1-1-2017


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MOLECULAR CHARACTERIZATION OF THE OPIOID RECEPTORS:
DESIGN, DEVELOPMENT, AND PRECLINICAL EVALUATION OF SALVINORIN A-
BASED MOLECULAR PROBES.

A dissertation submitted to the graduate degree program in Biomolecular Sciences and the Graduate Faculty of the University of Mississippi, School of Pharmacy, in partial fulfillment of requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences.

By

ADAM W. KEASLING

MAY 2017
ABSTRACT

The field of salvinorin chemistry represents a novel and emerging field of opioid research. The novelty is derived from the lead pharmacophore: salvinorin A — a neoclerodane diterpenoid natural product isolated from Salvia divinorum. Salvinorin A represents a pharmacologically unique compound in that it is the first known non-nitrogenous KOR subtype-selective agonist, exhibits a comparatively safe physiological profile with no reports of toxicological effects in clinical trials, and, most importantly, has a steadily growing body of literature indicating potentially useful clinical applications (e.g. antinociceptive, anti-addictive, antipruritic, neuroprotective, etc.). This has encouraged the development of analogues as essential molecular probes to elucidate the structure-activity-relationship of the salvinorin-class.

In this study, we expand the current field of salvinorin chemistry through the design, development, and preclinical evaluation of a series of C(22)-fused heteroaromatic salvinorin A analogues. Our in vitro models include: opioid receptor competitive radioligand binding affinity and functional [35S]GTP[γS] binding activity assays; while our in vivo models include: antinociceptive, antidepressant, and anxiolytic related assays. This resulted in three analogues exhibiting EC50 sub-200 nM functional activity, of which two displayed antinociceptive activities, with one also demonstrating antidepressant-like activity. As such, this study further supports the importance of the continued development of new salvinorin A analogues as essential research tools to ascertain potential three-dimensional ligand binding requirements, functional activities, and pharmacological consequences mediated through the clinically important opioid receptors.
DEDICATION

This work is dedicated to those who reminded me what human nature is.
# LIST OF ABBREVIATIONS AND SYMBOLS

$[^{35}\text{S}]\text{GTP}[^{\gamma}\text{S}]$  $[^{35}\text{S}]$Guanosine 5’-(γ-thio)-triphosphate

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADME/T</td>
<td>Absorption, distribution, metabolism, excretion, and toxicity</td>
</tr>
<tr>
<td>AK-1401</td>
<td>2-$O$-salvinorin B 1$H$-indole-2-carboxylate</td>
</tr>
<tr>
<td>AK-1402</td>
<td>2-$O$-salvinorin B benzo[$b$]thiophene-2-carboxylate</td>
</tr>
<tr>
<td>AK-1403</td>
<td>2-$O$-salvinorin B benzofuran-3-carboxylate</td>
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<tr>
<td>AK-1404</td>
<td>2-$O$-salvinorin B 1$H$-indole-3-carboxylate</td>
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<tr>
<td>AK-1405</td>
<td>2-$O$-salvinorin B benzo[$b$]thiophene-3-carboxylate</td>
</tr>
<tr>
<td>AK-1406</td>
<td>2-$O$-salvinorin B 1$H$-indene-2-carboxylate</td>
</tr>
<tr>
<td>AK-1407</td>
<td>2-$O$-salvinorin B 1$H$-indene-3-carboxylate</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>ASP*</td>
<td>4-(4-diethylaminostyryl)-N-methylpyridinium iodide</td>
</tr>
<tr>
<td>BCE</td>
<td>Before Current Era</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB$_1$</td>
<td>Cannabinoid receptor type-1</td>
</tr>
<tr>
<td>CE</td>
<td>Current Era</td>
</tr>
<tr>
<td>CPP</td>
<td>Conditioned place preference</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DCC</td>
<td>1,4-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(dimethylamino)pyridine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DZP</td>
<td>Diazepam</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal regulated kinase-1/2</td>
</tr>
<tr>
<td>FST</td>
<td>Forced swim test</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>GNTI</td>
<td>5’-guanidinonaltrindole</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>hDOR</td>
<td>Human delta (δ) opioid receptor</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>hKOR</td>
<td>Human kappa (κ) opioid receptor</td>
</tr>
<tr>
<td>hMOR</td>
<td>Human mu (μ) opioid receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>HRMS</td>
<td>High-Resolution Mass Spectrometry</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable bowel syndrome</td>
</tr>
<tr>
<td>ICSS</td>
<td>Intracranial self-stimulation</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IMI</td>
<td>Imipramine</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IT</td>
<td>Intrathecal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MM-GBSA</td>
<td>Molecular mechanics with generalized born surface area</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>Sodium sulfate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NET</td>
<td>Norepinephrine transporter</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>nor-BNI</td>
<td>Nor-binaltrohamine</td>
</tr>
<tr>
<td>OFT</td>
<td>Open field test</td>
</tr>
<tr>
<td>P.O.</td>
<td>Per os</td>
</tr>
<tr>
<td>P38 MAPK</td>
<td>P38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>P-3l</td>
<td>2-\emph{O}-salvinorin B benzofuran-2-carboxylate</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure activity relationship</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin transporter</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>$t_R$</td>
<td>Retention time</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>TST</td>
<td>Tail suspension test</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VLC</td>
<td>Vacuum liquid chromatography</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
First and foremost, I must thank Dr. Ikhlas A. Khan for allowing me to join his group and supporting me during my tenure at the University of Mississippi. Additionally, and perhaps most importantly, for his willingness to serve as my major advisor, provide the opportunity to pursue the following vein of research, and support the collaborations necessary for its completion. I would also like to thank my committee members Dr. Samir A. Ross, Dr. Kenneth J. Sufka, and Dr. Jordan K. Zjawiony for providing invaluable mentorship during this time.

I must thank Dr. Stephen J. Cutler as the Director of the Center of Biomedical Research Excellence Center of Research Excellence in Natural Products Neuroscience (COBRE-CORE-NPN) for allowing me to receive training in the In Vitro Pharmacology Core (Core C) and, most importantly, thank Samuel Hans, Janet Lambert, and Sara Pettaway for providing both training and the subsequent in vitro data discussed in this dissertation.

I also would like to thank Dr. James O. Fajemiroye for his interest, and subsequent investment of time and resources, towards the in vivo characterization of selected analogues in the discussed antinociceptive, antidepressant, and anxiolytic related assays. Additionally, I want to thank Dr. Pankaj Pandey and Dr. Robert J. Doerksen for their interest, and subsequent investment of time and resources, in the computational modeling and ADME/T calculations discussed in this dissertation.

Lastly, I must thank Dr. Christopher McCurdy as Chair of the Department of Biomolecular Sciences for nominating me for the Dissertation Fellowship that supported me during my final semester and afforded me the time necessary to compile this dissertation.
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CHAPTER 1: *Salvia divinorum*

In 1939 anthropologist Jean Bassett Johnson reported the use of hallucinogenic mushrooms by the Mazatec in Mexico, as well as, the use of an infusion of leaves from a plant called *hierba María* for similar purposes (Johnson, 1939). However, he was unable to secure a suitable herbarium specimen to allow for botanical taxonomy to be established. It was not until 1962 that Robert Gordon Wasson, while exploring the Sierra Mazateca in Oaxaca Mexico and studying the Mazatec use of hallucinogenic mushrooms, was able to both secure a suitable herbarium specimen, as well as, substantiate the previously reported activity of prepared infusions of the plant material by participating in its ceremonial use:

The effect of the leaves came sooner than would have been the case with the mushrooms, was less sweeping, and lasted a shorter time. There was not the slightest doubt about the effect, but it did not go beyond the initial effect of the mushrooms – dancing colors in elaborate, three-dimensional designs. Whether a larger dose would have produced a greater effect, I do not know. (Wasson, 1962)

Ritual preparation consists of pairing “flawless” leaves together, 50-100, and masticating the leaves for effect. Alternatively, and the method used by Wasson, is to grind the leaves with water, straining and consuming the resulting infusion for effect (Wasson, 1962). The ceremonious use of this plant, described by both Wasson and earlier investigators such as Blas Pablo Reko, indicate that the purpose is divinatory in nature (Reko, 1945 and Wasson,
A curandero would initiate the ritual to divine the cause of disease in participating patients. It is the divinatory use of this plant and, especially, the use of infusions prepared from its leaves that led Wasson to propose that *Salvia divinorum* may represent the ancient Aztec plant *pipiltzintzintli* (Wasson, 1963). Beyond the use of this plant for divinatory purposes it is also used medicinally by the Mazatecs to treat diarrhea, headaches, rheumatism and a Mazatec illness known as *panzón de barrego* [sic] (swollen belly). Additionally, it is used in patients that are near death to revive and alleviate them of their illness (Johnson, 1939 and Valdés III *et al.*, 1983).

