established to induce tolerance, described as the decreased efficacy of an opioid agonist after repeated or prolonged administration of a specific dose. (Morgan, & Christie, 2011). Drug interactions with opioid receptor(s), dose of drug and frequency of drug administration are the considerable factors for the development and extent of the tolerance. There are several reasons opioid tolerance develops, including upregulation of drug metabolism, desensitization of receptor signaling, and downregulation of receptors. (Cahill et al., 2016) Opioid-induced tolerance is problematic and challenging to manage. Hospitalized patients require longer hospital

stays, have higher readmission rates, and have higher mortality rates. (Gulur et. al., 2014) Increased opioid doses given to counter tolerance can result in opioid-induced hyperalgesia. (Cahill et al., 2016)

1.6.6. Opioid-induced hyperalgesia. Opioid-induced hyperalgesia (OIH) is defined as a state of increased pain sensitivity following the long-term use of high-dose opioids and occurs when neoplastic modifications happen in both peripheral and central nervous system. (Lee et al., 2011, Tompkins & Campbell, 2011). The molecular mechanisms that cause OIH are not well-established yet, but there are several proposed mechanisms. OIH may result when tolerance develops by molecular adaptations in MOR-expressing neurons that can change the interactions between cells and activate the independent oppositional system. (Zeng et al., 2006; Vera-Portocarrero et al., 2007) Opioid-induced cell apoptosis may contribute to the development of hyperalgesia; in particular, loss of GABA neurons via apoptosis may lead to changes in spinal neuron circuits. (Mao et al., 2002) This sensitization is a paradoxical response and patients become more sensitive to certain painful stimuli during the opioid treatment. The pain experienced in OIH can be very similar to the patient's original pain. OIH shows a distinct, definable, and characteristic phenomenon that can prove about the loss of opioid efficacy in some patients. (Lee et al., 2011; Tompkins & Campbell, 2011)

1.6.7. Opioid withdrawal and dependence. Opioid treatments can result in withdrawal symptoms, including the development of an altered physiological state involving autonomic and somatic hyperactivity. Dependence is a physical state that occurs during withdrawal following repeated administration of opioid drugs, producing persistent physical–somatic withdrawal symptoms. (Higgins et al., 2018) In general, physical

dependence produces a disorder in which the patient is not able to reduce or quit opioid use because withdrawal symptoms become too severe. (Collett, 1998) Importantly, physical dependence can result in greater long-term opioid use and can lead to addiction.

1.6.8. Opioid-induced immunologic effects. In the 1980s, scientists demonstrated cellular immune suppression and decreased resistance to bacterial infection in guinea pigs after administering morphine. Opioids increased the incidence of infections in heroin addicts and act as a cofactor in the pathogenesis of human immunodeficiency virus. While some exogenous opioids can generate immunosuppression, their endogenous counterparts like endorphins induce immune activation. (Stephanou et al., 1991; Cantacuzene, 1898) Immunosuppression leads by opioids have different mechanisms which produce different immune profile. Codeine, methadone, morphine, fentanyl, sufentanil, and remifentanil produce strong immunomodulating effect whereas oxycodone, tramadol, buprenorphine and hydromorphone produce weak immunomodulating effect. Morphine regulates adaptive and innate cells, like NK cells, macrophages, mast cells, B cells and T cells. Additionally, morphine's action is connected to central nervous system structures and the HPA axis suppressed NK cell cytotoxicity and lymphoproliferation. (Haroutounian, 2018) The lowest immunosuppressive agent is buprenorphine which considered as a first-line analgesic. (Davis, 2012) Since acute and chronic opioid administration can be a reason of the inhibitory effects on antibody and cellular immune responses, natural killer cell activity, cytokine expression, and phagocytic activity. The immunologic effects of opioids are controlled by central and peripheral mechanisms. (Stephanou et al., 1991; Peterson et al., 1998; Chuang et al. 1995) Central opioid receptors can facilitate peripheral immunosuppression by involving the hypothalamic- pituitary-adrenal axis and the

autonomic nervous system. Peripheral immune cells under the effect of cytokines, can release endogenous opioids modulating analgesia and inflammatory responses. (Chuang et al. 1995; Trescot et al., 2008)

1.6.9. Opioid-induced hormonal changes. Opioid administration produces hormonal effects in both men and women. These effects on hormonal function, called opioid endocrinopathy (OE), also occurs when the serum hormone levels return to normal after drug withdrawal. (Trescot et al., 2008) Opioids can affect different hormones, including testosterone, estrogen, luteinizing hormone, gonadotrophin releasing hormone, dehydroepiandrosterone and dehydroepiandrosterone sulfates, adrenocorticotropin and corticotropin-releasing hormone, and cortisol. Sexual disorders such as erectile dysfunction and decreased libido, depression, and decreased energy levels are common adverse effects for men. (Datta et al., 2008) One to four hours after acute administration of opioids, testosterone levels are significantly lowered, and it takes around 24 hours to return to normal levels. (Daniell, 2002) When opioids are administered chronically, it results in tonic decreases in both total and free testosterone levels. (Datta et al., 2008) There are other similar hormonal side effects for women, including depression, dysmenorrhea, sexual dysfunction, and potentially reduced bone mineral density. (Daniell, 2008)

1.6.10. Opioid-induced sleep disturbances. Opioid-related sleep disturbances include disorders of initiating and maintaining sleep, disorders of excessive somnolence, disorders of sleep–wake schedule, and dysfunctions associated with sleep, sleep stages, or partial arousals. (Walker et al., 1990) These disturbances are commonly experienced by cancer patients. (Moore & Dimsdale, 2002) While sleep disturbances can result from insomnia or pain, there is no evidence correlating pain severity and sleep disturbances.

(Trescot et al., 2008) There is some evidence that opioids can increase the number of sleep-wake transitions, reducing total sleep time and efficacy. (Koren et al., 2006; Kurz & Sessler, 2003) There are many neurotransmitters that regulate sleep and waking, including noradrenaline, serotonin, acetylcholine, dopamine, histamine, gamma-aminobutyric acid (GABA), the pituitary hormones, and the neurohormone melatonin. Drugs that can alter signaling by these neurotransmitters can affect sleep. Opioid drugs can alter the balance of these neurotransmitters, but how opioids exactly disrupt the sleep is still unclear. (Trescot et al., 2008)

1.6.11. Opioid abuse and addiction. Opioid addiction is a chronic, relapsing disorder characterized by a strong and habitual desire to use opioid drugs when medically unnecessarily. People can become addicted even when administered opioid drugs as prescribed, because opioids have very high possibility for causing addiction. (Morgan, & Christie, 2011). Opioids are neuroactive substances that alter neurotransmitter functions, inducing positive changes in mood (euphoria) or reducing negative dysphoric moods. (Lankenau, 2002). Opioid-induced euphoria can lead to misuse and abuse of medications. Prolonged use of these substances leads to tolerance, physical dependence, sensitization, craving, and relapse. (Leshner, 1997)

1.7. The “Opioid Epidemic”

The “opioid epidemic” is a major public health concern arising from the over-prescription of opioids for relieving pain and the growth in use, abuse, and overdose of opioids, significantly impacting patient health and economy. This opioid epidemic is not the first drug crisis in US: over a century ago, doctors frequently prescribed morphine to their patients to alleviate pain, causing the first opiate epidemic. (Courtright, 2001)

According to the Centers for Disease Control and Prevention, there are three different waves in the modern American opioid epidemic can be considered for rising the death of opioid overdose. The first wave started in the 1990s, when the opioid prescribing increased gradually. The second wave is marked by increased overdose deaths involving heroin in 2010. The third wave began in 2013 due to significant increases in overdose deaths involving synthetic opioids—mainly those involving illicitly manufactured fentanyl (IMF). The IMF market has changed over time. IMF can also be found in combination with heroin, counterfeit pills, and cocaine. (Centers for Disease Control and Prevention, 2018)

In 2016, 11.5 million Americans were misusing opioid prescriptions, more than 2.1 million had a diagnosable opioid use disorder, and more than 42,000 people died from opioid overdoses. (Department of Health and Human Services, 2018) The US Department of Health and Human Services declared a public health emergency for this opioid crisis in October 2017. (US Department of Health and Human Services, 2017) Over the last two decades, hundreds of thousands of lives have been lost and millions more people and their families affected by opioid epidemic. The use of opioids is important for pain management but must be weighed against the costs of opioid use disorder and deaths. The CDC has taken actions to raise awareness and reduce the practices of opioid prescription. In 2016, the Comprehensive Addiction and Recovery Act (CARA) was signed into law, consisting of six pillars to overcome the opioid crisis: prevention, treatment, recovery, law enforcement, criminal justice reform, and overdose reversal. (Florence et al., 2016; CARA, 2018)

Opioid misuse, abuse, and overdose deaths are increasing US as well as the whole world. These increases started in the late 1990s and accelerated since. According to the

Centers for Disease Control and Prevention (CDC), the age-adjusted rate of overdose deaths nationally rose by 9.6% from 2016 (19.8 per 100,000) to 2017 (21.7 per 100,000). Opioids were involved in 70,000 overdose deaths nationally in 2017. This number represents 67.8% of all drug overdose deaths in the United States. Synthetic opioids are primarily responsible for current drug overdose-related deaths. (CDC, 2019)

1.8. Treatments for Addiction

Treatment options of opioid addiction are limited. Behavioral therapy and pharmacotherapy can be used either in individually or combination (Carroll & Onken, 2005), but treatments combining medication along with counseling and support lead to improved recovery (Eitan et al., 2017). Treatment can be started with counseling, opioid replacement therapy, and gradual discontinuation of the drug. Discontinuation of the drug to quickly can produce serious withdrawal syndrome. For managing that situation, drug detoxification is the option for the physicians (NIDA, 2020).

1.8.1. Behavioral therapies. Behavioral therapy includes support for people to give up drugs of abuse by offering them incentives to stay away from those abusive compounds (Petry & Carroll, 2013; Tuten, 2012). There are several different types of behavioral therapies available for addiction treatment, including cognitive behavioral therapy, contingency management, community reinforcement approach, and motivational enhancement therapy (Carroll & Onken, 2005; NIDA, 2020).

1.8.1.1. Cognitive behavioral therapy. The main goal of Cognitive-Behavioral Therapy (CBT) is to move the patient towards abstinence; its effects are durable and improve after the end of treatment (Carroll et al., 1994; Carroll et al., 2000). The focus of

this therapy is on relapse prevention, countering the maladaptive behavioral patterns that underlie substance abuse. Patients learn different skills to identify and correct the problematic behaviors. Eventually, those skills can be effective to stop drug abuse and other related problems (NIDA 2020; Carroll & Onken, 2005). Computer-based CBT systems are under development to treat drug abuse-related complications broadly (Carroll et al., 2008).

