Method Development and Pharmacokinetic Study of JY08, A Dual Opioid-NPFF Receptor Ligand

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METHOD DEVELOPMENT AND PHARMACOKINETIC STUDY OF JY08, A DUAL OPIOID-NPFF RECEPTOR LIGAND

By
Sydney Rebecca Harrison

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2018

Approved by

_________________________
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_________________________
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Reader: Dr. Kenneth Sufka
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I would like to acknowledge Dr. Bonnie A. Avery for her continual guidance and encouragement throughout the past few years, from Mississippi to Florida. Under your leadership, I have been able to not only cultivate a deeper knowledge but also a passion for pharmaceutical research.

I would like to express my sincerest gratitude to Abhisheak Sharma and Tamara King who welcomed me into the Avery lab and guided me throughout my entire work. You were both essential mentors in this process, and I am grateful for your patience and teachings.

I would like to thank Dr. Chalet Tan for undertaking the role of advisor. I would also like to thank both Dr. Tan and Dr. Kenneth Sufka for the time and valuable input they have provided as members of my committee.

I would like to acknowledge Dr. Christopher McCurdy and John Yi for their collaboration and the synthesis and characterization of JY08. Without their foundational work, this project would not have been possible.

Lastly, I would like to thank my friends and family for their unwavering support throughout the past four (or twenty-one) years.

“There are places I’ll remember all my life, though some have changed.”

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ABSTRACT

SYDNEY REBECCA HARRISON: Method Development and Pharmacokinetic Study of JY08, A Dual Opioid-NPFF Receptor Ligand
(Under the direction of Dr. Bonnie A. Avery)

Within the United States and worldwide, a rapidly developing public health crisis is at hand due to the abuse of opioids. With high percentages of Americans experiencing both acute and chronic pain and limited treatment routes, opioid analgesics are highly prescribed in clinical practice. These drugs act on central nervous system to activate opioid receptors in regions of the brain regulating pain and reward. However, these compounds are associated with many unwanted adverse effects including tolerance, dependence, hyperalgesia, and addiction. Thus, there is a need to for the development of new drug candidates that can serve as analgesics for long-term use without the unwanted side effects. The neuropeptide FF (NPFF) system is known to modulate the antinociceptive properties of the opioid system. With this knowledge, the development of compounds that can serve both as a NPFF receptor antagonist and an opioid receptor agonist has become a recent focus in analgesic drug discovery and development. The novel compound JY08 was designed as a dual-activity ligand that acts as an agonist at the mu-opioid receptor and antagonist at the NPFF receptor in order to combat the tolerance and hyperalgesia conditions commonly associated with long-term opioid use. Ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) bioanalytical methods were developed for the quantification of JY08 through pharmacokinetic, solubility, and metabolic stability studies.
# TABLE OF CONTENTS

ABSTRACT............................................................................................................iv  
LIST OF ABBREVIATIONS......................................................................................vi  
LIST OF FIGURES....................................................................................................vii  
INTRODUCTION......................................................................................................1  
METHODS................................................................................................................8  
RESULTS AND DISCUSSION.....................................................................................17  
CONCLUSION..........................................................................................................28  
BIBLIOGRAPHY.......................................................................................................29
LIST OF ABBREVIATIONS

NPFF- Neuropeptide FF

OUD- Opioid use disorder

UPLC- Ultra-high performance liquid chromatography

HPLC- High performance liquid chromatography

MS- Mass spectrometry

CNS- Central nervous system

PNS- Peripheral nervous system

OIH- Opioid-induced hyperalgesia

PK- pharmacokinetics

IS- internal standard

AUC- area under the curve

$C_{\text{max}}$- plasma peak concentration

$T_{\text{max}}$- time to $C_{\text{max}}$

MRT- mean residence time

$V_d$- volume of distribution

$T_{1/2}$- half-life
LIST OF FIGURES

Figure 1. Structure of JY08.................................................................6

Table 1. Prediction of Oral Bioavailability.................................6

Table 2. UPLC-MS/MS Gradient 1........................................9

Table 3. UPLC-MS/MS Gradient 2.........................................10

Table 4. MS Parameters for JY08 and WA475....................17

Table 5. MS Parameters for JY08 and CM398.....................18

Figure 2. MRM Spectra of (A) JY08 and (B) IS (CM398)....19

Figure 3. Representative MRM Chromatograms of (A) JY08 Qualifier Ion (B) JY08 Quantifier Ion and (C) CM398.........................20

Graph 1. JY08 Calibration Curve in Rat Plasma......................20

Graph 2. Phase I Metabolic Stability of JY08.........................22

Table 6. Measured Metabolism in Rat Liver Microsomes........22

Table 7. Pharmacokinetic Parameters of JY08 Pilot Study.......24

Graph 3. Pilot Oral Pharmacokinetic Study of JY08..............24

Graph 4. Pilot IV Pharmacokinetic Study of JY08...............24

Table 8. Pharmacokinetic Parameters of JY08 Full Study........25

Graph 5. Full Oral Pharmacokinetic Study of JY08..............26

Graph 6. Full IV Pharmacokinetic Study of JY08.................26

Figure 4. Putative Metabolites of JY08.................................27
1. INTRODUCTION