The herbarium specimen obtained by Wasson was provided to Carl Epling and Carlos D. Játiva-M. who identified it as an undescribed species in the Lamiaceae family designating the new species as *Salvia divinorum* (Epling and Játiva-M., 1962) (Figure 1, line drawing; Figures 2 and 3, photographs of cultivated specimen; Figure 4, photograph of specimen in natural habitat). Botanically this species is characterized as a perennial herb averaging approximately a meter in height. Mature leaves are approximate 14 cm long (Figure 5A), ovate, acuminate, basally rounded, crenate-serrate with hairs in sinuses along the margins, glabrate but hirtellous along the lower
veins that attenuate into a petiole 2-3 cm long. The inflorescence is characterized by being bluish, slightly pubescent, in full panicles on branches approximately 35 cm long, with a violet calyx tube, approximately 15 mm long with superior lip 1.5 mm long and three impressed veins, lip 6 mm tall, inferior lip shorter and incurved.
Figure 2. Photograph of cultivated *Salvia divinorum* plant illustrating general growth habit.
Figure 3. Photograph of cultivated *Salvia divinorum* plant illustrating characteristic square stem and oppositely decussate leafing pattern.
Figure 4. Photograph of *Salvia divinorum* plant in natural habitat that illustrates the toppling growth pattern observed in this species.

[Creative Commons image: CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=157218]
The corolla tube is white, sigmoid, 22 mm long, with superior lip 6 mm tall, inferior lip shorter and incurved. The stamens are inserted near the mouth of the tube: style hirtellous, with posterior branch long, obtuse, flat, anterior carinate branch (Epling and Játiva-M., 1962; Schultes and Hofmann, 1973; Valdés III et al., 1987). Additionally, it was widely thought that this plant may represent a cultivar as it was essentially unknown outside of curandero cultivated patches isolated in the Sierra Mazateca highlands (Ott, 1993). However, this appears to be due to a characteristically low seed-set and limited seed viability observed in this irregularly flowering plant as seed-set was first demonstrated in 1987 (Valdés III et al., 1987). Laboratory cultivation experiments revealed low pollen viability (approximately 56% failure) from pollinated stigmas resulting in low seed-set (Jenks et al., 2011 and Reisfield, 1993).

![Figure 5. [A] Photograph of Salvia divinorum leaf, fresh. [B] Photograph of lyophilized Salvia divinorum leaf material coarsely ground prior to extraction.](image)
Salvia divinorum, while reported at several locations ranging from 500 to 1500 meters in altitude, has a limited geographical distribution within the highlands of the Sierra Mazateca in Oaxaca, Mexico (Figure 6) (Casselman et al., 2014 and Reisfield, 1993). This range is typified by tropical montane cloud-forests (i.e. persistent cloud cover resulting in high humidity). As an herbaceous plant Salvia divinorum represents an understory member whose populations are generally in partial to full shade conditions in nutrient-rich, moist soils often found near water courses. Either naturally, by toppling over (Figure 4), or aided by curanderos Salvia divinorum is propagated primarily vegetatively through clonal growth.

The first live collection of Salvia divinorum that was successfully propagated outside of Oaxaca was deposited by Sterling Bunnell at the University of California, Los Angeles Botanical Garden in 1963 and consisted of a single specimen (Siebert, 2003). Due to the notable low seed-set rate, usual method of clonal propagation, and limited number of reported live collections it is
suggested that this plant is likely the progenitor of the vast majority of propagated *Salvia divinorum* currently available outside of Oaxaca, Mexico. Intraspecific genetic studies of five populations obtained from within Sierra Mazateca revealed a limited genetic diversity, as well as, placed the species outside of the Salvia “core” subgenus Calosphace with *Salvia venulosa* being supported as its closest related species by both ITS and cpDNA phylogenetic assessment (Jenks *et al.*, 2011). Additionally, the authors indicated that the species appear reproductively capable but that anthropogenically-mediated clonal propagation may negatively affect this capacity. However, they concluded that further studies are needed into the sexual reproductive capacity and genetic diversity of the species.

Phytochemical investigation into the active constituents of *Salvia divinorum* began with Albert Hofmann in 1964, due in part to his close association with Wasson, having accompanied the 1962 expedition (Wasson, 1962). However, more important was Hofmann’s unparalleled expertise with hallucinogenic compounds (Figure 7): having discovered lysergic acid diethylamide (LSD; initial synthesis in 1938 and re-examination in 1943), and more relevantly, his experience working with other Mazatecan hallucinogenic species from Mexico, including the identification of psilocybin and psilocin as active constituents from the hallucinogenic mushroom *Psilocybe mexicana* (subsequently, isolated from numerous *Psilocybe* species; Nahuatl: *teonanácatl*) and the identification of lysergic acidamide as the active constituent from *Turbina corymbosa* seeds (Nahuatl: *ololiuhqui*) (Hofmann, 1964).
This pattern of alkaloids consistently being the perceptiotropic constituent suggested that a similar pattern may be observed with *Salvia divinorum*. Starting with an infusion prepared in Mexico and preserved with alcohol Hofmann attempted, and failed, to isolate the causative alkaloid upon return to his laboratory in Basel, Switzerland. When he reassessed the alcohol preserved infusion via experimenter self-administration he discovered that the material was inactive — concluding that the perceptiotropic principle was unstable in solution and that “the problem of the magic plant ska Maria Pastora still awaits solution” (Hofmann, 1979).
CHAPTER 2: Salvinorin A

Hofmann’s Solution

The solution to Hofmann’s Salvia divinorum problem continued to remain elusive until 1984 when Leander J. Valdes, III and collaborators utilized an in vivo bioassay-guided approach toward fractioning lyophilized leaf material (Figure 5B) (Valdes, III, et al. 1984). Employing a modified open field test (OFT), column fractions of the extracted leaf material were assessed at each stage of silica gel chromatographic purification for altered activity in mice. This process led to the isolation of a neo-clerodane diterpenoid which they designated as divinorin A. During the publication process they learned that the compound, divinorin A, had been previously reported by Alfredo Ortega in 1982 as salvinorin A (Ortega et al., 1982). The Ortega group had utilized a blind-fractionation process to isolate salvinorin A (Figure 8) and had not evaluated the compound pharmacologically.

However, it wasn’t until 1994 that the first pharmacological evaluation of salvinorin A in humans was reported (Siebert, 1994).
In this study, Daniel J. Siebert, with six volunteers, assessed both traditional *Salvia divinorum* leaf infusions (gastrointestinal absorption versus buccal absorption) and pure salvinorin A (buccal absorption versus vaporized inhalation absorption). This study was important as it was the first to verify the perceptiotropic effects of salvinorin A in humans. Additionally, it served to establish the initial pharmacology of the compound in humans. Siebert discovered that salvinorin A is an extremely potent perceptiotropic substance, which is active at 200-500 μg when vaporized and inhaled (they tested doses up to 2.6 mg with no adverse effects observed).