1.8.1.2. Contingency management interventions/motivational incentives.

Contingency management (CM) is an effective treatment approach in which patients receive rewards to stop taking drugs (McGovern & Carroll, 2003). There are two kinds of CM: voucher-based reinforcement and prize incentives CM. In voucher-based reinforcement, the patient receives incentive vouchers upon confirming a drug-free urine sample. Initially they receive low base amount of incentives, but it increases by confirming drug free urine sample for consecutive tests. Positive urine samples require the patient to start over from the baseline low incentives. Vouchers can be used for buying food items, movie tickets, or other items for leading healthy life. (Bickel et al., 1997; NIDA 2020) The program prize incentives CM provides cash prizes instead of vouchers. If participants test negative for drugs in urine or breath weekly for at least three months, and attend counseling sessions and target activities, they can win \$1-100 prizes by raffle draw (Bickel et al., 1997; NIDA 2020). A significant number of patients have remained abstinent from opioids or cocaine through this CM service (Petry et al., 2005; Prendergast et al., 2006).

1.8.1.3. Community reinforcement approach.

The community reinforcement approach is a psychosocial intervention that includes recreational, familial, social, and vocational reinforcers with material incentives. These activities reinforce a non-drug-using

lifestyle and the goal of the treatment includes habituating the patient to a drug-free life (NIDA, 2020). This approach enhances the importance of family relationships, developing different skills, new recreational activities, and social networks. The computer-based version of the community reinforcement approach is effective for opioids and/or cocaine-dependent patients (Higgins et al., 2003; NIDA, 2020). This computer-based version is also effective for adolescents (Brooks et al., 2010).

1.8.1.4. Motivational enhancement therapy (MET). This therapy is based on counseling to reinforce lifestyle alterations and reduced drug use. The purpose of this therapy is to induce rapid and internal motivational change in the patient and encourage abstinence. Individual sessions include an initial assessment battery session, stimulating discussion session, two to four individual treatment sessions, and motivational interviews. The principle of this interview is to build up strength to give up the abusive drugs (NIDA, 2020; Ball et al., 2007).

1.8.2. Addiction pharmacotherapy. Pharmacotherapy is an important step for treating opioid addiction, providing a beneficial effect when applied concurrently with behavioral therapy. Two general treatment patterns are available, opioid maintenance and detoxification (Stotts et al., 2009).

1.8.2.1. Methadone. Methadone is a well-established option for opioid maintenance pharmacotherapy, used all over the world with a long track record (Kreek et al., 2010; Mattick et al., 2009). Treatment with methadone provides significantly higher rates of treatment retention and lower rates of illicit opioid use compared with placebo or no treatment (Mattick et al., 2009). Methadone is a potent analgesic and it has a good oral bioavailability (75%). Though methadone is an opioid agonist, it has some dissimilarities

with the other available opioid analgesics. Oral methadone has a longer half-life than heroin—this is one reason for using this as an opioid replacement (Stotts et al, 2009). There are some limitations of methadone for patients with chronic renal diseases and pregnant women, for whom there is a chance the fetus may develop methadone dependence. Methadone pharmacotherapy works best in combination with behavioral therapy (Alinejad et al., 2016).

1.8.2.2. Buprenorphine. Buprenorphine is a narcotic drug derived from thebaine, used as a potential analgesic in many countries. Buprenorphine acts as a partial agonist at MOR and is approximately 30 times more potent than morphine, highly lipid soluble, well-absorbed sublingually, but it has low bioavailability (Center for Substance Abuse Treatment, 2004). For the treatment of opioid addiction, buprenorphine can be used in two ways: long-term maintenance or detoxification from opioids. Its partial MOR agonist properties reinforce patient compliance with regular administration (Barnett et al., 2001). The most important characteristics of buprenorphine is that it does not produce euphoria and it can significantly decrease opioid withdrawal effects. That is why primary care physicians can safely prescribe buprenorphine for the case of opioid withdrawal (Kahan et al., 2011).

1.8.2.3. Naltrexone. Naltrexone is a long-acting opioid antagonist which does not produce euphoria or addiction (Potenza, 2006). It is successfully used to reverse accidental heroin overdoses and treats opioid dependence. The main characteristics of the naltrexone is it can prevent a relapse to opioid use after heroin detoxification (Minozzi et al., 2011). For some patients, the main treatment goal is detoxification; methadone or buprenorphine detoxify slowly, but naltrexone has a faster detoxification capacity. Clonidine, an alpha 2

adrenergic agonist, is often used as a combination therapy with naltrexone for rapid opioid detoxification (Gowing et al., 2000). This treatment option seems extremely efficient, but different studies disagree about the claimed efficacy for opioid addiction treatment (Minozzi et al., 2011).

1.9. Economic Effects of Opioid Addiction

The economic consequences of opioid misuse and opioid use disorder has significantly impacted healthcare costs and public health. An analysis of an administrative database of a pharmacy claims shows that opioid abusers' annual healthcare costs are 8 times higher, and drug costs are 5 times higher, than nonabusers. (White et al., 2005) In 2007, a total of \$55.7 billion costs was associated with prescription opioid abuse, including \$25 billion in healthcare costs, \$25.6 billion in workplace costs, and \$5.1 billion in criminal justice costs. Approximately \$23.7 billion of healthcare costs are due to medical and prescription expenses. (Birnbaum et al., 2011) In 2013 the situation was even worse: the estimated costs rose to \$78.5 billion, \$22 billion more than in 2007. (Florence et al., 2016) Patients repeatedly receiving opioid therapy for severe pain have an increased morbidity. (Ballantyne, 2007) Healthcare cost can be lowered if opioid allocates and used properly. Mismanagement and misconceptions are the key to increase costs. Proper allocation and reduction of improper use of opioid can be lowered the health care costs. (Lipman & Webster, 2015)

1.10. Methods for Determination of Analgesic Activity

There are various methods to evaluate the analgesic activity of different drugs. These methods follow the general strategy that analgesic drugs can alter the effects of painful

stimuli (Davies et al., 1946). To screen for analgesic agents, nociceptive stimuli are administered to the animals prior to administration of an analgesic. These painful stimuli produce animal responses indicative of painful sensations, including jumps, withdrawing, or licking or shaking of the paws, tail flick, skin twitch, or flight (Pircio et al., 1975). Popular methods for determining analgesic activity are explained below:

1.10.1. Writhing test. Analgesic activity or anti-nociceptive activity of synthesized compound can be evaluated by a chemical method called the writhing test. In this method, different irritant compounds, like phenylquinone or acetic acid, are injected into the abdominal regions of mice or rats, inducing painful feelings, and increasing the frequency of writhing. After injecting an analgesic compound, the frequency of abdominal writhing should decrease significantly (Cruz, 1996; Gawade, 2012; Achar et al., 2010). This test is appropriate for testing the analgesic profile of the peripherally acting drugs, like chlorpromazine, antihistamine and meprobamate. But in this test, evaluation of analgesic duration is difficult, because the frequency of writhing decreases over time (Franklin & Abbott, 1989; Siegmund, 1957).

1.10.2. Hot plate test. The hot plate test is another way to evaluate acute, cutaneous, thermal pain sensitivity. This test believed to evaluate a supraspinally organized nociceptive response because of the involvement of higher brain functions (Eddy & Leimbach, 1953). In this principle, rodents are placed onto a hot surface for a specific time frame and observed for nocifensive activity, like paw licking or jumping. Administration of an analgesic compound can increase the latency time to licking or jumping (Woolfe & MacDonald, 1944; O'Callaghan & Holzman, 1975). The hot plate test is relatively

complicated compared to other thermal assays because rodents show complex and subtle behavioral activities (Espejo & Mir, 1993)

1.10.3. Von Frey tests. Von Frey tests are the set of tests to detect the noxious stimulus of a rodent due to stimulation of nociceptors. In this test, 50mm long a number of varying diameters Von Frey hair or fibers has been used. (Carter et al., 2010) Animal stands on an elevated mesh platform and von Frey hair inserted through the mesh to poke the animal's hind paw. Normal reaction for the animal including withdrawing or licking or shaking the paws. If animals show any of these kinds of reaction considered as a positive response. The exact force of the fiber is determined by its thickness. (Deuis et al., 2017; Minett et al., 2014)

1.10.4. Tail flick test. The tail flick test is one of the most common tests antinociceptive assays. Based on exposing rodents to a phasic thermal stimulus of high intensity and measuring the latency of the avoidance response (D'Amour & Smith, 1941). This model can be used for measuring acute nociception and it is not an injury model. (Irwin et al., 1951) In this method, radiant heat is applied to the tail of the animals, and the nociceptive sensitivity is determined by the tail–flick latency (D'Amour, Smith, 1941; Hole & Tjølsen, 1993). If this latency is prolonged by administering any drug or drug combination, that indicates analgesic activities of the test drug. But in this model, spinal transection above the lumbar level fails to block the tail–flick response, therefore it may not measure pain directly but rather the spinal nociceptive reflex (Ren & Han, 1979).

1.10.5. Formalin test. The tail formalin test is a popular test to evaluate inflammatory pain due to injury. This model is useful to measure clinical pain because it affects inflammatory, neurogenic, and central mechanisms of nociception (Hunskar and Hole, 1987; Tjølsen and Hole, 1997). In this model, a dilute solution of formalin is injected onto the planter surface of a rodent's hindpaw. Observation of the rodent's stereotypical behaviors, such as flinching, licking, and biting of the affected hindpaw, are the measurements of inflammatory pain. These effects last 15-60 minutes (Lariviere et al., 2002). This model is preferred over other models because both acute and tonic pain can be measured (Ibironke & Odewole, 2012).