1.1 The opioid epidemic

The United States is currently in the midst of an ongoing opioid epidemic. On October 26, 2017, President Donald Trump declared the national opioid crisis as a public health emergency (1). The US has experienced a 17-year increase in opioid overdose deaths due to both prescription and illicit opioids (2). Weiner et al. identified three driving forces associated with the growing opioid epidemic: the moral imperative for providers to treat pain and relieve suffering, the under-treatment of pain, and pharmaceutical industries (3). The epidemic’s antecedents can be traced back to the late 1990s during which the American Pain Society recognized pain as the fifth vital sign (4). As a result of these efforts and the associated moral imperative, physicians became more liberal in the prescription of opioids for chronic, noncancer pain (4). As pain was being adopted as the fifth vital sign, OxyContin was first hitting the markets. It was soon to become top 30% of total analgesic sales and become the most abused prescription opioid on the market (4). The manufacturer, Purdue Pharma, was assertively marketing OxyContin while distorting the risk for addiction associated with the drug (5). Mass prescription of opioid analgesics for acute and chronic pain soon followed these events, contributing to much of the crisis currently being experienced in the United States.

As the leading cause of US injury-related death, opioid overdoses were attributed to over 42,000 deaths in 2016, five times higher than experienced in 1999 (6). On average, 115
Americans die each day due to opioid abuse and misuse (2). However, overdoses are not the only risk related to opioids; the abuse, misuse, and addiction related to these drugs are each hallmarks of the current epidemic. In 2014, 2 million individuals claimed to abuse or be dependent on prescription opioids (7). Additionally, over 1000 patients are treated each day in emergency departments for opioid misuse (8). Coinciding with these increases in use, increases in the diagnosis of opioid-use disorder have been observed with 2.0 million people over the age of 12 years being affected in 2016 (9). Factor in the increased risk of infectious disease due to unsafe injecting and other psychiatric comorbidities, the US is currently experiencing one of the greatest public health crises of its time. The opioid crisis reaches beyond the United States, with 29.5 million people globally suffering drug use disorders—the majority of these due to opioids (10).

Recently the National Institute of Health (NIH) has launched an initiative to address this public health crisis focusing on three areas: overdose-reversal interventions, treatments for opioid addiction, and non-addictive treatments for chronic pain (11). With the majority of opioid abusers starting their path to dependence and addiction through prescription medications, the population that depends on opioid analgesics for daily living are particularly vulnerable in this epidemic.

1.1.1 Chronic pain

As defined by the International Association for the Study of Pain (IASP), pain is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (12). The IASP defines chronic pain as “pain that persists beyond normal tissue healing time, which is assumed to be three months” (12). Chronic non-cancer pain is a multifactorial condition that can be attributed
to neuropathic pain, fibromyalgia, lumbar pain, osteoarthritis, and many others. Chronic pain is reported to affect approximately 30% of Americans and 20% of people worldwide (11,13). The condition is also the cause for 15-20% of physician visits (13). The recommended first line of treatment for chronic pain includes antidepressants, anticonvulsants, and topical lidocaine (11). The IASP recommends opioids as a second-line chronic pain treatment (11). However, the use of opioids for the treatment of chronic pain is very common, with an estimated 20% of patients presenting with pain or a pain-related condition receiving an opioid prescription (14). Long-term use of opioids has been associated with many adverse effects including gastrointestinal issues, addiction, tolerance, cardiovascular complications, and hyperalgesia. Because of current treatment limitations and the US Congress declaration of 2000-2010 Decade of Pain and Control Research, the prescription of opioid analgesics for pain management has been rapidly rising in clinical practice. Addressing the needs of patients with chronic pain through the development of safer, more effective treatments is a necessary step in ultimately overcoming the opioid crisis.

1.2 The opioid system

The opioid receptor system consists of three subtypes widely distributed throughout the CNS and PNS: mu, kappa, and delta. Opioid medications primarily exert their analgesic effects through binding to the mu-opioid receptors (15). Within the CNS, mu-opioid receptors are present at high concentrations in areas regulating nociception (periaqueductal gray, thalamus, cingulate cortex, and insula), pain-induced emotional response (amygdala), and reward pathways (ventral tegmental area and nucleus accumbens) (15). Repeated use of opioids is known to be accompanied with an array of
unwanted adverse effects. Tolerance is a primary effect of frequent opioid use. Continuous administration of opioid agonists leads to a suppression of endogenous opioids and decrease in analgesic efficacy. This phenomenon is often observed in individuals with chronic pain disorders or opioid use disorders, two of the populations most dependent on opioid analgesics. The physiological process of tolerance involves a reduction in endogenous opioid production in the body. Thus, higher doses of exogenous opioids are needed with each subsequent administration in order to achieve the same analgesic effect, and for those suffering from opioid use disorder, often to reach the same euphoric effect. Tolerance also involves the desensitization and internalization of opioid receptors, further decreasing agonist efficacy (16). Opioid-induced hyperalgesia (OIH) has also been recognized as an issue due to long-term opioid use. Though commonly confused with tolerance, OIH is a separate phenomenon associated with opioid use. This disorder leads to an increased sensitivity to pain stimuli. Thus, patients taking opioid analgesics for the treatment of pain will experience increasing pain sensations, resulting in the use of higher doses of opioids as with tolerance. The mechanism of OIH is thought to be a counter-balance by endogenous pronociceptive peptides such as dynorphin A and neuropeptide FF. Neuropeptide FF (NPFF) and its receptors have been found to play a role in pain transmission, interacting with the opioid system (17). These anti-opioid modulating systems are observed to be overstimulated during chronic opioid administration. Dual ligands for the opioid and NPFF receptors have recently become a target of drug discovery and design in the development of treatments for chronic pain without the classic opioid-related adverse effects.