Pharmacologically they established that vaporization/inhalation effects are markedly different from the more traditional route of administration. The traditional route (i.e. buccal absorption) results in perceptiotropic effects that are as described in the earlier literature: *mild* hallucinogenic activity that takes approximately ten minutes before initial effects are felt, that plateau for about one hour before gradually subsiding over the next hour. This contrasts sharply when administered via vaporization and inhalation: the full effects are best described as *dissociatively* hallucinogenic and is experienced in approximately thirty seconds and last for five-to-ten minutes. In an attempt to establish the pharmacological target of salvinorin A Siebert collaborated with David Nichols to screen at a series of likely neuroreceptors, such as the dopamine and serotonergic receptors. However, they found no significant activity at any of the screened receptors (Siebert, 1994).

It wasn’t until 2002 when Siebert collaborated with Bryan L. Roth of the National Institute of Mental Health Psychoactive Drug Screening Program (NIMH-PDSP) that a pharmacological target was elucidated. Employing a competitive radioligand binding assay against fifty cloned human G-protein-coupled receptors (GPCRs) they discovered, surprisingly,
that salvinorin A not only bound to the opioid receptors, but was subtype selective, exhibiting potent selectivity for the kappa opioid receptor (KOR) subtype (Figure 9). This was followed with functional assessment utilizing a $[^{35}\text{S}]\text{GTP}[^{\gamma}\text{S}]$ assay at KOR that revealed salvinorin A was a potent agonist with an EC$_{50}$ of approximately 1 nM. This represents the first report of a non-nitrogenous subtype-selective opioid receptor agonist (Roth et al., 2002). It also represents the solution to “the problem of the magic plant ska María Pastora” posed by Hofmann that was forty years in the making and the beginnings of the field of salvinorin chemistry.

**Figure 9.** Screening of Salvinorin A in competitive radioligand binding assays against fifty cloned human GPCRs at 10μM, demonstrating selectivity at hKOR. LSD is shown as comparison. Roth et al. 2002. Reprinted with permission.
The Opioid System — A Clinically Important Target

The opioid system is crucially involved in the modulation of antinociception and associated behaviors. Chronic pain affects an estimated 100 million Americans, has associated costs exceeding $600 billion per year in lost productivity and medical expenses, and the currently available drug therapies consist primarily of classical opioid analgesics such as morphine and closely related analogues (Prisinzano, 2013). Compounding the issue, admissions for treatment of prescription opioid abuse has increased by an astounding 400% over the past 15 years and the death rate (estimated by the CDC at 90 Americans per day in 2015) from opioid drug use exceeds all other drug classes (Ling et al., 2011; Nielsen and Bruno, 2011; Rudd et al., 2016). Beyond legal production it is estimated that annual production of illicit opioids exceeds $800 billion (Rinner and Hudlicky, 2012). In spite of these problems, opioids continue to be the most prescribed, and abused, class of drugs (Negri et al., 2013).

Extending beyond antinociception, numerous studies have indicated involvement of this system in a diverse array of critical behavioral states, including: addiction, anxiety, and depression — further reinforcing the clinical importance of this system (Al-Hasani and Bruchas, 2011; Lalanne et al., 2014; Ling et al., 2011). The opioid system is composed of four G-protein-coupled receptors (GPCRs) that belong to the Rhodopsin-like, Class A, subfamily that include opioid receptors (OR): delta (DOR), kappa (KOR), mu (MOR), and nociception (NOR) — with an increasing number pharmacologically defined subtypes for each resulting from alternative splicing, post-translational modification, and/or receptor oligomerization (Al-Hasani and Bruchas, 2011; Stein, 2016; Urbano et al., 2014; Ying-Xian et al., 2003).

The opioid receptors are widely expressed throughout both the peripheral and central nervous systems, as well as throughout diverse systems such as: cardiovascular, gastrointestinal,
and reproductive (Feng et al., 2012). Physiologically, DOR, KOR, and MOR are each modulated by several endogenous neuropeptides that are subtype cognate (Table 1). For example, DOR is activated by enkephalins (Figure 10), KOR by dynorphins (Figure 11) and MOR by endorphins (Figure 12) — however, this is a preferential affinity as there is some degree of non-selectivity exhibited by these representative endogenous ligands across the

<table>
<thead>
<tr>
<th>Primary Target</th>
<th>Sequence (Three-Letter-Code)</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOR</td>
<td>Tyr-Gly-Gly-Phe-Met</td>
<td>[Met⁵]-encephalin</td>
</tr>
<tr>
<td>DOR</td>
<td>Tyr-Gly-Gly-Phe-Leu</td>
<td>[Leu⁵]-encephalin</td>
</tr>
<tr>
<td>KOR</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys</td>
<td>dynorphin A</td>
</tr>
<tr>
<td>KOR</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Gln-Phe-Lys-Val-Val-Thr</td>
<td>dynorphin B</td>
</tr>
<tr>
<td>KOR</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Gln-Phe-Lys-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Leu-Phe-Asp-Val</td>
<td>leumorphin</td>
</tr>
<tr>
<td>MOR</td>
<td>Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr</td>
<td>α-endorphin</td>
</tr>
<tr>
<td>MOR</td>
<td>Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu</td>
<td>β-endorphin</td>
</tr>
<tr>
<td>MOR</td>
<td>Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu</td>
<td>γ-endorphin</td>
</tr>
<tr>
<td>MOR</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys</td>
<td>α-neo-endorphin</td>
</tr>
<tr>
<td>MOR</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro</td>
<td>β-neo-endorphin</td>
</tr>
</tbody>
</table>

Compiled from the following references: Hazum et al., 1979; Janecka et al., 2004; Kangawa et al., 1981; Minamino et al., 1981; Nakao et al., 1983; Suda et al., 1984; Toll et al., 2016.
subtypes (Albert-Vartanian et al., 2014; Phan et al., 2012). The nociceptin opioid receptor (NOR) represents an atypical opioid receptor in that it possesses limited pharmacological response to classical opioids, despite assignment based on amino acid sequence homology, its cognate endogenous peptide has been identified as a unique heptadecapeptide ligand designated as nociceptin (Meunier et al., 1995).

Complications in clinically targeting opioid receptor subtypes, and their variants, arises from the high degree of amino acid residue homology observed between DOR, KOR, MOR, and NOR — with greater than 70% homology observed in the II, III, VII transmembrane (TM) helices (TM2, TM3, TM7), 50% homology between I, V, VI (TM1, TM5, TM6), and 24% homology within IV (TM4) (Toll et al., 2016). Despite this high degree of homology, the receptor subtypes exhibit a range of pharmacological distinctiveness including desirable properties such as analgesia and anxiolytic effects or undesirable properties such as anhedonia or impaired coordination.

![Figure 10](image)

**Figure 10.** Structures of representative encephalin-class endogenous opioid peptides.
Figure 11. Structures of representative dynorphin-class endogenous opioid peptides.
Figure 12. Structure of representative endorphin-class endogenous opioid peptide.
Morphine Chemistry — Origins of Classical Opioids

It is the pursuit of these desirable drug-induced properties that has fostered research into small molecule ligands targeting the opioid receptors and to a degree that far outdistances that of current peptide research. This is, in large part, due to the rather lengthy history of ethnomedicinal use of the plant *Papaver somniferum* — otherwise known as opium poppy. Some of the earliest archaeological evidence indicating human cultivation of *Papaver somniferum* dates back to approximately 5700 BCE from the site of an early European Neolithic settlement discovered submerged under Lake Bracciano, Italy (Merlin 2003). Evidence of the use of this plant continues during the Bronze Age (circa 1550-1100 BCE) with the discovery of numerous poppy capsule artifacts (Figure 13) present throughout mainland Greece indicating a

![Figure 13. Greek poppy capsule artifact. Merlin, 2003. Reprinted with permission.](image)
likely significance beyond mere food substance and a connection with ritual and/or medicinal use of the plant. This was later substantiated in the writings of Hippocrates of Kos during the Classical Greece period (circa 500-400 BCE) (Kritikos and Papadaki, 1967). However, the first written record of the medicinal use of *Papaver somniferum* was much earlier, being found in the Ebers Papyrus from Egypt, written circa 1550 BCE, that indicated the use of this plant for sedative effects (Atanasov *et al.*, 2015).