1.11. Need for New Analgesia & Strategy

Opioids and NSAIDs have been used to treat pain for a long time. More effectiveness and less adverse effects are the considerable factors to develop new analgesic drugs. Since the choice of opioids are limited, so it is necessary to develop a new analgesic drug without or low abuse liability and side effects. In the middle of nineteenth century, morphine, a weak base, or alkaloid started use for minor surgical procedures, postoperative and chronic pain. (Brownstein, 1993) In 1939, meperidine discovered serendipitously which got the different structure than morphine. (Eisleb & Schaumann, 1939) In 1946, another compound like morphine synthesized called methadone. (Scott & Chen, 1946) After more than 100 years, morphine's structure established, and total synthesis done in the laboratory. Bentley, 1987; Gates & Tschudi, 1956) In current studies, after analyzing structure activity relationship in 4,5a-epoxymorphinan skeleton (*Figure 1*) some modifications in the structure of morphine helped to create a novel analgesic. (S. Majumdar et al., 2012; Pasternak & Pan, 2013)

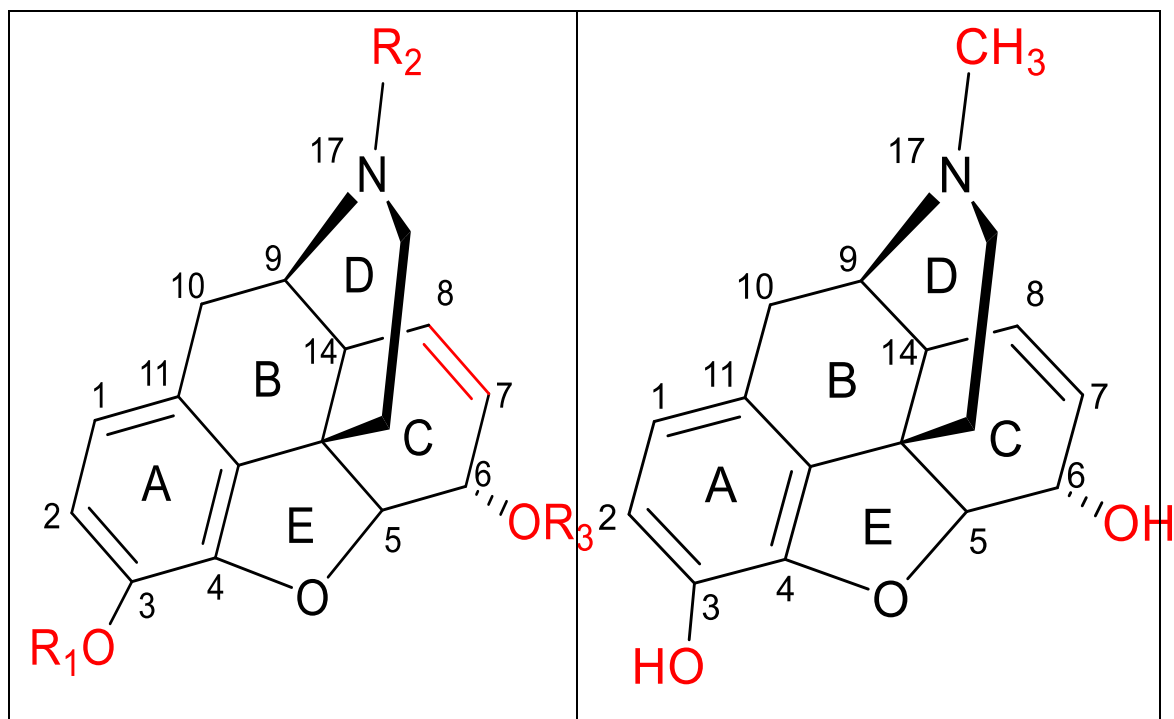


Figure 1. 4,5 α -Epoxy-morphinan Template and Morphine. 4,5 α -epoxy-morphinan template (left) and morphine (right). The SARs of morphinan compounds have been primarily created by altering substituents at the three R groups. (Pasternak & Pan, 2013)

Chapter 2

Discriminative Stimulus Effects of a Novel Atypical mu Opioid Receptor Agonist, 3-Iodobenzoylnaltrexamide (IBNtxA)

2.1. Introduction

In order to identify novel opioids with better analgesic activity, limited or no side effects and no abuse potential, a group of scientists from Memorial Sloan-Kettering Cancer Center, New York, synthesized radiolabeled opioid derivatives. During this research, they characterized an atypical novel opioid, 3-Iodobenzoylnaltrexamide (IBNtxA), synthesized as a 6 β -naltrexamine derivative of naltrexone (Figure #) with the following substitutions: methylcyclopropane at the R₁ positions, hydrogen at the R₂ position, and 3-iodobenzene at the R₃ position (Majumdar et al., 2012).

Further explorations into the pharmacological and chemical properties of IBNtxA found that IBNtxA is more potent than morphine animal models of analgesia. (Majumdar et al., 2011) and the tail flick model of analgesia (Grinnell et al., 2014). Other investigations found that IBNtxA had fewer side-effects compared to morphine. In mice, IBNtxA did not produce respiratory depression after administration of up to a 5-fold greater dose than its analgesic ED₅₀. After chronic administration, it did not produce any physical dependence and cross-tolerance to the morphine. (Grinnell et al., 2014) IBNtxA also produced less slowing of intestinal transit, and no place preference when they tested single dose (Majumdar et al., 2011b; Majumdar et al., 2012, Grinnell et al., 2014).

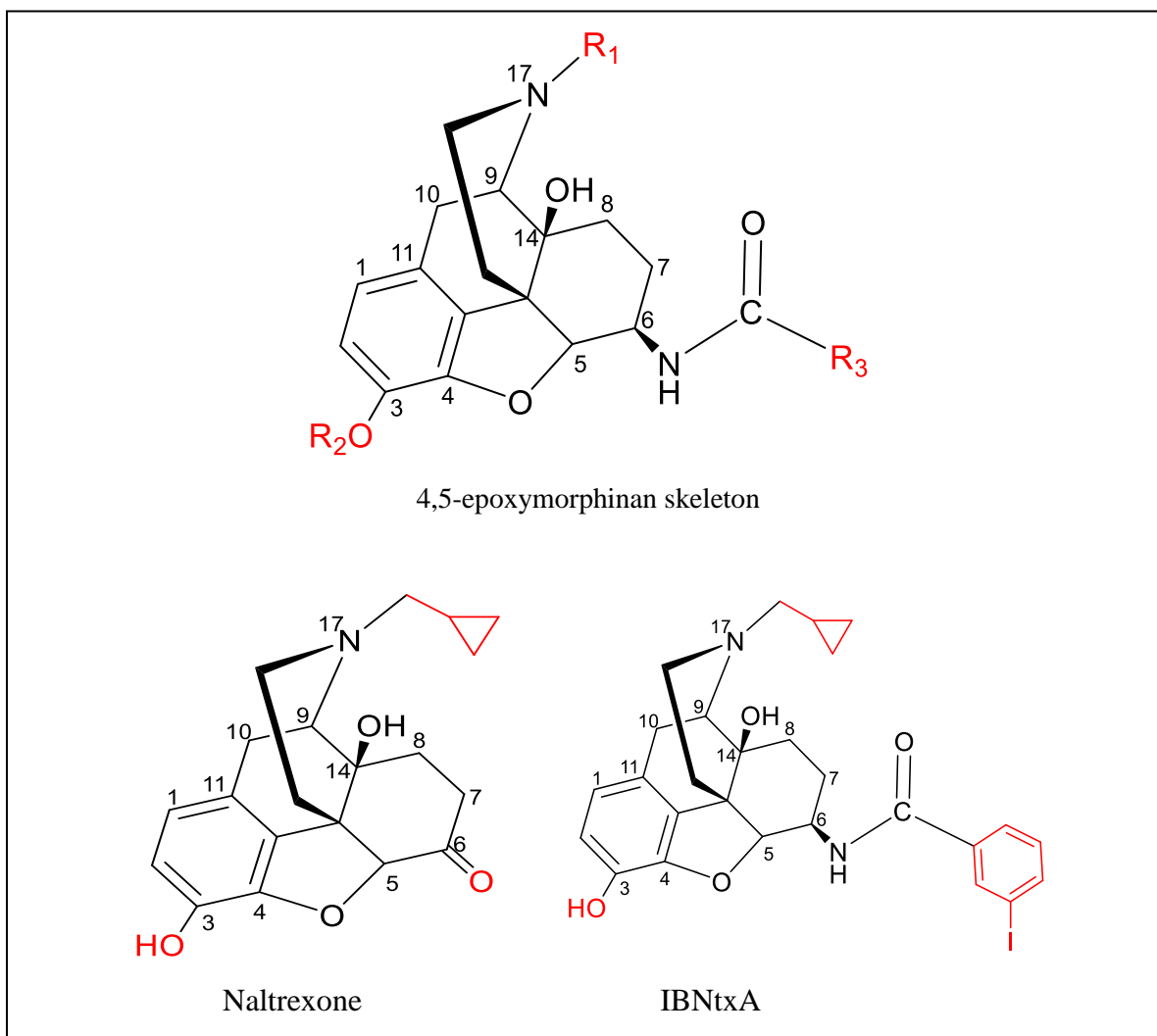


Figure 2. SARs of 4,5-Epoxy-morphinan Skeleton. SARs of 4,5-epoxy-morphinan skeleton wherein the replacement of R₁ at N-17 position by methyl cyclopropane, R₂ at C-3 position by hydrogen and double bond with oxygen at C-6 position, produces opioid antagonist, naltrexone. Change in 4,5-epoxy-morphinan skeleton at R₁ and R₂ same as naltrexone but replacement of R₃ by 3-iodobenzene creates an atypical mu opioid receptor agonist IBNtxA which is a derivative of 6β-naltrexamine with higher analgesic effects but limited side effects than morphine and highly selective to 6TM/E11 MOR splice variant. Image from Majumdar et al., 2012.

A variety of genetic studies indicated that IBNtxA probably signals through truncated MOR splice variants—particularly exon 11-associated 6 transmembrane region splice variants (6TM/E11) (Majumdar et al., 2011). The loss of exon 11-associated MOR splice variants in knock-out (KO) mice caused a loss of IBNtxA-induced analgesia, but the analgesic effect of morphine was unchanged. When exon 1-associated MOR splice variants as well as DOR and KOR were knocked out, morphine analgesia was lost, but IBNtxA induced analgesia. These results indicate that IBNtxA may signal through 6TM/E11 (Majumdar et al., 2011). A later study evaluated molecular models of full-length and 6TM/E11 MORs in response to morphine and IBNtxA. Using homology modeling, docking and molecular dynamics, this study confirmed that morphine is unable to activate 6TM/E11 MORs whereas IBNtxA can activate 6TM/E11 MORs, and with higher affinity over the full-length MOR (Sader et al., 2018).

The characteristics and *in vivo* activities of 6TM/E11 are not well-established, though it's been hypothesized that it can affect the analgesic signaling of other MOR agonists, such as morphine, buprenorphine, and methadone. (Grinnell et al., 2014, Lu et al., 2015; Majumdar et al., 2011). Based on these studies, IBNtxA appears to be one of the first compounds that might be preferential for 6TM/E11 receptors and could serve as the starting point for developing new 6TM/E11-selective compounds.

In order to better understand the physiological effects of IBNtxA and probe whether it might be useful for evaluating 6TM/E11 signaling *in vivo*, it's necessary to expand our understanding of IBNtxA pharmacology. Drug discrimination is a useful paradigm for the assessment of psychoactive properties of drugs to evaluate the safety profile, pharmacology, and possible drug abuse and drug dependency. It has been used to test novel

compounds compared to the standard established drugs, including therapeutic psychoactive drugs, like antidepressants, anxiolytics, antipsychotics, opioids, cannabinoids, and other compounds (Swedberg & Giarola, 2015; Porter et al., 2018).

Drug discrimination testing can be performed using a wide variety of species, including mice, rats, pigeons, non-human primates, and humans. (Porter et al., 2018) Drug discrimination studies are useful for testing drug abuse liability and identification of underlying pharmacological actions and mechanisms of novel compounds because a test compound that substitutes for a training drug is understood to share the discriminative stimulus and pharmacological properties of that training drug. (Colpaert, 1999) This procedure requires extensive training of animals to learn to identify the effects of an administered training drug or a vehicle control (Porter et al., 2018). Once fully trained, test drugs can be administered and the behavioral response of the animal will be driven by the test drug's discriminative stimulus effects (Catania, 1971).