1.2.1 Neuropeptide FF
Neuropeptide FF belongs to the RF-amide peptide family and interacts with two Gi protein-coupled receptors, NPFFR1 and NPFFR2. These receptors are distributed throughout the central nervous system (CNS) with high density in the spinal dorsal horn and the periaqueductal gray, areas involved in pain modulation (18). The NPFF signaling system has is bimodal in pain perception, producing both pro-nociceptive and anti-nociceptive effect (17). The experienced effect is dependent upon the route of administration. Intra-thecal administration of NPFF is shown to potentiate opioid analgesia. In contrast, intra-cerebroventricular administration of NPFF is shown to decrease morphine-induced analgesia in rats (17).

1.3 JY08, a novel dual NPFF/opioid receptor ligand

JY08 was developed by the McCurdy research group at the University of Mississippi Department of BioMolecular Sciences. The compound was developed to create a dual-activity drug that acts as an analgesic while eliminating tolerance or hyperalgesic effects. JY08 is a non-peptide small-molecule with the classic guanidine and phenyl components of NPFF receptor antagonists. Structure-activity relationship (SAR) studies analyzing the effect of NPFF’s C-terminal amino acids on NPFFR binding and activity have shown that the amidated residues of phenylalanine and arginine on NPFF are crucial for activation of anti-opioid effects (19). Thus, the guanidine and phenyl groups on JY08 are able to mimic these interactions while maintaining low molecular weight and size to increase bioavailability. Additionally, the absence of a peptide bond prevents protease cleavage of the NPFF antagonist. A piperidine core was incorporated into the molecule based on past mu-opioid agonists developed by the McCurdy research group to facilitate opioid receptor binding. Based on the structure and Lipinski’s parameters regarding oral
bioavailability, JY08 meets 3 of 4 criteria and is predicted to be orally bioavailable (Table 1). The calculated LogP of JY08 also indicates the compound to have high lipophilicity, showing potential for JY08 to cross the blood brain barrier. These predications are to be tested later through *in vivo* studies.

![Structure of JY08](image)

**Figure 1. Structure of JY08**

<table>
<thead>
<tr>
<th>Lipinski’s Rules</th>
<th>JY08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>≤ 500</td>
</tr>
<tr>
<td>Number of hydrogen bond donors</td>
<td>≤ 5</td>
</tr>
<tr>
<td>Number of hydrogen bond acceptors</td>
<td>≤ 10</td>
</tr>
<tr>
<td>Calculated LogP</td>
<td>0 &lt; LogP &lt; 5</td>
</tr>
</tbody>
</table>

*Table 1. Prediction of Oral Bioavailability*

JY08 was tested in multiple cell assays. When tested against the delta-, kappa-, and mu-opioid receptors, it exhibited a $K_i$ of $1763 \pm 215$, $649.9 \pm 117.8$, and $34.29 \pm 5.38$ nM, respectively. JY08 also exhibited an EC$_{50}$ of $721.6 \pm 195.0$ nM for the mu-opioid receptor.
1.4 Preclinical studies of JY08

Pharmacokinetics and drug metabolism are essential components of the drug development process. In order to successfully develop a drug candidate, the pharmacokinetic principles of absorption, distribution, metabolism, and elimination (ADME) must be analyzed. Key parameters for pharmacokinetic analysis include oral bioavailability (F), half-life (t_{1/2}), volume of distribution (V_d), and clearance (CL). These screening processes can eliminate drug candidates that exhibit poor absorption, extensive first-pass metabolism, and/or too long/short half-lives. However, these processes can also identify drug candidates that have good oral bioavailability and distribution to target tissues. Thus, both in vitro and in vivo pre-clinical studies are necessary for the development of successful drug candidates for potential use in humans.
2. METHODS

2.1 Materials and reagents

The compound (JY08) and internal standards (WA475, CM398) were provided by Dr. Christopher McCurdy, initially synthesized by Dr. McCurdy’s research group, Department of BioMolecular Sciences, The University of Mississippi. LC-MS grade methanol, acetonitrile, and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid and trifluoroacetic acid were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). TRIS, magnesium chloride, and potassium phosphate were obtained from Sigma-Aldrich (St. Louis, MO, USA). NADPH was purchased from MP Biomedicals (Solon, OH, USA). Rat liver microsomes were purchased from XenoTech (Lenexa, KS, USA). Rat plasma was purchased from VWR (Suwannee, GA, USA).

2.2 Instrumentation and analytical conditions

Two LC-MS/MS instruments were used for analysis of the compounds. The instrumentation from Thermo Scientific was used for the provided calibration curve, metabolic stability studies, and solubility studies. Waters instrumentation was used for the analysis of pharmacokinetic studies in rats. Separate methods for JY08 and the respective internal standards (IS) were developed on each instrument to ensure validated analysis. Internal standards were chosen based on their chemical structure and function, which is preferably similar to the structure and function of JY08. The IS should be
compatible with the LC-MS/MS method developed for JY08 and produce strong, consistent signals within the run time.