Eventually this usage extended into analgesic effects by the Archaic Greece period (circa 800-500 BCE) preceding the Classical Greece period and possibly noted in The Odyssey:

…”she cast a drug into the wine of which they drank to lull all pain and anger and bring forgetfulness of every sorrow. (Homer, early 8th Century BCE)

The harvesting of the milky-white latex (Figure 14) (Greek *opoulos*; opium) that is collected by lancing unripe seed capsules of *Papaver somniferum* which upon drying results in a viscous, dark brown-black resinous material that is raw opium (Figure 15) (the Greek *μηκώνιον*; mekonion). However, it is likely that analgesic use extends even further in antiquity, as archeological evidence indicates that as early as 3000 BCE the Sumerians referred to the opium poppy as “hul gil” — the
plant of joy (Brownstein, 1993).

Additionally, during this time (circa 1300-1000 BCE) trade had seen the spread of *Papaver somniferum*, as both a food and medicinal substance, from the Asia Minor and Mediterranean regions throughout India, China, and Europe — essentially through all parts of the then known world. During this lengthy period, numerous routes of opium administration had been created; the two most widely used were dissolving the opium in solution such as wine (see The Odyssey reference above by Homer) or other alcohol (e.g. laudanum) or vaporization of the opium and inhalation of the opium vapors.

*Figure 15. Dried opium. [Public Domain image from the DEA, 2017]*
It wasn’t until the early 1800’s (CE) that advancements in the medicinal use of opium occurred. In 1806 Friedrich Wilhelm Sertürner established the initial pharmacochemical properties of a crystalline substance isolated from opium that he named *Morphium*, after the Greek god of Dreams (this compound was later referred to as Morphine). The sleep inducing and analgesic activity was established first in canine test animals and subsequently verified by experimenter self-administration (Atanasov *et al.*, 2015; Brownstein, 1993). This compound represents the first isolated alkaloid and established the field of alkaloid-chemistry and, more specifically, the field of morphine-chemistry. The structure of morphine remained elusive until 1952 when sufficient technological advancements finally allowed for the verification via total synthesis by Marshall D. Gates (Gates, 1952) of the structure (Figure 16) proposed in 1925 by Sir Robert Robinson (Gulland and Robinson, 1925) [For an excellent review summarizing the historical developments of the total-synthesis of morphine since 1952 see: Rinner and Hudlicky, 2012.]. This was the culmination of a 146-year endeavor that resulted in a two column publication that concluded: “With this, the first synthesis of morphine is complete.”

**Figure 16.** Structure of morphine.
However, the utility of morphine in relieving pain was not without undesirable side-effects, perhaps most notable has been that of addiction. The addictive use of opium has been reported since antiquity in some of the earliest documents relating to the opium trade throughout Asia, India, and Asia Minor (Brownstein, 1993). Even prior to the first total synthesis of morphine in 1952, efforts had been directed at semi-synthetic structural modification to limit or avoid addiction potential. The first reported success was in 1874 with the synthesis of an acetylated form of morphine (Wright, 1874). However, it wasn’t until 1893 that Felix Hofmann re-synthesized this same analogue for the Bayer Company, who decided upon a heroic name for the compound: Heroin (from the German heroisch). Bayer marketed Heroin (Figure 17) as lacking the narcotism (i.e. addictive properties) seen with morphine use — unfortunately this ultimately was found to be inaccurate (Hofmann, 2017).

Figure 17. Bayer Heroin bottle. [Public Domain image from Mpv_51, 2017]
Salvinorin A — A Novel Opioid

Pharmacological advancements within the field of morphine chemistry has yielded thousands of analogues; however the ability to clearly separate desirable drug-like properties such as antinociceptive activity from undesirable side-effects continues to remain elusive (Kieffer, 2016). In this pursuit, overwhelmingly, MOR has been the primary opioid receptor targeted for scientific study due to the effective antinociceptive activity of classical opioid agonists that primarily mediate through this receptor subtype. However, there are numerous adverse side-effects, beyond undesirable respiratory effects, MOR activation is complexed with the dopaminergic reward pathway promoting euphoria, ultimately resulting in both physiological and psychological dependence (Albert-Vartanian et al., 2016; Shook et al. 1990). Nevertheless, MOR agonists continue to be the primary pharmacologic antinociceptive class of drugs clinically used to treat pain. This has led to MOR agonists based on morphine-chemistry becoming the most misused class of pharmaceutical agents in the world, which has stimulated interest in targeting alternative opioid subtypes that are devoid of these physiologically adverse effects (Negri et al., 2013).

The kappa opioid receptor (KOR) subtype, which is the target of salvinorin A, is widely expressed throughout both central nervous tissues (brain and spinal cord), as well as, the peripheral tissues, such as the gastrointestinal tract. Within the brain KOR is highly expressed in the amygdala, hippocampus, hypothalamus, and striatum — regions associated with behavioral functions including cognitive processing, emotional function, pain and stress response (Urbano et al., 2014; Mailet et al.,2015). Furthermore, activation of KOR is generally not associated with the physiologically adverse effects observed in MOR activation (i.e. physical dependence or respiratory failure). This has generated interest in KOR becoming a potential clinical target for
pain management, as well as, for the treatment of psychiatric conditions including anxiety and depression. However, historically, KOR has been pharmacologically avoided due to associated undesirable side-effects including anhedonia, dysphoria, and perceptiotropic effects (e.g. hallucinations) (Lalanne et al., 2014; Maillet et al., 2015). Advancing within this paradigm there has been significant work to elucidate the mechanism of salvinorin A activity with relation to KOR due to the novel aspect of it being the first known non-nitrogenous opioid receptor agonist (Roth et al., 2002).

Early computational studies were restricted to X-ray crystallographic data limited in scope to “in-active” crystal structures derived from antagonist bound receptors (Roth et al., 2002). The most common crystal structure utilized for this computational work has been human KOR complexed with JDTic (PDB ID: 4DJH) (Polepally et al., 2014; Wu et al., 2012). To address this limitation, site-directed mutagenic studies were undertaken to establish potential key residues to aid in defining the salvinorin A binding pocket (Kane et al., 2006; Van et al., 2013; Yan et al., 2005). Separately, these studies corroborated the importance of residues Y320 and Tyr-139 of TM3 and residues Tyr-119, Tyr-312, Tyr-313, and Tyr-320 of TM7 as essential for the binding of salvinorin A in KOR — it has been proposed that this aromatic cluster may form a key binding pocket (Kane et al., 2008; Kane et al., 2006; Yan et al., 2005). Additionally, non-aromatic residues Gln-115 of TM2 and Ile-316 of TM3 were also shown to be significant in salvinorin A binding. In particular, loss of Ile-316 resulted in abolishment of salvinorin A binding at KOR (Kane et al., 2008; Van et al., 2013; Yan et al., 2005). These residues were contrast with the

![Figure 18. Structure of U-69,593.](image)
prototypical U-69,593 (Figure 18) KOR subtype selective agonist and the endogenous peptide dynorphin A, both of which displayed different requirements at these residues (Kane et al., 2006; Yan et al., 2005).