In this study, we investigated the discriminative stimulus effects of IBNtxA (3'-iodobenzoyl-6 β -naltrexamide) compared to other opioid receptor ligands to better understand the subjective effects of IBNtxA and more thoroughly evaluate its abuse liability.

2.2. Materials and Methods

This experiment used drug-naïve adult male C57BL/6 mice obtained from Charles River Laboratories (Wilmington, MA). The animals were housed in the temperature- and humidity-controlled vivarium located in Cooper Medical School of Rowan University. This vivarium has a barrier facility and animals kept under a 12 h light/dark cycle (lights on at 0700, off at 1900). Animals were group housed (four animals/cage) in polycarbonate

cages with *ad libitum* food and water and enrichment provided by paper Bio-Huts and/or nestlets. Mice arrived at the facility approximately 28 days of age and were equilibrated to the facility for a minimum of seven days before beginning testing. One group (7 animals) of mice were used for the drug discrimination studies. Though mice have adequate access to water and air, but they were food restricted for 6-12 hours prior to experiments. Animals were trained with IBNtxA 3mg/kg and DMSO vehicle (10% DMSO and 90% saline). After couple of months training, well-trained animals were tested with different doses of novel drug IBNtxA, μ opioid receptor agonist (Morphine), partial agonist at μ and nociceptin opioid receptor and antagonist at δ and κ receptors (Buprenorphine), κ opioid agonist without μ opioid antagonist effects (U-50488), potent and selective non-peptide δ opioid receptor agonist (SNC 162) and Selective and potent nociceptin opioid receptor agonist (SCH 221510)

2.3. Animals

The C57BL/6 strain of mice is a typical inbred strain, most widely genetically modified laboratory mice for biomedical, pharmaceutical, translational science or any animal study research (*Figure 3*). These animals are widely used in different studies because of their availability and robustness. This strain of animal was first developed by C.C. Little in 1921 which was eventually handed over to Charles River in 1974 from NIH. (Chia et al., 2005; River, 2018; Sarna et al., 2000) They are deep brown or almost black (*Figure*) in color. Their important characteristics is, they are highly sensitive to noise and odors; not docile like CD-1 mice and more likely to bite. They are barbering in nature, and dominant mice can remove hair and whisker of housemates. (Sarna et al., 2000, Willott et al., 1995) These animals are highly susceptible to addiction, atherosclerosis and age-related hearing loss.

(Willott et al., 1995) Like CD-1 mice, this strain also grows with time, reaching full weight after fifteen weeks; we started to weigh them after five weeks, and the average approximate weight was 15-19 g.



Figure 3. C57 Mouse. Image of deep brown or black colored C57BL/6 genetically designed animal. These mice are odors sensitive; are highly susceptible to addiction, atherosclerosis, and age-related hearing loss. They are barbering in nature, prone to engage fighting with inmates, resulting hair removal and sometimes possible injuries. (River, 2018; Sarna et al., 2000; Zurita et al., 2011)

2.4. Drugs

IBNtxA was synthesized at Rowan University by using a multi-step laboratory synthesis. Initially commercial naltrexone (Tocris) was converted into naltrexamine. This naltrexamine reacted with 2,5-dioxopyrrolidin-1-yl-3-iodobenzoate and purified to produce the IBNtxA used for this experiment. This synthesis was performed in the laboratory of Dr. Gustavo Moura-Letts.

Morphine sulfate was purchased from Henry Schein (Melville, NY). Cocaine HCl was purchased from Sigma Aldrich (St. Louis, MO). Buprenorphine, naltrexone, U-50488,

SCH 221510, and SNC 162 were purchased from Tocris (Minneapolis, MN). All drugs were administered via intraperitoneal (i.p.) injection at a volume of 10 mL/kg to the animals. Since body weight is an important factor to measure the dose of drug, dilutions were premixed to provide a given mg/kg dose prior to every test. For example, a 20 g mouse would receive a 1 mg/kg drug dose via the injection of a 0.20 mL volume of a 0.1 mg/mL drug solution. IBNtxA was delivered in a 10% DMSO vehicle, prepared via stepwise mixing with 10% dimethyl sulfoxide (DMSO) and 90% physiological saline. All other drugs were readily dissolved in the same 10% DMSO vehicle. All drugs were kept secure inside a locker with a regulated inventory procedure under the control of Dr. Bradford Fischer, who holds controlled substances licenses from the State of New Jersey and the U.S. Drug Enforcement Agency.

2.5. Apparatus

For drug discrimination training and testing, mouse operant chambers (Med Associates, Fairfax, VT) were used. (*Figure 4*) Each apparatus was positioned in sound-attenuating cabinets and connected to a computer running MED-PC software (version 4). The drug discrimination apparatus was a small box made of transparent acrylic and containing two nose poke holes. One hole was designated as the “drug side” and the other was designated as the “vehicle side” (*Figure 5*). A liquid dipper was located between those two nose poke holes, connected via tubing to a pump and syringe that discharged vanilla Ensure for 3 seconds (delivering an approximate 0.1 mL volume) as a palatable food reward. The nose poke holes were equipped with infrared beams; when animals nose poked on either side, the infrared beam was broken, and a signal was sent to the operating MED-PC software.

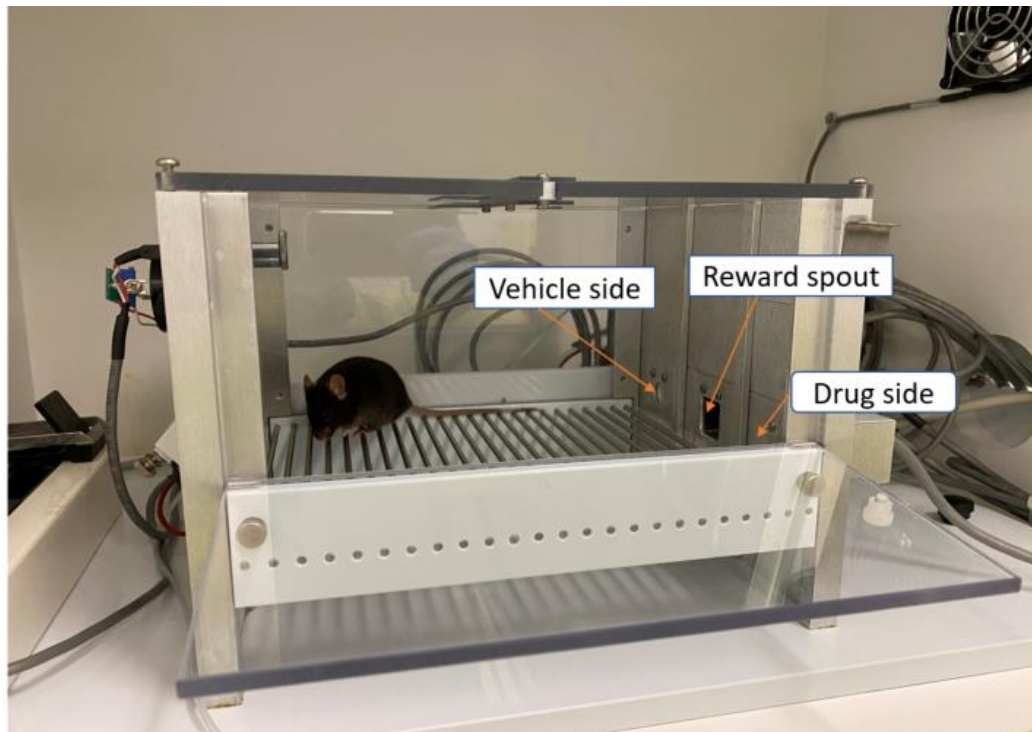


Figure 4. Drug Discrimination Testing Apparatus. Image of drug discrimination testing apparatus from our research lab. There are two nose poke holes and mice can easily poke those holes for finding rewards. The activity of animals is tracked by infrared and then signal is sent to MED-PC software to analyze and present on the monitor. The left hole is vehicle-paired; if animals knock this hole on vehicle-training session is considered as correct response and the right hole is drug-paired; on drug-training session if animals knock this hole, is considered as correct response. For every five correct nose pokes, animals receive one single reward which is three seconds Ensure Plus syrup discharge through reward spout. During substitution test day any nose pokes to either side are considered for reward. The speaker on top of image is a sound generator which produces a tone during reward delivery.

2.6. Procedure

Before training/testing, animals were food restricted up to 18-20h. Though animals do not have any available food, but they have *ad lib* access to water. The overall training procedure is represented in the overall procedure is represented by Figure. During training, each animal was injected with either 3 mg/kg IBNtxA or vehicle control. To earn the food reward, animals were required to complete a specific set of correct responses: the required number of correct responses to achieve a reward is known as the fixed ratio (FR). Training initially started with an FR1 and increase up to FR5 based on their training improvement. I took almost 2 months to reach FR5. An FR5 training paradigm, animals required to complete 5 correct nose pokes in a row to earn a reward. MED-PC software controlled the entire system. All rewards were accompanied by light and tone stimuli during the duration of the 3-second reward delivery.

Mice were trained initially to nose poke for Ensure rewards using a fixed-ratio 1 (FR1) schedule, in which a single nose poke on either side initiated reward delivery and associated cues. Following successful nose poke training, in which mice received at least 90 of 100 possible rewards in a 1-hour time period, animals were trained to discriminate between DMSO vehicle (10% DMSO and 90% saline) and 3 mg/kg IBNtxA. During the training phase, mice received i.p. injections of 3 mg/kg IBNtxA or DMSO vehicle and were placed in the operant chamber 15 minutes prior to the start of training, with the start of the session indicated by a house light turning on. IBNtxA and vehicle were given with a pseudorandom order of training to avoid day-of-the-week training effects. For all chambers, IBNtxA was programmed to be associated with the right nose poke hole, and DMSO vehicle with the left nose poke hole. In order to earn an Ensure reward, animals were required to complete

an unbroken FR response in the correct nose poke hole. Over the course of training, the FR requirement was increased until mice were correctly nose poking >90% on a FR5 schedule; mice were considered to have successfully learned the DD procedure at a given FR level when $\geq 90\%$ of the initial 10 nose pokes in a given training session matched the desired response. All training sessions lasted for 60 minutes or until 100 rewards were earned. Before and after all training and testing procedures, each test apparatus and floor insert were cleaned with 70% isopropyl alcohol and allowed to dry completely.