2.2.1 Method development on Thermo Scientific UPLC-MS/MS systems

Accela ultra-high performance liquid chromatography system (Thermo Scientific, Waltham, MA, USA) equipped with a quaternary solvent manager, vacuum degasser, thermostatted column sleeve, an auto-sampler, a Waters Acquity UPLC™ CSH C18 column (1.7 μL, 2.1 x 100 mm; Milford, MA, USA) and was used for chromatographic separation. The mobile phase was a gradient method (Table 2) using 0.02% v/v trifluoroacetic acid in water and 0.02% v/v trifluoroacetic acid methanol. The mobile phase was pumped at a flow rate of 0.3 mL/min for a run time of 3.5 minutes. The column temperature was maintained at 40°C and the injection volume was 10 μL. Two washes were used in between runs to prevent carryover and sample contamination: strong needle wash (methanol) and weak needle wash (50:50 methanol: water (0.01% v/v formic acid)). Weak needle washes typically have similar compositions to the mobile phase and are used following the strong needle wash to ensure the strong wash is flushed from the needle and sample loop.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
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<td>5</td>
</tr>
<tr>
<td>3.5</td>
<td>95</td>
<td>5</td>
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</table>

Table 2. UPLC-MS/MS Gradient 1  
*A- 0.02% TFA in Water *B-0.02% TFA in Methanol

Mass spectrometric (MS) analysis was performed using TSQ Quantum Access MAX (Thermo Scientific, Waltham, MA, USA) equipped with an electro-spray ionization (ESI) source. The detection was achieved with ESI positive ionization using multiple reaction
monitoring (MRM). The protonated parent ion [M + H]⁺ and daughter ions were monitored for transitions m/z 403.22 > 268.27 and 403.22 > 224.97. WA475, internal standard, was monitored for transition m/z 406.23 > 161.08. The MS parameters were tuned to optimize the molecular ion signal. Spray voltage, tube lens offset, and skimmer offset were set at 3500, 127, and 0 V. Sheath gas pressure, ion sweep gas pressure, and aux gas pressure were optimized to 40, 0, and 5 psi, respectively. Capillary temperature was set to 325°C.

2.2.2 Method development on Waters UPLC-MS/MS systems

Chromatographic separations were carried out on Acquity ultra-high performance liquid chromatography system (Waters, Milford, MA, USA) equipped with a binary solvent manager, vacuum degasser, thermostatted column compartment and an auto-sampler. A Waters Acquity UPLC™ CSH C18 column (1.7 μm, 2.1 x 100 mm; Milford, MA, USA) was used for chromatographic separations. A gradient method (Table 1) using aqueous ammonium formate buffer (5 mM, 0.25% v/v formic acid) and acetonitrile was applied for chromatographic separation. The mobile phase was pumped at a flow rate of 0.28 mL/min for a run time of 4.0 minutes. The column temperature was maintained at 40°C and the sample temperature was maintained at 10°C. Injection volume was 2 μL. Two washes were used in between runs: strong needle wash (80:20 acetonitrile: water) and weak needle wash (20:80 acetonitrile: water).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
<th>Curve</th>
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<tr>
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</tr>
<tr>
<td>3.4</td>
<td>95</td>
<td>5</td>
<td>6</td>
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</table>
Mass spectrometric analysis was performed using Xevo TQ-S Micro (Waters, Milford, MA, USA) equipped with an ESI source. The detection was achieved with ESI positive ionization using multiple reaction monitoring. The protonated parent ion \([M + H]^+\) and daughter ions were monitored for transitions \(m/z\) 403.20 > 268.15 and 403.22 > 225.04. CM398 was used as an internal standard for all analyses on Waters instrumentation due to better signals and method compatibility. IS was monitored for transition \(m/z\) 396.14 > 232.10. The following MS parameters were tuned for optimization of the signal. Capillary voltage was set to 0.5 kV. Source temperature and desolvation temperature were set to 150 and 500°C, respectively. Cone gas flow and desolvation gas flow were set to 50 and 900 L/h, respectively.

2.3 Preparation of calibration standards

Initial stock solution of JY08 and IS (WA475, CM398) were prepared in methanol to yield concentrations of 1 mg/mL. Appropriate volumes of stock solutions were serially diluted with methanol to obtain working standard solutions of JY08 and IS ranging from 20 to 4000 ng/mL. The working standard solutions were stored at -20°C.

For calibration standards, samples at 1, 2.5, 10, 50, 100, and 190 ng/mL were prepared by 2 μL of the appropriate stock to 198 μL of 2:1 0.02% v/v TFA in methanol: 0.02% v/v TFA in water containing IS (3 ng/mL) for a total volume of 200 μL.

For pharmacokinetic sample analysis, calibration standard samples at concentrations of 1, 2.5, 5, 25, 50, 100, 190 and 200 ng/mL were prepared by spiking 1 μL of the appropriate stock to 19 μL of rat plasma.
2.4 Plasma sample clean-up

The rat plasma samples were stored after collection at -80℃ and thawed at room temperature prior to analysis. Protein precipitation with acetonitrile containing the IS (CM398, 20 ng/mL) and formic acid (0.25% v/v) was used for the extraction of JY08 from rat plasma samples. An aliquot of 20 μL of plasma was quenched with 100 μL of quenching solution. Samples were vortexed for 10 minutes at 2000 rpm on a BenchMixer multi-tube vortex mixer (Benchmark, San Francisco, CA, USA). Samples were then centrifuged at 4℃ for 10 minutes at 15000 rpm using Eppendorf centrifuge 5427-R (Eppendorf, Hauppauge, NY, USA). A fixed aliquot (~80 μL) of the supernatant was then taken from centrifuged samples for analysis via UPLC-MS/MS.