Together these studies were used to establish putative key binding regions of salvinorin A within KOR which suggested that the tricyclic core may vertically span (Figure 19) the receptor between TM2 and TM7 (Kane et al., 2006; Yan et al., 2005). Perhaps most interestingly, these residues differ significantly from the classical opioid ligand binding pocket centrally located in the receptor, spanning TM3 and TM6, characterized by conserved Asp-138 and Asp147 residue interactions — potentially indicating an unique binding mode for salvinorin A within the opioid

![Figure 19. Putative binding orientation of salvinorin A structure within KOR (Kane et al., 2006).](image)
receptor (Kane et al., 2008; Manglik et al., 2012; Surratt et al., 1994; Yan et al., 2005; Yuan et al., 2015).

Expanding beyond a structural understanding of salvinorin A significant work has been undertaken to build upon the initial pharmacological results reported by Bryan L. Roth in 2002 that established salvinorin A as the first known non-nitrogenous opioid receptor agonist that was subtype selective for KOR (Roth et al., 2002). Their study used a competitive radioligand binding affinity assay that employed $[^3]$H-bremazocine and assessed salvinorin A at KOR reporting a $K_i$ of 4 nM. This was then followed by a functional assay that utilized $[^35]$S-Guanosine 5’-(γ-thio)-triphosphate ($[^35]$S-GTP[γS]) as a measurable radioligand to assess the activation of the receptor through binding of guanosine triphosphate (GTP) for which they reported an $EC_{50}$ of 1 nM for salvinorin A. This activity was subsequently corroborated in a 2004 study that reported similar values for salvinorin A at KOR with a binding affinity $K_i$ of 18 nM and functional assessment of $[^35]$S-GTP[γS] binding activity reported at an $EC_{50}$ of 0.63 nM (Chavkin et al., 2004).

Toxicological assessment of salvinorin A (0.4 - 6.4 mg/kg, IP — a dose range significantly greater than used by humans) for acute physiological and chronic histological effects in mice established that there were no significant alterations in at any endpoint (physiological measures: pulse pressure, galvanic skin response, cardiac conduction, body temperature; histological measures: spleen, liver, kidney, brain, bone marrow, and blood). Researchers concluded that the data suggested that potential toxicity from salvinorin A may be quite low (Mowry et al., 2003). Furthermore, this lack of acute toxicity has been substantiated in a number of subsequent clinical trials (table 2). The lack of overt toxicity has been largely consistent in the literature, with one exception. In 2016 cytotoxicity of salvinorin A was
reported for the first time from an in vitro MTT-based cell viability assay utilizing N27, A549, Caco-2, HepG2, COS-7, and HEK-293 cell lines that was both dose-dependent (0.1, 1, 10, and 50 μM) and time-dependent (12-60 hours) across all cell lines (Marinho et al., 2016).

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>Dose range</th>
<th>Number of participants</th>
<th>Physiological Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaporization/ inhalation</td>
<td>200-500 μg 6</td>
<td>No negative effects reported.</td>
<td>Siebert 1994</td>
<td></td>
</tr>
<tr>
<td>Vaporization/ inhalation</td>
<td>0.375-21μg/kg 4</td>
<td>No significant change to heart rate or blood pressure.</td>
<td>Johnson et al., 2011</td>
<td></td>
</tr>
<tr>
<td>Sublingual</td>
<td>1-4 mg 8</td>
<td>No effects, poor bioavailability by sublingual administration.</td>
<td>Mendelson et al., 2011</td>
<td></td>
</tr>
<tr>
<td>Vaporization/ inhalation</td>
<td>100-1,017 μg/kg 30</td>
<td>No significant change to body temperature or systolic blood pressure.</td>
<td>Addy et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Vaporization/ inhalation</td>
<td>0-12 mg 10</td>
<td>No significant change to blood pressure, heart rate, or euphoria.</td>
<td>Ranganathan et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Vaporization/ inhalation</td>
<td>0.375-21μg/kg 8</td>
<td>No significant change to blood pressure or heart rate. No resting or kinetic tremors were observed.</td>
<td>MacLean et al., 2013</td>
<td></td>
</tr>
</tbody>
</table>

Due to the high affinity and efficacy of salvinorin A at KOR a discriminative study was undertaken in rhesus monkeys (Macaca mulatta) (Butelman et al., 2004). This study revealed that subcutaneous administration of salvinorin A (0.001-0.032 mg/kg, SC) produced drug discriminative effects in rhesus monkeys similar to the high-efficacy KOR agonist U-69,593. It also demonstrated that KOR antagonist 5’-guanidinonaltrindole (GNTI) failed to antagonize the
salvinorin A effect — possibly indicating that salvinorin A may possess a unique binding pose within KOR distinct from that of classical opioids. Further *in vivo* pharmacological assessment of salvinorin A (2 mg/kg, IV) in a mouse climbing model of motor coordination reported a significant, but short-lived (≤ 15 minutes, with peak effects within five minutes), incoordination similar to the U-69,593 KOR agonist control (Fantegrossi *et al.*, 2005). This study observed a reduction in motor-incoordination when salvinorin A groups were pretreated with nor-binaltrophenamine (nor-BNI; KOR antagonist). More interestingly however, this reduction was not observed in the U-69,593 control group — once more bolstering the proposal that the binding properties of salvinorin A within KOR may be unique.

The assessed *in vivo* pharmacological activities that are modulated by salvinorin A continues to increase, this includes: antiproliferative, antipruritic, anti-inflammatory, anti-protozoal, gastrointestinal hypermotility, and neuroprotective effects in brain ischemia-models — amongst others (Aviello *et al.*, 2011; Calzada *et al.*, 2015; Lozama *et al.*, 2011; Vasiljevik *et al.*, 2014; Wang *et al.*, 2005; Xin *et al.*, 2016). However, our focus will be towards modulatory effects of salvinorin A in models of antinociception, conditioned place aversion, and depression.

Pharmacological characterization of salvinorin A has demonstrated KOR-mediated antinociceptive activity in mice. The first published report was in 2006 utilizing both chemo-nociceptive and thermo-nociceptive assays (McCury *et al.*, 2006). This study used two thermo-nociceptive models: tail-flick and hotplate, both revealing dose- and time-dependent antinociceptive effects. Specifically, that salvinorin A possesses a short duration of activity, peaking at ten minutes with a rapid return to baseline. This pattern of activity was also observed in their chemo-nociceptive assay. Subsequent studies corroborated this pattern of
antinociceptive activity in mice, including the short duration of activity (Ansonoff et al., 2006; John et al., 2006).

Based upon previously establish effects of KOR agonists (e.g. U-69,593) that promote aversive effects in place preference models, decrease drug reward effects through altered dopamine levels and effect decreased cocaine self-administration, corresponding pharmacological characterization was undertaken with salvinorin A as an atypical opioid (Chiara and Imperato, 1988; Devine et al., 1993; Donzanti et al., 1992; Heidbreder et al., 1993). A 2005 study by the Zhang group used high doses of salvinorin A (1-3 mg/kg, IP) and established that this treatment lowered dorsal striatum dopamine levels, measured in vivo in mice (C57BL/6J mice) via microdialysis over a three hour duration post-treatment, in the caudate nucleus and putamen. Moreover, this salvinorin A mediated reduction of striatal dopamine was completely blocked by pretreatment with KOR antagonist nor-BNI. They also reported that at this dose range salvinorin A induced both conditioned place aversion and decreased locomotor activity in the test mice. They concluded that that the inhibitory effect on striatal dopamine levels by salvinorin A administration may contribute to the observed conditioned place aversion and lowered locomotor rates (Zhang et al., 2005). This conditioned place aversion was subsequently demonstrated in a rat model using male Sprague-Dawley rats in 2014, notably at a lower dose range (0.3-1 mg/kg, IP) than the previously used mouse model (Sufka et al., 2014).