After meeting FR5 training criteria, mice were tested with varying doses of IBNtxA (0.33-3.0 mg/kg), morphine (0.33-10 mg/kg), U-50488 (0.33-10 mg/kg), buprenorphine (0.10-1.0 mg/kg), SNC162 (3-18 mg/kg), SCH 221510 (1-10 mg/kg) and cocaine (3-10 mg/kg) given via i.p. injection. For each test, mice were given a drug injection and placed in the operant chamber 15 minutes prior to the start of testing session, with the start of the session indicated by a house light turning on. Drug discrimination was measured by the first response (drug side or vehicle side) after the start of the session, after which the session was immediately ended with no rewards given. This limited, stringent testing procedure was adopted after initial studies determined that IBNtxA discrimination training was easily disrupted by rewards earned while exposed to some tested drugs (possibly owing to IBNtxA being a relatively weak discriminative stimulus). This procedure allowed much quicker (~1 week) re-establishment of drug discrimination between tests, but it also results in data that are less typical of DD reports in the literature: instead of reporting the proportion of overall nose pokes (drug-paired vs. vehicle-paired) following testing, we report the proportion of animals whose initial responses were on the drug-paired vs.

vehicle-paired side. Likewise, because we cannot report standard drug effects on response rates (e.g., nose pokes/sec), we report time to initial nose poke at the start of the session.

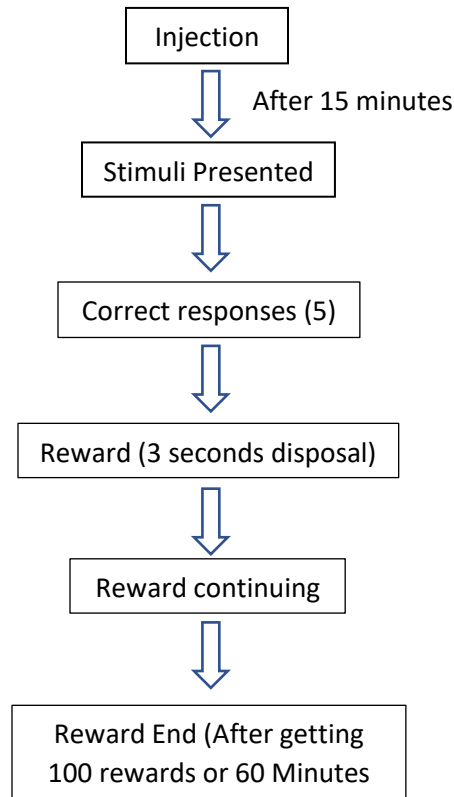


Figure 5. Outline of drug discrimination training. From the top of this image, after receiving an injection (i.p.), animals are placed inside operant chamber. The trial starts 15 minutes later. Each subject receives a reward of three seconds liquid food dispensed via a reward spout for every five correct responses. The training sessions automatically end after either earning 100 rewards or after 60 minutes have elapsed. (adapted from Solinas et al., 2006)

A group of animals contained Seven C57BL/6 mice has been trained. The animals were trained with vehicle (10% DMSO and 90% Saline) and IBNtxA 3 mg/kg. To train mice properly and unbiasedly, the pattern of training was always being changed in each day for overcoming any possible effect of training schedule pattern which might affect discrimination study.

The animals that could not reach the standard training with minimal 80% initial correct, 80% total correct response and 80% reward were excluded beforehand. Furthermore, every animal was kept in close observation for any health issue such as weight loss, stool condition, any possible injuries etc. Sick animals were separated from other mates and treated with required medicines. The animals with better health were trained and tested only.

2.7. Statistical Analysis

Following equations were used for analysis:

For training.

$$\% \text{ of total correct responses} = \left(\frac{\text{Total correct responses}}{\text{Total correct responses} + \text{Total incorrect responses}} \right) \times 100$$

$$\% \text{ of initial correct responses} = \left(\frac{\text{Initial correct responses}}{\text{Initial correct responses} + \text{Initial incorrect responses}} \right) \times 100$$

2.8. Time to First Response

Drug discrimination was measured by the first response (drug side or vehicle side) after 15 minutes of the session started. This is important to study drug discrimination, because how the animals feel like after the administration of a drug. An immediate response confirmed the appropriate training dose and the possible range of dose which might be

tested for a drug. This limited, stringent testing procedure was adopted after initial studies determined that IBNtxA discrimination training was easily disrupted by rewards earned while exposed to some tested drugs (possibly owing to IBNtxA being a relatively weak discriminative stimulus).

2.9. Results

Seven mice were trained to discriminate 3 mg/kg IBNtxA from 10% DMSO vehicle. IBNtxA proved to be a weak discriminative stimulus and training took approximately 90 days in order for all animals to meet training criteria (> 80% correct responses). Initial experiments revealed that drug trials in which animals could respond for 30 minutes substantially disrupted subsequent discrimination training and required extended re-training between drug tests. Therefore, drug tests were redesigned to count only the initial (unrewarded) nose poke after test drug delivery 15 minutes prior to the start of the trial. Only results from this second iteration are presented in Figure 3; panel A indicates the first nose poke response for each tested drug dose and panel B indicates the time to initial nose poke response, an indication of whether a drug dose was behaviorally disruptive.

Initial test trials with DMSO vehicle and 3 mg/kg IBNtxA (black squares) indicated that this method could reliably distinguish vehicle from training drug. Dose responses were then recorded for IBNtxA (0.3-3 mg/kg), MOR agonist morphine (0.33-10 mg/kg), KOR agonist U-50488 (0.33-10 mg/kg), DOR agonist SNC162 (3-18 mg/kg), NOP agonist SCH 221510 (1-10 mg/kg), and MOR partial agonist/NOP agonist buprenorphine (0.10-1 mg/kg). To test generalizability, the non-opioid cocaine (3-10 mg/kg) was tested as well.

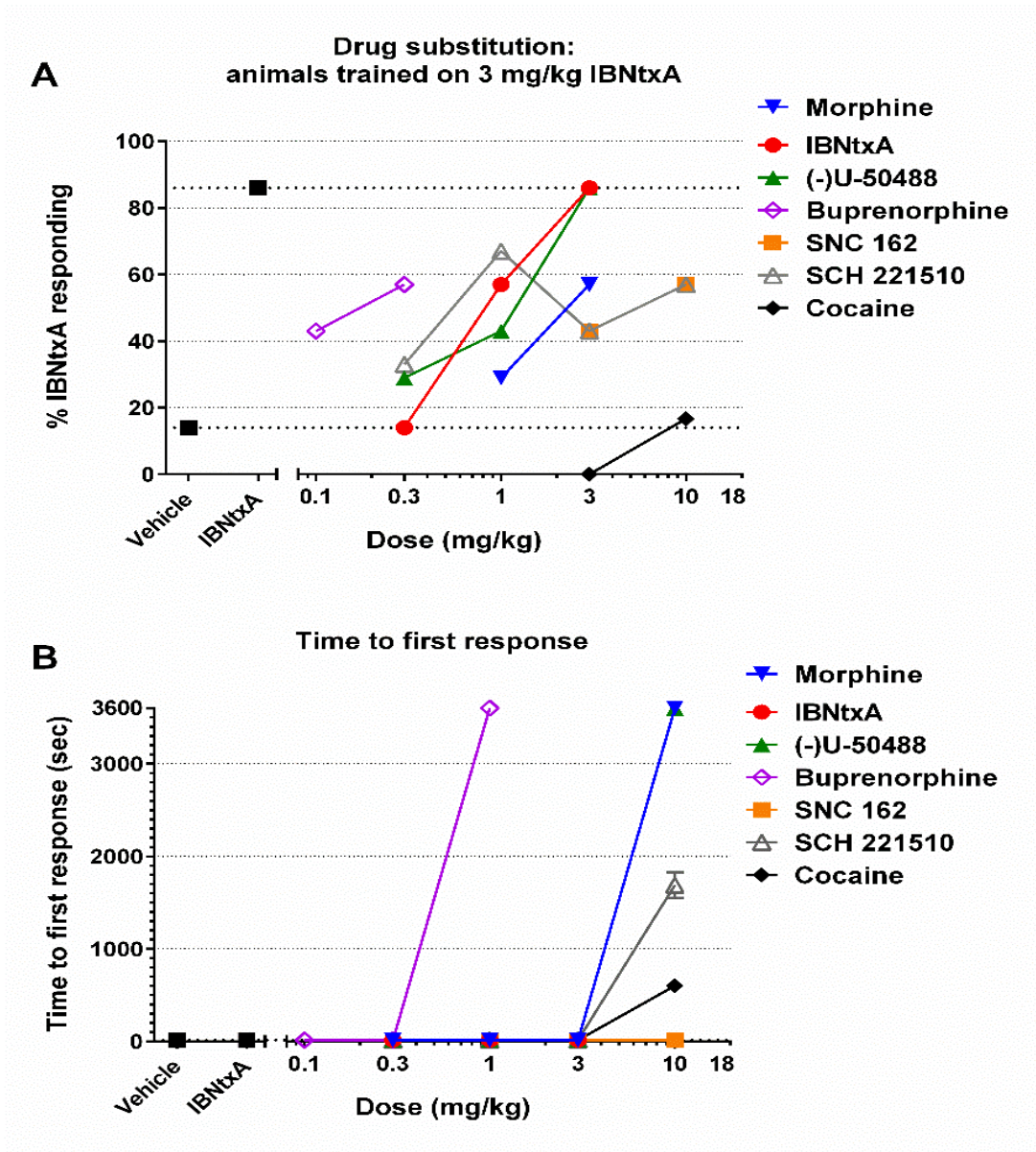


Figure 6. Drug Discrimination Results. Drug discrimination results for animals trained to distinguish 3 mg/kg IBNtxA from vehicle. A. IBNtxA and KOR agonist U-50488 fully substitute for IBNtxA. MOR agonist morphine, DOR agonist SNC162, NOP agonist SCH 221510, MOR partial agonist/NOP agonist buprenorphine partially substitute for IBNtxA. The psychostimulant cocaine does not substitute for IBNtxA. Because these data ultimately represent the proportion of mice who chose the IBNtxA-paired nose poke hole for their first response, there are no error bars. B. Behavioral disruption of drug responding as determined by the time to first drug response. The highest tested doses of buprenorphine, morphine, SCH 221510, and cocaine each induced a substantial behavioral disruption. These results are presented as means \pm SEM.

IBNtxA dose-dependently and fully substituted for itself. MOR agonist morphine, DOR agonist SNC162, NOP agonist SCH 221510 and MOR partial agonist/NOP agonist buprenorphine each partially substituted for IBNtxA, while the psychostimulant cocaine did not substitute for IBNtxA (Figure 7A). At the highest doses tested, morphine (10 mg/kg), SCH 221510 (10 mg/kg), and buprenorphine (1 mg/kg) each disrupted responding (Figure 7B). KOR agonist U-50488 fully substituted for IBNtxA, indicating that KOR signaling effects are likely crucial to the *in vivo* characteristics of IBNtxA. First drug response time is important because behavioral disruption of drug response is determined by using the first drug response time. The highest doses of morphine (10 mg/kg), SCH 221510 (10 mg/kg), cocaine (10 mg/kg), and buprenorphine (1 mg/kg) showed the disrupted response and for 10 mg/kg morphine and 1 mg/kg buprenorphine, drug substitution data couldn't be presented. The reason for this because of the animals' failure of nose poke on either the drug or vehicle side. That is why drug substitution data are not available for 10 mg/kg morphine and 1 mg/kg buprenorphine.