2.5 Solubility studies

The most common route of administration for drugs is oral ingestion (20). Oral bioavailability is dependent on several factors including aqueous solubility, dissolution, and first-pass metabolism (20). Thus, solubility studies are utilized to determine if compounds are sufficient candidates for oral administration by examining the aqueous solubility at physiological pH.

Solubility of JY08 was determined in phosphate buffer at a pH of 7.4 (n=2). Stock solution of JY08 13 mg/mL was made fresh in potassium phosphate buffer (0.1 M, pH 7.4, 100 μL). Samples were placed on a tube revolver for 24 hours at room temperature (25℃). After 24 h, samples were centrifuged and the supernatant was collected. This supernatant was then diluted three times. For a dilution factor of 1000, supernatant (2 μL) was added to methanol (1998 μL). For a dilution of factor of 50,000, 10 μL of the
first dilution was added to methanol (490 µL). For a dilution factor of 500,000, 10 µL of the second dilution was added to 90 µL of methanol. Each sample was then analyzed via UPLC-MS/MS for JY08 content.

2.6 Metabolic stability studies

Metabolic stability studies are important for the in vitro analysis of a drug’s pharmacokinetic parameters. The liver is a primary site of drug metabolism (21). High rates of metabolism can impact the oral bioavailability of a drug, while low rates can impact toxicity. Metabolism in the human body primarily occurs through two phases. Phase I modifications (hydrolysis, oxidations, reductions, etc.) are most commonly performed by the cytochrome P450 enzymes. Phase II modifications involve conjugation reactions performed by transferases. Phase I metabolic stability studies were conducted on JY08 using rat liver CYP450 microsomes. Metoprolol, a compound with a well-defined metabolic profile, was used for positive and negative control samples.

To a 96-well plate, rat liver microsomes (0.5 mg/mL, 10 µL) were added. TRIS buffer (50 mM, pH 7.4, 168 µL) was then added, followed by magnesium chloride in TRIS (500 mM, 20 µL). NADPH (40 mM, 20 µL) was added to positive controls and JY08 sample only. The 96-well plate was then placed in an incubator held at 37°C for 10 minutes. See procedure below for specific reaction mixture.

For negative and positive control samples, metoprolol (1 µM, 2 µL) was added to the specified wells and placed back in the incubator. For JY08 sample, JY08 (1 µM, 2 µL) was then added to the specified wells and placed back in the incubator. At the designated time points of 0, 5, 10, 15, and 20 minutes, aliquots of 40 µL were taken from each
reaction mixture and placed in a separate 96-well plate containing a quenching solution of ice-cold methanol with IS (3 ng/mL WA475). Samples were then centrifuged at 12000 rpm for 15 minutes. The supernatant (50 μL) was collected and added to 100 μL of 0.02% v/v TFA in water for analysis via LC-MS.

2.7 Pilot pharmacokinetic study of JY08 in rats

Pharmacokinetic animal studies were performed using cannulated, male Sprague-Dawley rats (n=2) obtained from Envigo (East Millstone, NJ, USA) with an average body weight of 240.5 ± 12 g. All experimental procedures were approved and performed in accordance with the guidelines set in place by the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi. The rats were housed in metabolic cages and allowed free movement and access to water throughout the experiment. Rats were fasted 12-15 hours prior to the experiment. For pilot studies, both single oral dosing (20 mg/kg) and IV bolus (1 mg/kg) of JY08 (n=1, each) was tested. The oral dose was prepared by dissolving JY08 (41.3 mg) in distilled water (7 mL) and administered as a weight-based dose using an oral gavage syringe. IV bolus dose was prepared by dissolving JY08 (3 mg) in saline (3 mL) and injected through the caudal vein. Sample collection for IV was performed at pre-dose and 5 min, 10 min, 20 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h post-dose. For oral pharmacokinetic study, blood samples were collected pre-dose and 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h post-dose. At each time point, 0.1 mL of blood was taken from each animal using a fresh syringe. Following each collection, cannulas were flushed with 0.1 mL of heparinized saline. Blood samples were collected in micro-centrifuged tubes and
centrifuged for 10 minutes to separate plasma and cell components. Plasma was then placed into a clean tube and stored at -80°C until analyzed.