However, a 2007 conditioned place preference study by Daniela Braida in zebrafish (Danio rerio) reported that at a significantly lower dose range of 0.2-0.5 μg/kg of salvinorin A promoted place preference in the associated drug-compartment. The highest dose assessed (80 μg/kg) in this study produced aversive effects in zebrafish (Braida et al., 2007). In 2008 the Braida group translated this conditioned place preference study to male Wistar rats and reported
a rewarding effect by salvinorin A at 0.1-40 μg/kg (SC) doses and an aversive effect at 160 μg/kg (SC) (Braida et al., 2008). They also utilized a non-competitive self-administration paradigm and observed rewarding effects at doses 0.1-0.5 μg/intracerebroventricular (ICV) infusion, but aversive effects at 1μg/infusion. Additionally, they were able to antagonize the reward effect of salvinorin A, as expected, by pretreatment with KOR antagonist nor-BNI, but more interestingly with cannabinoid receptor type-1 (CB₁) antagonist rimonabant. However, subsequent work in 2010 by the Walenty group utilizing radioligand binding affinity, [³⁵S]GTP[γS] functional assessment, and calcium channel signaling revealed that this apparent cannabinoid mediated activity by salvinorin A was not by direct activation — which corresponds to earlier studies which reported no direct affinity/activation of cannabinoid receptors by salvinorin A (Chavkin et al. 2004; Roth et al., 2002; Walenty et al., 2010).

In 2001 it was observed that the overlapping distribution of KOR expression with dopamine transporters (DAT) in striatal axons was strategically located to potentially modulate dopamine uptake (Svingos et al., 2001). This study presented the first ultrastructural data showing KOR localization and potential involvement in presynaptic regulation of dopamine. Further work supporting the involvement of KOR dopamine modulation in conditioned place aversion utilized conditional knock-out mice (DAT Cre-KOR KO) and determined that KOR expression on dopaminergic neurons was essential for KOR-mediated conditioned place aversion behavior (Chefer et al., 2013). Based upon the previously observed effect of decreasing dopamine-levels and promotion of conditioned place aversion by salvinorin A it was proposed that the mechanism of this activity may be through DAT/KOR modulation (Kivell et al., 2014).

Utilizing transfected EM4 cells the Kivell group in 2014 observed that salvinorin A only promoted DAT activation in DAT/KOR co-expressing cells via measured change of 4-(4-
diethylaminostyryl)-N-methylpyridinium iodide (ASP+) substrate accumulation in the test cells, this accumulation was not observed in cells that only expressed DAT. Additionally, this altered DAT function in DOR/KOR co-expressing cells was reversible with nor-BNI treatment. They measured that this effect was differential between amine transporters as they observed a decreased rate of ASP+ substrate accumulation with the serotonin transporter (SERT) and no alteration with the norepinephrine transporter (NET).

Employing a surface biotinylation assay they reported that salvinorin A up-regulated DAT surface expression in DAT/KOR co-expressing EM4 cells and that this effect was linked to the extracellular signal regulated kinase-1/2 (ERK1/2) pathway as inhibition with PD98059 (an ERK1/2 inhibitor) blocked this up-regulated DAT surface expression. Immunoblotting revealed that salvinorin A promoted increased phosphorylation of ERK1/2, but not P38 mitogen-activated protein kinase (P38 MAPK) signaling in their DAT/KOR co-expressing EM4 cells. Inhibition of ERK1/2 by PD98059 prevented salvinorin A modulation of DAT ASP+ accumulation, while inhibition of P38 MAPK by SB203580 (a P38 MAPK inhibitor) failed to alter salvinorin A DAT effects which may indicate an ERK1/2 dependent mechanism of action for the observed dopamine modulation by salvinorin A (Kivell et al., 2014).

With the established trend of KOR agonist induced dysphoria and the dopamine modulation effects of salvinorin A it was posited that this may represent an approach towards altering addictive responses observed with drugs of abuse, such as cocaine (Chartoff et al., 2008). An initial study observed that in male Sprague-Dawley rats acute treatment with salvinorin A (2 mg/kg, IP) resulted in attenuation of locomotor stimulant effects from cocaine. Salvinorin A (0.3 mg/kg, IP) attenuation of locomotor stimulant effects from cocaine in Sprague-Dawley rats was also observed by the Morani group in 2012, but they further reported that
salvinorin A treatment produced fewer adverse effects (such as sedation, aversion, and depression) as commonly seen with other KOR agonist (e.g. U-69,593) (Morani et al., 2012). Following this vein, use of a male Sprague-Dawley rat self-administration model found that salvinorin A treatments (0.3-1 mg/kg, IP) attenuated cocaine self-administration, and had no effect on operant response for sucrose reinforcement (Morani et al., 2009). This work was subsequently translated to rhesus monkeys in 2014, where not only did salvinorin A attenuate cocaine self-administration, but MOR agonist remifentanil self-administration as well, in a dose-dependent manner (Freeman et al., 2014).

Lastly, for our specific pharmacological interest in salvinorin A there is the potential modulatory role in depression. Numerous studies have indicated involvement of the opioid system, and KOR in particular, in a diverse array of critical behavioral states, including: addiction, anxiety, and depression (Al-Hasani and Bruchas, 2011; Lalanne et al., 2014; Ling et al., 2011). Furthermore, there is an accumulated body of evidence demonstrating that KOR activation can produce depression-like effects both in model organisms and, more importantly, in humans (Carlezon et al., 2006; Ebner, et al., 2010; Lalanne et al., 2014; Pfeiffer et al., 1986). In 2006 it was reported that in forced swim tests (FST) and intracranial self-stimulation (ICSS) tests, both standard depression-like models, in male Sprague-Dawley rats that administration of salvinorin A (0.5-2.0 mg/kg, IP) would promote depression-like effects (Carlezon et al., 2006). This study also observed that salvinorin A decreased striatal dopamine levels, but not serotonin levels, within the nucleus accumbens — which is thought to play a critical role in reward response related to addiction, as well as, depression and anxiety (Bewernick et al., 2010; Lalanne et al., 2014; Olsen 2011). These results were corroborated in 2010 by the Ebner group also utilizing the ICSS model in male Sprague-Dawley rats and administering salvinorin A at two
doses 0.25 mg/kg and 2.0 mg/kg (IP), however they found only the high dose of 2.0 mg/kg promoted the depression-like effects (Ebner, et al., 2010). They also reported a corresponding decrease in striatal dopamine levels within the nucleus accumbens.

However, a 2009 study observed that salvinorin A (01-160.0 μg/kg, SC) produced anxiolytic-like effects in male Sprague-Dawley rats when assessed using an elevated plus maze, anti-depressant-like effects in male Sprague-Dawley rats in a FST, and anti-depressant-like effects in male Albino Swiss mice assessed utilizing a tail suspension test (TST) — effects which were antagonized by nor-BNI treatment (Braida et al., 2009). Ultimately, these studies (Table 3) illustrate the pharmacological complexity observed in the study of salvinorin A: not simply in various models/model organisms, but also with the range of observed effects that dose and route of administration potentiate. Together this research demonstrates that additional pharmacological characterization of salvinorin A and elucidation of mechanisms of activity are needed.