2.10. Discussion

IBNtxA have a crucial analgesic effect to alleviate moderate to severe pain. During cancer or major surgery, patient needs more potent analgesics. The effects of IBNtxA is compared to the effects of IBNtxA. KOR agonist U-50488 fully substituted for IBNtxA, indicating that KOR signaling effects are likely crucial to the *in vivo* characteristics of IBNtxA. Since, IBNtxA partially substituted by DOR, MOR, and NOP agonists, and fully substituted by a KOR agonist in the drug discrimination assays indicate that these receptors may each contribute to IBNtxA-mediated analgesia. This is the first time to test the discriminative stimulus properties of IBNtxA. Though the discriminative stimulus

properties of 6TM/E11 agonism are not established yet. But it is known that opioid agonists have important effects mediated by 6TM/E11 activation (Majumdar et al. 2011b; Marrone et al. 2016). There are some limitations of this study. In this test, all tests performed by using male mice, not female mice. There are some sex-mediated differences in opioid receptor expression and signaling, manifesting in differential effects in tests of analgesia in humans and rodents and important differences in rodent models of drug abuse and relapse (Becker & Chartoff, 2019; Craft, 2008; Dahan et al., 2008; Lee & Ho, 2013). IBNtxA showed full KOR agonism in male mice but sex difference may impact in analgesia and abuse liability. (Chartoff & Mavrikaki, 2015) The animals housed in light/dark cycle, but experiment performed only in the light part. So, the inactive part of the mouse diurnal cycle may provide variable opioid receptor expression. (Mitchell et al. 1998) and opioid receptor activation can itself alter circadian rhythms (Pacesova et al. 2015; Webb et al. 2015). If the tests performed in dark cycle, there is a possibility to get different results. In further, other derivatives of IBNtxA and other opioid analgesics can be tested.

Chapter 3

Synergistic Analgesic Effects of Morphine and the Novel $\alpha 2/3$ -Preferring GABA_A

Receptor Positive Allosteric Modulator MP-III-024

3.1. Introduction

Pain is a complex phenomenon involving numerous neurotransmitters and their end target receptors. The currently available analgesics to treat pain include opioids, however this class of drugs also carries dose-limiting adverse effects and the potential risk vs benefit must be considered when prescribing (Mao, 2015). Opioid receptors are distributed both within and outside the central nervous system (CNS), and mediate effects producing both therapeutic properties such as pain relief and a broad spectrum of adverse effects including sedation, respiratory depression and constipation, as well as tolerance and physical dependence following chronic use.

3.1.1. GABA. γ -aminobutyric acid (GABA) works as a chemical messenger in the CNS where it functions as an inhibitory neurotransmitter. Among the two major classes of GABA receptors, ionotropic GABA type A (GABA_A). This GABA_A receptors are in the ligand gated ion channels family. (Ferando & Mody, 2014) GABA_A receptors include $\alpha\beta\gamma 2$ isoforms on which benzodiazepine-type drugs function as positive allosteric modulators (Fritschy, 1997). These receptors incorporated in postsynaptic membrane and mediate transient and fast synaptic inhibition within milliseconds. GABA_A receptors also located at the extrasynaptic places mostly surrounded by GABA and occurs long term inhibition. (Rudolph & Knoflach, 2011) These GABA_A receptors are differentially distributed within the CNS, and receptors containing $\alpha 2$ and $\alpha 3$ subunits next to the $\gamma 2$ subunit ($\alpha 2$ -containing GABA_A and $\alpha 3$ -containing GABA_A receptors, respectively) are expressed in dorsal horn

spinal pathways and have been implicated in nociceptive transmission. Previous work has demonstrated antihyperalgesic effects of intrathecally administered benzodiazepines (Knabl et al., 2008, 2009; Witschi et al., 2011) as well as systemically delivered compounds with functional selectivity for $\alpha 2$ GABAA and $\alpha 3$ GABAA receptors (Knabl et al., 2008; Di Lio et al., 2011, Paul et al., 2013; de Lucas et al., 2015, Fischer et al., 2017).

The expression of both opioid, $\alpha 2$ GABAA and $\alpha 3$ GABAA receptors in nociceptive pain pathways raises the possibility of interactive effects of concurrent administration of compounds that modulate each receptor. If greater than additive effects are detected on endpoints related to pain reduction, combination therapy may be useful to treat pain-related disorders. Recently a novel benzodiazepine-type compound methyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024) was described (Fischer et al., 2017). MP-III-024 displayed preference for $\alpha 2$ GABAA and $\alpha 3$ GABAA receptors and produced antihyperalgesic effects with limited off-target effects as measured with operant responding and locomotor activity. MP-III-024 also demonstrated a similar time course and duration of action relative to morphine making it ideal for combination studies.

The use of dose-addition analysis is one method used to provide a quantitative evaluation of drug interactions and can be used to differentiate effects that are additive from effects that are subadditive or supra-additive (synergistic) (Fischer, 2011). In the present study, dose-addition analysis was used to evaluate $\alpha 2$ GABAA receptor/ $\alpha 3$ GABAA receptor-opioid interactions. The effects of combinations of MP-III-024 and the prototypical mu opioid agonist morphine were examined in CD1 mice using two different assays. To assess the extent to which interactive effects occur on an endpoint related to

inflammatory pain, the zymosan A model of mechanical hyperalgesia was used. Second, the acute thermal antinociceptive effects of MP-III-024, morphine and their combinations were evaluated in the hot plate procedure. Drug interactions were assessed using a fixed-proportion design, since this has been recommended for the study of drug interactions (Fischer 2011) and has been used to study similar drug mixtures on similar endpoints

3.2. Materials and Methods

All experiments used adult male CD-1 mice 10 weeks of age obtained from Charles River Laboratories (www.criver.com). Animals were housed in the temperature- and humidity-controlled vivarium with constant access to air and water, under a 12h light/dark cycle (lights on at 7:00 AM). Mice were grouped in polycarbonate cages with ad libitum food and water and enrichment provided by paper Bio-Huts and/or nestlets. Mice were habituated to the colony room environment for 2 weeks prior to any experimental manipulation and exposed to the testing environment and handled for 2 days prior to initiation of an experiment. All testing procedures were conducted between 11:00 AM and 3:00 PM. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of Rowan University and all testing adhered to the “Guide for the Care and Use of Laboratory Animals” (National Research Council, National Academy of Sciences, Washington, D.C., USA, 2011).

3.3. Animals

CD-1 laboratory mice are widely used in biomedical and pharmaceutical research. (*Figure 8*) Most of the currently used mice are the progeny of nine Swiss mice, two male and seven female albino mice, which were imported to the USA in 1926 by Dr. Clara Lynch of the Rockefeller Institute for Medical Research, now named Rockefeller University (Chia

et al, 2005). In 1948, new Ha/ICR mice were initiated from previously imported Rockefeller “Swiss” mice at the Institute for Cancer Research (ICR) in Philadelphia. (Chia et al., 2005)

We started experiments when animals were around 35 days old, at which the average weight of mice was approximately 22-25 g. When we were working with CD-1 mice, in our observation, they were usually easy to handle but were quite variable in their activity during the first week, especially during drug administration. The reason behind their aggressive behavior during the first few days was likely the time need for adaptation to human and drug administration.



Figure 7. CD-1 Outbred Mouse. Image of white colored CD-1 outbred mouse. These mice are docile in behavior and widely used in biomedical research. They are normal wild type mice, grow over time and gains maximum weight in fifteen weeks. (River, 2018)

3.4. Drugs

The novel benzodiazepine analog methyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024) was synthesized at the Department of Chemistry and Biochemistry at the University of Wisconsin-Milwaukee. Morphine was purchased from Sigma-Aldrich (St. Louis, MO). Drugs were suspended in 0.5% methyl cellulose and 0.9% NaCl and administered intraperitoneally in a total volume of 10 ml/kg body weight.

3.5. Hot Plate Testing

3.5.1. Background. Antinociception during the hot-plate procedure was assessed using a hot plate analgesia meter (25.3 × 25.3 cm; Columbus Instruments, Columbus, OH, USA) maintained at $56 \pm 0.1^\circ\text{C}$. The antinociceptive response was evaluated by recording the latency to lick or shuffle the hind paw(s) and/or to jump from the hot plate surface. A predetermined cutoff time of 20 s was defined as a maximal response and was employed to prevent tissue damage. The antinociceptive response was measured twice at 30 and 15 min prior to the beginning of drug administration and these data were averaged to yield one baseline value.

3.5.2. Apparatus. The hot plate analgesia meter (Columbus Instruments, OH, USA) for small laboratory animals were used for this analgesic test. (*Figure 9*) The hot plate could continuously provide 55°C temperature on an aluminum surface, with a digital built-in thermometer to maintain surface temperature to 0.1°C precision and a timer with a 0.1 sec precision. The square shaped surface plate was enclosed by a clear acrylic cage to confine animals during testing. Pushes on start/stop button related to the timer, which displayed the time on the screen which was recorded manually.



Figure 8. Hot Plate Apparatus. Image of hot plate apparatus with a CD-1 mouse from our research lab. The hot plate has an aluminum flat surface maintains 56-degree Celsius temperature. There is a switch to turn on and a built-in timer.

3.5.3. Procedure. The hot plate was set at 56 °C to observe the effects of drugs on animals. Certain behavioral changes, paw licking, flutter, and jumping, were considered as an animal's pain feeling. (Rezaee-Asl, Sabour, Nikoui, Ostadhadi, & Bakhtiarian, 2014) Latency time after placing mice on the metallic hot plate provided the threshold level of animals. Prior to injecting the drug, each mouse was weighed and tested for two baseline studies where the animal was not injected with any drug or vehicle. After baseline studies, testing drug was administrated and animals were placed on hot plate in 15, 30, 45, 60, and/or 75/90 minutes time intervals to collect the latency time. If any behavioral change like paw licking, flutter or jumping had been observed, the mouse was immediately removed from hot plate and latency time was recorded. Animals were removed from hot plate after 20 seconds even though there was no considerable behavioral change to avoid tissue damage and this specific time is known as maximum latency time. Any animal which

showed more latency time more than 20 seconds was excluded from further investigation. (Menéndez, Lastra, Hidalgo, & Baamonde, 2002; Rezaee-Asl et al., 2014)

3.5.4. Statistical analysis. The anti-nociceptive effect for each dose was calculated as the % of the Maximal Possible Effect (% MPE) using the following formula:

$$\% MPE = \left(\frac{\text{Latency time after drug administration} - \text{Mean latency time of baseline studies}}{\{\text{Maximal latency time (20)} - \text{Mean latency time of baseline studies}\}} \right) \times 100$$

3.6. Von Frey Test

3.6.1. Background. Antihyperalgesic effects were studied following inflammation evoked through subcutaneous injection of 0.06 mg zymosan A suspended in 20 μ l 0.9% NaCl into the plantar surface of the right hindpaw. The non-injected left hindpaw was used as control. Mechanical sensitivity was then assessed 24 h after zymosan A injection by applying von Frey filaments of increasing stiffness (5-26 g) to the mid plantar surface of the hind paws until the filament bends (IITC Life Sciences, Woodland Hills, CA). A positive response evoked a paw withdrawal behavior and mechanical threshold was defined as the minimum force necessary to elicit a positive withdrawal response.