2.8 Full pharmacokinetic study of JY08 in rats

Pharmacokinetic animal studies were performed using cannulated, male Sprague-Dawley rats received from Envigo (East Millstone, NJ, USA) with an average body weight of 275 ± 25 g. All experimental procedures were approved and performed in accordance with the guidelines set in place by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida. The rats were housed in metabolic cages and allowed free movement and access to water throughout the experiment. Rats were fasted 15 hours prior to the experiment. Male rats received either a single oral (n=6) or IV (n=6) administration of the JY08 formulation. The oral dose was administered using an oral gavage syringe. IV bolus was administered through the caudal vein. Sample collection following single IV bolus (1 mg/kg) was performed pre-dose and 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, and 36 h post-dose. Sample collection following oral dosing was performed pre-dose and 5 min, 10 min, 15 min, 20 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h post-dose. Please note that blood volume taken from each animal did not exceed 10% of the total blood volume for each animal. At each time point, approximately 0.1 mL of blood was sampled from each animal using a fresh syringe. Following each collection, cannulas were flushed with 0.1 mL of heparinized saline. Blood samples were collected in micro-centrifuged tubes and centrifuged for 10 minutes to separate plasma and cell components. Plasma was then collected and stored at -80°C until analyzed.
2.9 Pharmacokinetic analysis

Data analysis of pharmacokinetic studies was performed with Phoenix Software (Certara Inc., MO, USA) using non-compartmental analysis. Concentration-time data and pharmacokinetic parameters are represented as mean ± standard error mean (SEM). Peak plasma concentration ($C_{\text{max}}$) and time to reach peak concentration ($T_{\text{max}}$) were determined based on concentration-time data. Area under the plasma concentration-time curve from 0 to last measured concentration ($\text{AUC}_{\text{last}}$) was calculated through the linear trapezoidal method. Half-life ($t_{1/2}$) was calculated as a function of the slope (elimination constant) from the plotted curves.
3. RESULTS AND DISCUSSION

3.1 UPLC-MS/MS optimization

3.1.1 Thermo Scientific

Positive electrospray ionization of JY08 and WA475 produced their respective molecular ions \([\text{M + H}^+]\) at \(m/z\) 403.22 and 406.23. Capillary voltage (3.5 kV), tube lens offset (127 V), capillary temperature (325 °C) and sheath gas pressure (40 psi) were tuned to obtain the most intense signal for both compounds. Fragmentation produced several daughter ions for JY08 and IS, establishing qualitative and quantitative ions for monitoring transitions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ions</th>
<th>Dwell (sec)</th>
<th>Cone Voltage</th>
<th>Collison Energy Voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JY08- Qualitative</strong></td>
<td>403.22 &gt; 268.27</td>
<td>0.21</td>
<td>88.0</td>
<td>25.0</td>
</tr>
<tr>
<td><strong>JY08- Quantitative</strong></td>
<td>403.22 &gt; 224.97</td>
<td>0.21</td>
<td>88.0</td>
<td>28.0</td>
</tr>
<tr>
<td><strong>WA475- IS</strong></td>
<td>406.23 &gt; 161.08</td>
<td>0.21</td>
<td>70.0</td>
<td>34.0</td>
</tr>
</tbody>
</table>

*Table 4. MS Parameters for JY08 and WA475*

During chromatographic method development, both isocratic and gradient methods were explored with the intent to develop a quick, efficient method with optimal retention times and peak shapes for both JY08 and IS. Various chromatographic conditions were employed using multiple columns (Acquity UPLC BEH C\(_{18}\), CSH C\(_{18}\), Cyano, Phenyl Hexyl) and mobile phase compositions (methanol, acetonitrile, formic acid, and ammonium acetate buffer) in both gradient and isocratic methods. It was determined that an Acquity UPLC CSH C\(_{18}\) column and a gradient mobile phase of 0.1% v/v formic acid
in water and methanol optimized the chromatographic separation of both JY08 and WA475 (IS). Retention times for JY08 and WA475 were 2.40 and 2.65 minutes, respectively. This method produced sharp, clean peaks allowing for a LOD of 1 ng/mL.

3.1.2 Waters

The electrospray ionization of JY08 and the IS produced their respective protonated molecular ions, [M + H]+, at m/z 403.20 and 396.14 under positive ionization mode. To obtain the most intense molecular ion signal, several source parameters were optimized. These include capillary voltage (0.5 kV), source temperature (150°C), desolvation temperature (500°C), cone gas flow (50 L/hr), and desolvation gas flow (900 L/hr). The protonated molecular ions of each compound undergo fragmentation in the collision cell of the mass spectrometer. Several daughter ions are produced from each compound and those with the greatest abundance and stability are selected for monitoring transitions. Fragment ions m/z 268.15 and m/z 225.04 were selected as the qualitative and quantitative transitions for JY08, respectively. Fragment ion m/z 232.10 of the IS was selected. Several parameters were optimized to both compounds and their fragment ions as described below in Table 5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ions</th>
<th>Dwell (sec)</th>
<th>Cone Voltage</th>
<th>Collison Energy Voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>JY08- Qualitative</td>
<td>403.20 &gt; 268.15</td>
<td>0.110</td>
<td>88.0</td>
<td>28.0</td>
</tr>
<tr>
<td>JY08- Quantitative</td>
<td>403.20 &gt; 225.04</td>
<td>0.110</td>
<td>88.0</td>
<td>28.0</td>
</tr>
<tr>
<td>CM398- IS</td>
<td>396.14 &gt; 232.10</td>
<td>0.110</td>
<td>70.0</td>
<td>26.0</td>
</tr>
</tbody>
</table>

Table 5. MS Parameters for JY08 and CM398
Chromatographic analysis of JY08 and the IS was initiated with the intent to develop a simple method with clean separation in a short run time. Various chromatographic conditions were tested using various columns (Acquity UPLC CSH and BEH C\textsubscript{18}) and mobile phase compositions (methanol, acetonitrile, formic acid, ammonium acetate, and ammonium formate buffers) in both gradient and isocratic methods. It was determined that an Acquity UPLC CSH C\textsubscript{18} column and a gradient mobile phase of 0.25% formic acid in 5 mM ammonium formate buffer and acetonitrile optimized the chromatographic separation of both JY08 and the IS. Retention times for JY08 and CM398 were 1.87 and 1.91 minutes, respectively. This method produced sharp, clean peaks allowing for a LOD of 1 ng/mL.