<table>
<thead>
<tr>
<th>Model Organism</th>
<th>Test Model</th>
<th>Activity</th>
<th>Dose</th>
<th>Route of Administration</th>
<th>Time-Course</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J mice</td>
<td>CPP</td>
<td>Aversive</td>
<td>1.0-3.2 mg/kg</td>
<td>IP</td>
<td>30 min</td>
<td>Zhang et al., 2005</td>
</tr>
<tr>
<td>Male Swiss mice</td>
<td>Tail-flick test</td>
<td>Antinociceptive</td>
<td>1.0-4.0 mg/kg</td>
<td>IP</td>
<td>20 min</td>
<td>McCurdy et al., 2006</td>
</tr>
<tr>
<td>Male Swiss mice</td>
<td>Hotplate</td>
<td>Antinociceptive</td>
<td>1.0 mg/kg</td>
<td>IP</td>
<td>10 min</td>
<td>McCurdy et al., 2006</td>
</tr>
<tr>
<td>Male Swiss mice</td>
<td>Acetic acid</td>
<td>Antinociceptive</td>
<td>0.5-2.0 mg/kg</td>
<td>IP</td>
<td>15 min</td>
<td>McCurdy et al., 2006</td>
</tr>
<tr>
<td>Model Organism</td>
<td>Test Model</td>
<td>Activity</td>
<td>Dose</td>
<td>Route of Administration</td>
<td>Time-Course</td>
<td>Reference</td>
</tr>
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<td>-------------------------</td>
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</tr>
<tr>
<td>C57BL6/Jx129S6 mice</td>
<td>Tail-flick test</td>
<td>Antinociceptive</td>
<td>7.5 μg</td>
<td>ICV</td>
<td>15 min</td>
<td>Ansonoff et al., 2006</td>
</tr>
<tr>
<td>CD-1 mice</td>
<td>Tail-flick test</td>
<td>Antinociceptive</td>
<td>13.9-23.1 nmol</td>
<td>IT</td>
<td>10 min</td>
<td>John et al., 2006</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>FST</td>
<td>Depressive-like effect</td>
<td>0.25-2.0 mg/kg</td>
<td>IP</td>
<td>15 min</td>
<td>Carlezon et al., 2006</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>ICSS</td>
<td>Attenuates</td>
<td>0.5-2.0 mg/kg</td>
<td>IP</td>
<td>1 hr</td>
<td>Carlezon et al., 2006</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>CPP</td>
<td>Rewarding</td>
<td>0.2-0.5 μg/kg</td>
<td>Dissolved</td>
<td>15 min</td>
<td>Braida et al., 2007</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>CPP</td>
<td>Aversive</td>
<td>80 μg/kg</td>
<td>Dissolved</td>
<td>15 min</td>
<td>Braida et al., 2007</td>
</tr>
<tr>
<td>Wistar rats</td>
<td>CPP</td>
<td>Rewarding</td>
<td>0.1-40.0 μg/kg</td>
<td>SC</td>
<td>30 min</td>
<td>Braida et al., 2008</td>
</tr>
<tr>
<td>Wistar rats</td>
<td>CPP</td>
<td>Aversive</td>
<td>160.0 μg/kg</td>
<td>SC</td>
<td>30 min</td>
<td>Braida et al., 2008</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>Cocaine induced locomotor activity</td>
<td>Attenuates</td>
<td>2.0 mg/kg</td>
<td>IP</td>
<td>2 hr</td>
<td>Chartoff et al., 2008</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>Cocaine induced locomotor activity</td>
<td>Attenuates</td>
<td>0.3 mg/kg</td>
<td>IP</td>
<td>1 hr</td>
<td>Morani et al., 2009</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>Cocaine self-administration</td>
<td>Attenuates</td>
<td>0.3-1.0 mg/kg</td>
<td>IP</td>
<td>2 hr</td>
<td>Morani et al., 2009</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>FST</td>
<td>Anti-depressant-like effect</td>
<td>0.1-160.0 μg/kg</td>
<td>SC</td>
<td>15 min</td>
<td>Braida et al., 2009</td>
</tr>
<tr>
<td>Model Organism</td>
<td>Test Model</td>
<td>Activity</td>
<td>Dose</td>
<td>Route of Administration</td>
<td>Time-Course</td>
<td>Reference</td>
</tr>
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</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>Elevated plus maze</td>
<td>Anxiolytic-like effects</td>
<td>0.1-160.0 μg/kg</td>
<td>SC</td>
<td>20 min</td>
<td>Braida et al., 2009</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>TST</td>
<td>Anti-depressant-like effect</td>
<td>0.1-160.0 μg/kg</td>
<td>SC</td>
<td>6 min</td>
<td>Braida et al., 2009</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>ICSS</td>
<td>Attenuates</td>
<td>2 mg/kg</td>
<td>IP</td>
<td>3 hr</td>
<td>Ebner, et al., 2010</td>
</tr>
<tr>
<td>Male Sprague-Dawley rats</td>
<td>CPP</td>
<td>Aversive</td>
<td>0.3-1.0 mg/kg</td>
<td>IP</td>
<td>15 min</td>
<td>Sufka et al., 2014</td>
</tr>
<tr>
<td>Rhesus monkeys</td>
<td>Cocaine self-administration</td>
<td>Attenuates</td>
<td>0.1 mg/kg/inj</td>
<td>IV</td>
<td>*Ten choice sessions</td>
<td>Freeman et al., 2014</td>
</tr>
</tbody>
</table>
CHAPTER 3: Salvinorin Analogues

In developing this pharmacological understanding of salvinorin A necessary work has been undertaken to explore the chemistry of this compound. The first report of successful total synthesis of salvinorin A occurred in 2007 by David A. Evans, consisting of a 29 step reaction with an overall yield of 0.8% (Scheerer et al., 2007). This was subsequently improved by Hisahiro Hagiwara in 2008, whose group achieved a reduction to 20 steps with a total yield improvement to 0.95% (Nozawa et al., 2008). In 2009 the Hagiwara group reported a second-generation synthesis scheme that further reduced the number of steps to 13 and increased the total yield to 2.8% (Hagiwara et al., 2009). However, in terms of cost effectiveness, the current synthetic routes are still not competitive with the crude, but effective, isolation of salvinorin A from the host plant Salvia divinorum, which typically yield in excess of 3% (Munro and Rizzacasa, 2003; Sufka et al., 2014).

In addition, there are a growing number of natural products that have been isolated from Salvia divinorum; however special interest has continued to be focused on those possessing neoclerodane diterpenoid structures. To date there have been an additional nine salvinorin compounds isolated, designated salvinorin A-J (Figure 20). However, with the exception of salvinorin B, most have yet to be pharmacologically characterized to any significant degree (for further information see references in Table 4).
Table 4. Natural salvinorin analogues

<table>
<thead>
<tr>
<th>Natural Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salvinorin A</td>
<td>Grundman et al., 2007; Hanson, 2010; Ortega et al., 1982; Shirota et al., 2006; Valdes et al., 1984</td>
</tr>
<tr>
<td>Salvinorin B</td>
<td>Grundman et al., 2007; Shirota et al., 2006; Valdes et al., 1984</td>
</tr>
<tr>
<td>Salvinorin C</td>
<td>Grundman et al., 2007; Shirota et al., 2006</td>
</tr>
<tr>
<td>Salvinorin D</td>
<td>Bigham et al., 2003; Munro and Rizzacasa, 2003; Shirota et al., 2006</td>
</tr>
<tr>
<td>Salvinorin E</td>
<td>Munro and Rizzacasa, 2003; Shirota et al., 2006</td>
</tr>
<tr>
<td>Salvinorin F</td>
<td>Munro and Rizzacasa, 2003; Shirota et al., 2006</td>
</tr>
<tr>
<td>Salvinorin G</td>
<td>Shirota et al., 2006</td>
</tr>
<tr>
<td>Salvinorin H</td>
<td>Shirota et al., 2006</td>
</tr>
<tr>
<td>Salvinorin I</td>
<td>Shirota et al., 2006</td>
</tr>
<tr>
<td>Salvinorin J</td>
<td>Kutrzeba et al., 2009</td>
</tr>
</tbody>
</table>

Figure 20. Natural salvinorin analogues.
Beyond these naturally occurring analogues, in exploring the structure-activity relationship of salvinorin A there have been more than 300 analogues synthesized (for an excellent review up to 2011 see: Cunningham et al., 2011). The majority of these analogues have only been utilized as molecular probes targeting KOR, strictly being assessed for KOR binding affinity. However, increasingly there has been a wider application of salvinoin A-based molecular probes being used to characterize DOR and MOR via binding affinities, as well as, measures of functional activity — with an increasing number progressing to applications within in vivo pharmacological models (Aviello et al., 2011; Calzada et al., 2015; Cunningham et al., 2011; Prisinzano and Rothman, 2008; Salaga et al., 2014; Vasiljevik et al., 2014; Wang et al., 2005; White et al., 2015; Xin et al., 2016; Yan et al., 2009). Nevertheless, the majority of research has focused on establishing a structure-activity relationship of the salvinorin A structure at KOR.