3.6.2. Apparatus. The von Frey platform was set up above eye level and placed in a place the room in order to allow to move around all sides of the platform without impeded access. The Von Frey test is basically a mechanical sensitivity test which consists a set of thin calibrated plastic filament that are applied to the plantar surface of the hind paw while testing. Von Frey filaments of increasing stiffness (0.008-300 g) are used to determine the threshold that produces a hind paw withdrawal response. The mechanical withdrawal threshold is well-defined as the minimum gauge Von Frey filament that causes a withdrawal reflex.

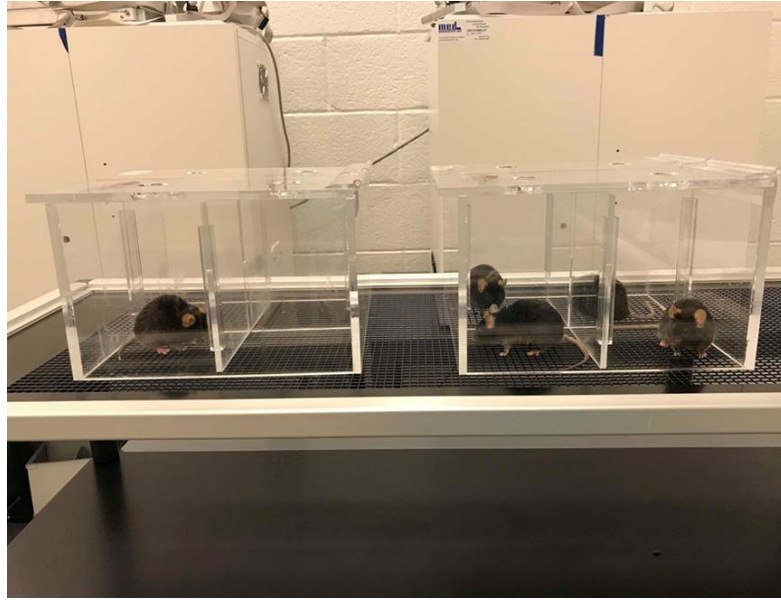


Figure 9. Von Frey Set Up. Image of the von Frey setup from our research lab. The setup has a mesh surface where mice can move freely.



Figure 10. Von Frey Filaments. Image of von Frey filaments from our research lab.

3.6.3. Procedure. Prior to injecting the drug, each mouse was weighed and tested for two baseline studies where the animal was not injected with any drug or vehicle. After baseline studies, testing drug was administrated and animals were placed on wide gauge, wire mesh surface in 15, 30, 45, 60, and/or 75/90 minutes time intervals and Von Frey filaments of increasing stiffness had been applied until the filament bends. If the mouse responds by flicking its paw away from the stimulus 3 times by the same size filament, the filament diameter size has been recorded. The process is repeated with increasing gauges of von Frey filaments that have different stiffness until stimulation forces a hind paw withdrawal.



Figure 11. Von Frey Testing Procedure. Image of von Frey testing procedure from our research lab. Using von Frey hair, poking plantar surface of the animal's footpad.

3.6.4. Statistical analysis. The mechanical threshold following drug administration was normalized to the baseline measurement of the non-injected left hind paw and expressed as a percentage of the maximal possible effect (%MPE) from the following formula:

$$\% MPE = \left(\frac{\text{post drug right paw threshold (g)} - \text{baseline right paw threshold (g)}}{\text{baseline left paw threshold (g)} - \text{baseline right paw threshold (g)}} \right) \times 100$$

3.7. Isobolographic and Dose-Addition Analysis

Interactions between MP-III-024 and morphine were assessed using both graphical and statistical approaches (Wessinger, 1986; Tallarida, 2000). Using the graphical approach, the distinction between subadditive, additive, or synergistic interactions were made with the use of isobolograms. In the current study, isobolograms were constructed by connecting the ED₅₀ of MP-III-024 alone plotted on the abscissa with the ED₅₀ of the morphine alone plotted on the ordinate to obtain an additivity line. The additivity line contains the loci of dose pairs that produce an ED₅₀ equal to the ED₅₀ of MP-III-024 or morphine alone. Dose pairs that fall below the additivity line suggest an ED₅₀ was reached with lesser quantities of the drugs, suggestive of synergism. In contrast, experimental points representing dose pairs that fall above the line are suggestive of subadditivity.

Drug interaction can be analyzed in different ways. Among them, a comparison of the ED₅₀ values for each mixture with the predicted additive ED₅₀ values is a good way to determine the potency of the prospective combination drug. ED₅₀ values for each mixture can be represented by Z_{mix} and predicted additive ED₅₀ values can be represented by Z_{add}. (Tallarida, 2011) Total drug dose of MP-III-024 and morphine which can produce a 50% maximum possible effect can be called Z_{mix}. If two drugs Morphine and MP-III-024 added together in a specific ratio, and if they did not do anything special, that is

predicted additive ED50 (Z_{add}). In this mechanical sensitivity assay, if both drugs were effective equally, an equation $Z_{add} = fA + (1 - f)B$ can be used to calculate Z_{add} values individually. In that equation, A is the ED50 for MP-III-024 alone, B is the ED50 for the morphine alone, and f is the fraction. For determining the proportion of MP-III-024 in each mixture equation $fA/[fA + (1 - f)B]$ can be used. This study examined effects produced by mixtures in which $f = 0.25, 0.5, \text{ and } 0.75$. When $f = 0.25$, the mixture contains a proportion of $[A/(A + 3B)]$ MP-III-024 and a mixture ratio of $[(A/B)/3]$ parts MP-III-024 to one-part morphine; $f = 0.50$ leads to a proportion of $[A/(A + B)]$ MP-III-024 in the mixture and a mixture ratio of (A/B) parts MP-III-024 to one-part morphine; and $f = 0.75$ leads to a proportion of $[A/(A + B/3)]$ MP-III-024 in the mixture and a mixture ratio of $[(A/B) \times 3]$ parts MP-III-024 to one-part morphine.

Isobolograms represents the analgesic effects in the hot plate assay (A) and the von Frey test (B) with morphine alone, or morphine in combination with MP-III-024. In combination study, after plotting the ED50 on isobologram, three either cases can be found. Points can be on additive line or lower-left side or upper-right side. If it is on the additive line, there are no significant effects of this combination. When it falls the lower left side, which represents a synergistic effect of the combination. On the other hand, falling on the upper-right side represents subadditive or counterproductive effects. Tested with the mixture in different ratios in both hot plate assay and the von Frey test, there is some leftward shift of the ED50 values on isobologram noticed. That's why more potency of this combination drug is expected.

3.8. Results

Figure 12 shows the dose response curves for morphine and MP-III-024 administered alone in both the hot plate and von Frey procedures. In the von Frey procedure, injection of zymosan A into the right hind paw reduced mechanical sensitivity relative to the non-injected left hind paw and paw withdrawal thresholds of the non-injected paw were unaffected (data not shown). In this assay (right panel) each compound produced dose- and time-dependent increases in mechanical sensitivity as expressed as %MPE. A statistical test for parallelism revealed that the morphine and MP-III-024 dose-effect curves were parallel ($p < 0.05$). These relative potency values were used to determine relative proportions of the compounds used in subsequent studies assessing MP-III-024/morphine mixtures. *Figure 12* (left) also shows the antinociceptive effects of morphine and MP-III-024. Morphine produced dose-dependent increases in latency to respond on the hotplate, and the resulting ED₅₀ value of 12 mg/kg. MP-III-024 was without effect in this assay, therefore, the relative potencies determined in the von Frey procedure were used to determine the relative proportions of the compounds in each mixture.

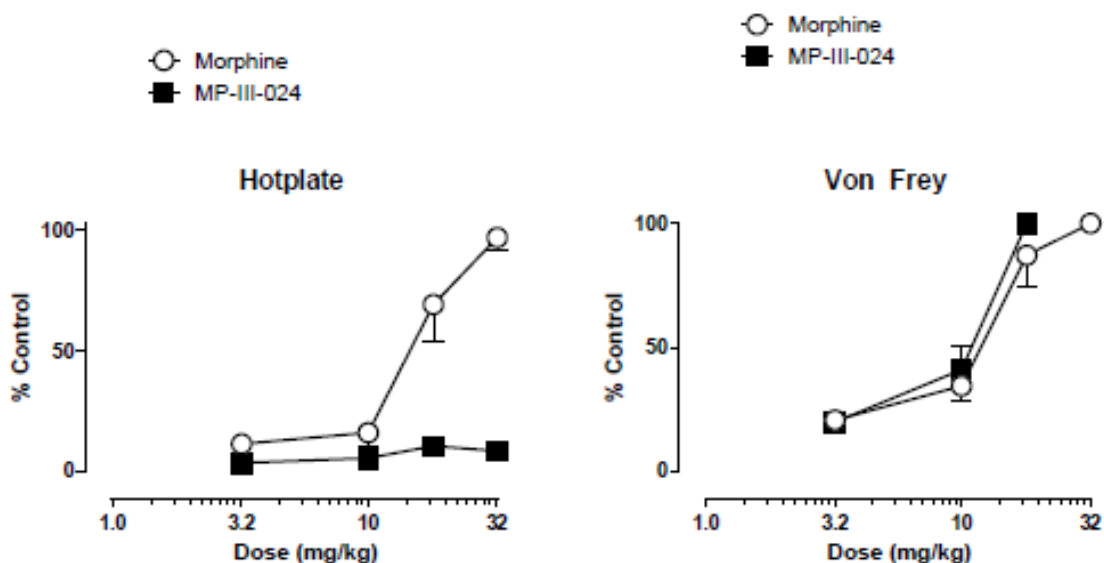


Figure 12. Dose-Effect Curves of Analgesic Effects (Single Drug). Dose-effect curves of analgesic effects in the hot plate assay (A) and the von Frey test (B). Morphine was a potent analgesic in both tests but MP-III-024 only produced analgesia in the von Frey test. On hot plate assay (A), MP-III-024 shows almost no analgesic effects with the 3.2mg/kg, 10mg/kg and 32mg/kg doses. Increasing doses of MP-III-024 does not show any significant effects. On the Von Frey assay (B), MP-III-024 and Morphine showed effectiveness simultaneously. Though not significantly, the effects of MP-III-024 is better than Morphine on Von Frey testing.