*Figure 2. MRM spectra of (A) JY08 and (B) internal standard CM398.*
3.2 Calibration curve

Calibration standards 1, 2.5, 5, 25, 50, 100, 190 and 200 ng/mL were run using UPLC-MS Gradient 1 (Table 3). Calibration curve was constructed by plotting the peak area ratio of JY08 to that of IS against the corresponding nominal concentration. The linear regression equation of the calibration curve obtained from 8 points was \( y = 0.0785x + 0.0183 \). Correlation coefficient was always \( \geq 0.99 \) during the analysis.

3.3 Solubility studies
The calculated solubility of JY08 at a pH of 7.4 is 3.75 mg/mL. The United States Pharmacopeia states that a substance is “slightly soluble” if 100-1000 parts of solvent are required to dissolve 1 part of solute (20). Thus, JY08 can be classified as slightly soluble. These values indicate JY08 partly in an ionized state at pH 7.4. The extent of ionization of a drug will determine the amount of absorption at a given pH within the body. Thus, at a physiological pH of 7.4, only unionized drug will be absorbed.

3.4 Metabolic stability studies

Metabolic stability samples were run on Thermo Scientific instrumentation using gradient method 1. The area under the curve (AUC) of each sample was used to calculate the percent remaining of each compound, using the IS (WA475) AUC as reference. The JY08 sample exhibited odd metabolism patterns, with an increase in concentration for three time points. This is most likely due to instrumentation or procedure error. However, repeated studies (not shown) displayed the same metabolic pattern. This metabolic pattern may indicate a slow metabolism of JY08 by CYP450 enzymes or primary metabolism of JY08 occurs by phase II enzymes. Positive control metoprolol sample exhibited a normal metabolism pattern, indicating that reaction components and enzymes were working properly. Negative control metoprolol sample was expected to stay at 100% throughout all time points; however, the decreases in concentration observed can be attributed to non-specific binding of compound to enzyme. Because of
the problems that occurred with this study, the phase I metabolic studies will be repeated by the Avery group at a later date.

Graph 2. Phase I Metabolic Stability of JY08

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>% remaining JY08</th>
<th>% remaining Metoprolol (NC)</th>
<th>% remaining Metoprolol (PC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>118.30</td>
<td>84.80</td>
<td>59.39</td>
</tr>
<tr>
<td>10</td>
<td>137.75</td>
<td>79.92</td>
<td>44.12</td>
</tr>
<tr>
<td>15</td>
<td>121.75</td>
<td>81.54</td>
<td>41.25</td>
</tr>
<tr>
<td>20</td>
<td>92.32</td>
<td>78.88</td>
<td>38.66</td>
</tr>
</tbody>
</table>

Table 6. Measured Metabolism in Rat Liver Microsomes

3.5 Pilot pharmacokinetic study

Following IV dosing in Sprague Dawley rats (n=1), JY08 exhibited biphasic and extravascular distribution with a volume of distribution ($V_d$) of 26.3 L/kg. This volume of distribution is larger than the total blood volume in rats (0.085 L/kg) (22). IV
injections follow multiphasic absorption patterns in which there are two phases. In the alpha phase, drug distribution shifts from systemic circulation (central compartment) to body tissues and organs (peripheral compartment). In the alpha phase, absorption is greater than elimination. In the beta phase, elimination is greater than absorption and a decrease in plasma drug concentration is seen due to metabolism and excretion mechanisms. Extra-hepatic metabolism and elimination was determined to be negligible with a clearance (CL) of 2.0 L/h/kg compared to average hepatic blood flow of 4.8 L/h/kg in rats (23).

The rat receiving an oral dose of JY08 (n=1) also displayed extensive extravascular distribution as indicated with a calculated $V_d$ of 26.3 L/kg. The study also displayed quick absorption, distribution, and elimination of JY08 from plasma and an oral bioavailability of 13.9%. The concentration-time curves resulting from the oral pharmacokinetic study of JY08 display multiple peak phenomenon (Graph 3). Oral concentration-time curves typically exhibit an initial absorption phase in which an increase in concentration is seen following administration as the drug enters systemic circulation. Multiple peaks in the curve may indicate multiple absorption phases of the compound occurring in the animal. Multiple peak phenomenon can be due to enterohepatic recirculation, gastrointestinal recirculation, delayed gastric emptying, and/or absorption differences in the intestines (24). Since multiple peaks are not apparent in the IV concentration-time curve, enterohepatic circulation is probably not the cause of the multiple absorption phases. This phenomenon could possibly be due to the multiple pKa points displayed by JY08. Drugs are only absorbed in unionized states. Thus, as the
surrounding pH changes throughout the GIT, JY08 will experience different ionization states causing discontinuous absorption.