Figure 13 shows the antihyperalgesic effects of morphine alone and in combination with MP-III-024. Each drug mixture produced dose-dependent decreases in response rates. Addition of MP-III-024 produced concentration dependent leftward shifts in the morphine dose-effect curve. *Figure 14* also shows the antinociceptive effects of morphine alone and in combination with MP-III-024. In this procedure, each drug mixture produced dose-dependent increases in antinociception, and addition of MP-III-024 again produced leftward shifts in the morphine dose-effect curve.

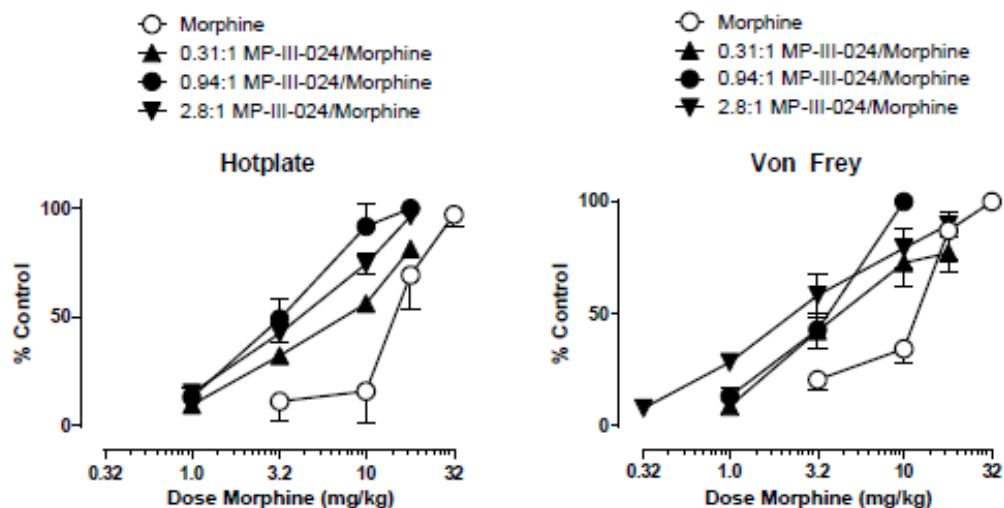


Figure 13. Dose-Effect Curves of Analgesic Effects (Drug combination). Dose-effect curves of analgesic effects in the hot plate assay (A) and the von Frey test (B) with morphine alone, or morphine in combination with MP-III-024. The addition of MP-III-024 induced a leftward curve shift in each assay. The effect of any combination is better than the effect of Morphine alone in both the platforms. Though MP-III-024 is not effective on Hot Plate at all, it might increase the activity of Morphine. As a result, the maximum effectiveness achieved by the relatively low combination doses than Morphine alone. This synergistic effect indicates the effect of combination doses and 0.94:1 combination shows negligible better result among other combinations.

The isobolographic graphical analysis of the drug combinations is shown in *Fig. 15*. In the von Frey procedure (right), the 0.31:1 MP-III-024/morphine mixture produced additive effects as these ED_{50} values fell close to the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (i.e., $Z_{add} = Z_{mix}$) (insert values). In contrast, the 0.94:1 and 2.8:1 MP-III-024/morphine mixtures produced supra-additive (synergistic) effects as these ED_{50} values fell below the line of additivity, and these observations were also confirmed with statistical dose-addition analysis. On the hot plate procedure (left panel). Graphical analysis of the MP-III-024/morphine mixtures indicates that each mixture produced supra-additive effects because these ED_{50} values fell to the left of the

line of additivity. Statistical comparison determined that the experimentally determined ED_{50} values (Z_{mix}) for these mixtures were significantly less than the predicted additive ED_{50} values (Z_{add}). If the combination drug does not have any synergistic effects, the points of ED_{50} values should fall on the additive line. When it produces some extra effects for the combination, the points of ED_{50} values should fall on the lower left quadrant of the additive line and if this combination slows down its potency, it should fall on the upper right quadrant of that line.

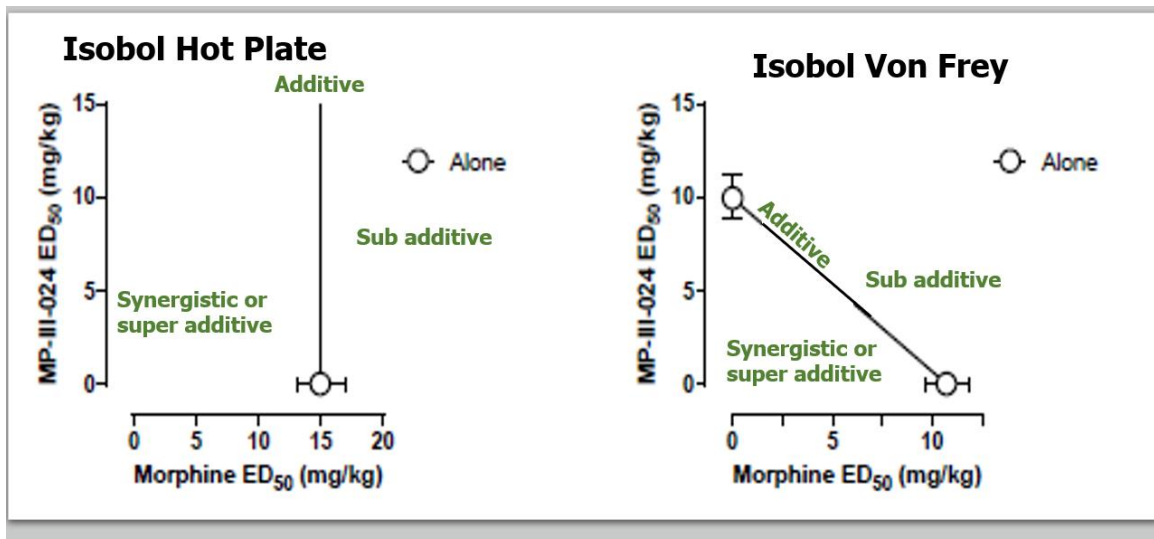


Figure 14. Isobolographic analyses. Straight line indicates the additive effects of the combination. Lower-left shift means the synergistic or super additive properties of drug combination and upper-right shift represents the counterproductive or sub additive effects of the combination.

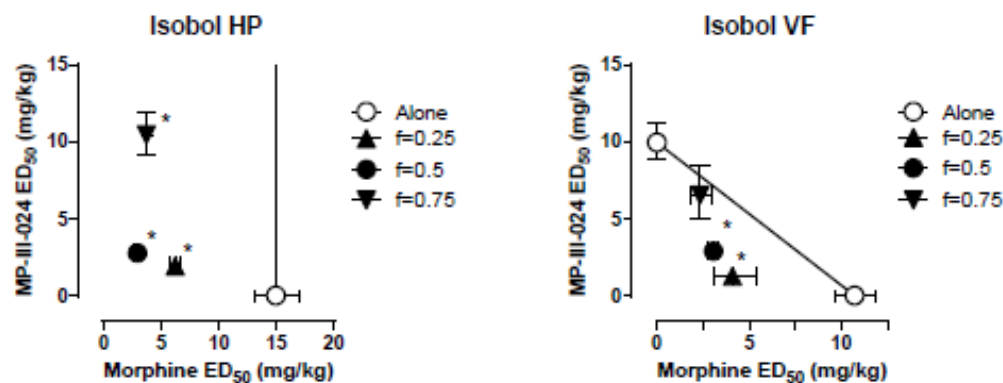


Figure 15. Isobolographic Analyses (Drug combination). Isobolographic analyses of analgesic effects in the hot plate assay (A) and the von Frey test (B) with morphine alone, or morphine in combination with MP-III-024. Because MP-III-024 was ineffective in the hot plate assay, the line of additivity is vertical in panel A at the determined ED₅₀ of morphine. All combinations of morphine with MP-III-024 significantly induced synergistic effects. In the von Frey assay, the line of additivity spans from the determined ED₅₀ values of morphine and MP-III-024; combinations of the two drugs fell into the lower left quadrant, indicating synergistic effects. Both the isobols show that E₅₀ values are into the lower left quadrant which means for getting a similar effect with Morphine alone, lower dose is needed with the combination therapy. High doses of analgesics are responsible for the high abusive effects. Low dose low or no abusive effects.

3.9. Discussion

In the von Frey procedure, each compound produced dose- and time-dependent increases in mechanical sensitivity, whereas only morphine was effective on the hot plate. In combination of 0.94:1 mixture of MP-III-094 and Morphine demonstrate the two drugs interact in a synergistic manner across both procedures.

The measurement of variations in withdrawal responses is an important tool used to assess changes in tactile sensitivity in rodent models of pain and inflammation. Using a von Frey apparatus to assess these changes in tactile sensitivity. On Hot plate, at 56 °C temperature, nociceptive behaviors including paw licking, flutter, and jumping can be observed, and

increased response latencies following drug administration are interpreted as an antinociceptive response. These two techniques show two different effects for the two different drugs. But in combination, synergistic effects suggest a new combination of analgesic by using low doses and obtain higher efficacy. There are some limitations of this study. In this test, all tests performed by using male mice, not female mice. There are some sex-mediated differences in opioid receptor expression and signaling, manifesting in differential effects in tests of analgesia in humans and rodents and important differences in rodent models of drug abuse and relapse (Becker & Chartoff, 2019; Craft, 2008; Dahan et al., 2008; Lee & Ho, 2013). The effect of the combination study may be different, if the other sex animal used. The animals housed in light/dark cycle, but experiment performed only in the light part. So, the inactive part of the mouse diurnal cycle may provide variable opioid receptor expression. (Mitchell et al. 1998) and opioid receptor activation can itself alter circadian rhythms (Pacesova et al. 2015; Webb et al. 2015). If the tests performed in dark cycle, there is a possibility to get different results. In future research plan is to test with different GABA_A PAMs like HZ-166, MP-III-080, MP-III-024 in various ratios.

Chapter 4

Conclusions

Opioids are generally prescribed to treat pain but with prolonged use, the effects of pain-relieving effects may lessen. Gradually a patient may develop dependence as well as withdrawal symptoms, which leads to possible addiction. The opioid epidemic necessitates the development of new potent analgesics or analgesic combinations without or limited abuse liability. Our investigations into the novel analgesic IBNtxA and combinations of morphine and MP-III-024 represent two avenues that can provide new pharmacotherapeutics to meet this challenge. IBNtxA has been identified as a novel analgesic that has no addictive properties. New drug combinations of morphine and MP-III-024 may provide synergistic effects. Isobolographic study played a crucial role in interpreting the potency of these combinations. In the future, other pharmacological properties, and pharmacokinetics of IBNtxA can be investigated. Combination of other GABA PAMs with Morphine or other Opioids, the safety profile of these combinations need to be studied.

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