After oral and IV dosing, mean residence time (MRT) for JY08 was found to be 6.4 and 4.9 h, respectively. MRT is used to represent the average amount of time the drug stays in a body. Since drugs administered intravenously do not undergo an absorption phase, it is expected for the residence time of IV administered drugs to be shorter than oral drugs. This concept applies to terminal half-life ($t_{1/2}$) as well (25).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Oral</th>
<th>Intravenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max1}}$ (ng/mL)</td>
<td>174.9</td>
<td>-</td>
</tr>
<tr>
<td>$C_{\text{max2}}$ (ng/mL)</td>
<td>158.4</td>
<td>-</td>
</tr>
<tr>
<td>$T_{\text{max1}}$ (h)</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>$T_{\text{max2}}$ (h)</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>AUC (ng•h/mL)</td>
<td>1357.5</td>
<td>489.5</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>6.4</td>
<td>4.9</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>11.33</td>
<td>8.93</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>34.1</td>
<td>26.3</td>
</tr>
<tr>
<td>Clearance (L/h/kg)</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>13.9</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 7. Pharmacokinetic Parameters of JY08 Pilot Study*
3.6 Full pharmacokinetic study

The full PK studies of JY08 in rats produced similar results to pilot studies. With a volume of distribution ($V_d$) of 20.4 ± 4.3 and 32.8 ± 6.8 L/kg for oral and IV doses, respectively, JY08 is extensively distributed in extra-vascular systems. Extra-hepatic metabolism and elimination were negligible with clearances (CL) of 1.1 ± 0.2 and 1.2 ± 0.2 L/h/kg for oral and IV, respectively. Similar to pilot study results, oral bioavailability was determined to be low at 9.1%. This low range may be due to first pass metabolism or poor absorption of JY08 in the gastrointestinal tract (25). Concentration-time curves of the oral pharmacokinetic study of JY08 display multiple peak phenomenon as also seen in the pilot study. The $C_{max}$ and $T_{max}$ for each absorption phase is listed in Table 8. The terminal half-life for oral and IV doses was 13.6 ± 2.7 and 32.8 ± 6.8, respectively.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Oral</th>
<th>Intravenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{max1}$ (ng/mL)</td>
<td>217.5 ± 40.0</td>
<td>-</td>
</tr>
<tr>
<td>Parameter</td>
<td>Value 1</td>
<td>Value 2</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>$C_{\text{max}2}$ (ng/mL)</td>
<td>210.1 ± 36.1</td>
<td>-</td>
</tr>
<tr>
<td>$T_{\text{max}1}$ (h)</td>
<td>0.4 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>$T_{\text{max}2}$ (h)</td>
<td>2.8 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>AUC (ng•h/mL)</td>
<td>1788.5 ± 265.1</td>
<td>988.1 ± 158.3</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>13.6 ± 2.7</td>
<td>21.6 ± 3.7</td>
</tr>
<tr>
<td>$V_d$ (L/kg)</td>
<td>20.4 ± 4.3</td>
<td>32.8 ± 6.8</td>
</tr>
<tr>
<td>Clearance (L/h/kg)</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>9.1</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 8. Pharmacokinetic Parameters of JY08 Full Study*

3.7 Putative metabolites of JY08

Using MetaboLynx software, metabolites of JY08 from *in vivo* plasma samples were identified via mass spectrometry. Phase I metabolism includes oxidation, reduction, hydrolysis, and dealkylation. As depicted in Figure X, both metabolites 1 and 3 underwent dealkylation reactions by phase I enzymes. Metabolite 4 underwent a hydroxylation reaction. In metabolite 2, the guanidine group of JY08 was cleaved. Other metabolites that are conjugation products were also present in lower abundance.
Analyzing the possible biotransformation routes of JY08 can reveal metabolic “weak spots” in compound structure that can be modified through drug design to develop a more successful drug \textit{in vivo}.

\textit{Figure 3. Putative Metabolites of JY08}
4. CONCLUSION

With the rapidly evolving opioid crisis and limited long-term analgesic therapies, there is a major need for the development of opioids that can combat the tolerance and hyperalgesia commonly experienced with long-term use of opioids. JY08, developed as a dual activity ligand at the mu-opioid receptor and neuropeptide FF receptor, has the potential to serve as a safer, more effective treatment for pain.

The pharmacokinetic principles of absorption, distribution, metabolism, and excretion were analyzed to determine the physiochemical properties of JY08 in vitro and in vivo. A simple and sensitive UPLC-MS method was developed for the analysis of these studies. A simple and efficient extraction method was developed for plasma sample preparation that required low plasma volumes, enabling repeated sampling in rodents.

The results of ADME studies determined that JY08 is quickly absorbed in rats. Single dose oral and IV pharmacokinetic studies revealed extensive extravascular distribution. In vitro metabolic studies were inconclusive but can be repeated to determine the primary pathway of metabolism of JY08. In vivo studies showed that JY08 is primarily dependent on hepatic elimination. When JY08 is dosed orally in rats, it exhibits multiple absorption phases and low oral bioavailability, most likely due to first pass effect or poor absorption. Though bioavailability is low, the pharmacokinetic profile of JY08 with a large volume of distribution and long half-life support the potential of JY08 as a drug candidate for the treatment of pain in the clinical setting.