Such research has revealed that the C(2)-position of salvinorin A represents a critical pharmacophore feature for interaction at KOR. This is best exemplified by the hydrolysis of

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**Figure 21.** Key pharmacophore features of salvinorin A. Keasling and Zjawiony, 2016. Reprinted with permission.
salvinorin A at the C(2)-position into the KOR-inactive natural product salvinorin B (Chavkin et al., 2004). The general pattern amongst the current molecular probes indicates that analogues whose functional C(2)-position substitutions are able to mimic the carbonyl oxygen characteristics of the lead, salvinorin A, exhibit the highest binding affinities toward KOR (Cunningham et al., 2011). Exploration of other prominent pharmacophore features (Figure 21) of the salvinorin A structure have generally shown that structural modifications to the C(1)-position [ketone], C(4)-position [carbomethoxy], C(12)-position [furan], and the C(17)-position [lactone] result in a reduced KOR binding affinity, with only minor alterations at these positions being tolerated (Cunningham et al., 2011; Lozama et al., 2011; Riley et al., 2014).

However, in 2005 while exploring the C(2)-position of salvinorin A via structural modification the Prisinzano group reported one analogue exhibiting a surprising pharmacological effect (Harding et al., 2005). The addition of an aromatic substitution at the C(22)-position (Figure 22), through the introduction of a benzoyl group at C(2), reduced the subsequent binding affinity towards KOR 47-fold ($K_i$ of 90 nM) and reduced the functional activity 33-fold ($EC_{50}$ of 1320 nM), as assessed utilizing a $[^{35}S]$ GTP[$\gamma$S] binding assay (Table 5). However, this isn’t surprising as most salvinorin A analogues exhibit a decreased or abolished KOR binding affinity. What was surprising was that this substitution induced a MOR binding affinity ($K_i$ of 12 nM) and functional activity ($EC_{50}$ of 500 nM), with the authors reporting this to be the first salvinorin A-based agonist exhibiting high binding affinity towards MOR (Harding et al., 2005).
Due to this novel activity, additional characterization was pursued in 2007, by which time the compound had been named *Herkinorin* (Groer *et al.*, 2007). This study revealed that Herkinorin promoted phosphorylation of MAP kinases ERK1/2 in a dose-dependent manner, similar to salvinorin A (see Chapter 2), but mediated through MOR and was able to be antagonized by treatment with naloxone. They also reported that this ERK1/2 activation was independent from β-arrestin-2 signaling and that Herkinorin does not promote the MOR recruitment of β-arrestin-2 (Figure 23). This is of interest as there is a growing body of research indicating β-arrestin-2 involvement in MOR regulation and that β-arrestin-2-KO mice exhibit reduced opioid tolerance development, improved opioid-induced antinociception, and abolished both respiratory suppression and morphine-induced constipation (Bohn *et al.*, 1999; Bohn *et al.*, 2002; Przewlocka, *et al.*, 2002; Raehal *et al.*, 2005). Furthermore, this study showed that Herkinorin did not promote MOR internalization (Figure 24). This is of interest as GPCR-
internalization has been associated with opioid-induced addiction and posited as a means of receptor regulation (Bohn et al., 2004; Connor et al., 2004; Gainetdinov et al. 2004).

**Figure 23.** Herkinorin effect on β-arrrestin-2 recruitment. Shows MOR mediated translocation of β-arrrestin-2-GFP by agonist treatments: DAMGO (1 μM) and Herkinorin (2 μM). Groer et al., 2007. Reprinted with permission.

**Figure 24.** MOR agonist-mediated internalization. Shows effect of agonist treatment on MOR-YFP in HEK-293 cells. Groer et al., 2007. Reprinted with permission.

Subsequent pharmacological characterization of Herkinorin (1-10 mg/kg, SC) in male Sprague-Dawley rats utilizing a formalin chemo-nociceptive model revealed dose-dependent antinociception in both phase one (neurogenic pain caused by direct stimulation of the nociceptors) and phase two (inflammatory pain caused by release of inflammatory mediators),
with these antinociceptive effects being antagonized by naloxone pretreatment (Lamb et al., 2012). Interestingly, they showed that contralateral administration failed to promote similar antinociceptive effects — possibly indicating a peripheral restriction of Herkinorin to the site of administration. They also utilized a tolerance development model to assess the effects of chronic administration of Herkinorin (10 mg/kg, SC) as measured using the previous formalin assay and found that it was still effective in both phases, potentially indicating a decreased tolerance liability for this pharmacophore (see Table 5 for in vivo summary of Herkinorin).

Following this vein, an additional series of salvorin A-based molecular probes were generated where C(22)-position aromatic substitutions were explored (Tidgewell et al., 2008). Of particular interest were the bicyclic addition of naphthalene-1 (Figure 25-1), naphthalene-2 (Figure 25-2) and the heteroaromatic addition of thiophene-2 (Figure 25-3) and thiophene-3 (Figure 25-4). While the steric bulk of the naphthalene addition was toleratad, the subtle ester-linkage shift from the substituent 1-position to the substituent 2-position resulted in a preferential binding affinity towards KOR or MOR, respectively (Table 5), with significantly reduced binding affinity at other opioid subtypes. In the heteroaromatic thiophene analogues the

**Figure 25.** C(22)-position naphthalene and thiophene analogues of salvorin A.
KOR/MOR binding affinity is far more balanced, without clear preferential affinity towards a single opioid receptor subtype. However, the preliminary activity is comparable to Herkinorin (Table 5).

Together this data potentially indicates that the steric bulk of bicyclic C(22)-substitutions on salvinorin A would not only be tolerated, but also that a subtle 1-/2-substituent-linkage alteration may provide an approach for developing opioid receptor subtype-preferential molecular probes. Additionally, the C(22)-heteroaromatic substitutions appears to be well tolerated and from the preliminary in vitro data (Table 5) may yield analogues with increased functional activity at KOR, as compared to the Herkinorin lead.
Table 5. *In vitro* data summary of aromatic analogues of salvinorin A.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Figure</th>
<th>Binding affinity ( K_i \pm SD, \text{nM} )</th>
<th>Functional Activity ([^{35}S]GTP[\gamma S]) binding, ( EC_{50} \pm SD, \text{nM} )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOR</td>
<td>KOR</td>
<td>MOR</td>
<td>KOR</td>
<td>MOR</td>
</tr>
<tr>
<td>Herkinorin</td>
<td>22</td>
<td>1170 ±60</td>
<td>90 ±2</td>
<td>12 ±1</td>
</tr>
<tr>
<td>naphthalene-1</td>
<td>25-1</td>
<td>&gt;10,000</td>
<td>410 ±40</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>naphthalene-2</td>
<td>25-2</td>
<td>&gt;10,000</td>
<td>5,490 ±640</td>
<td>180 ±20</td>
</tr>
<tr>
<td>thiophene-2</td>
<td>25-3</td>
<td>1,380 ±130</td>
<td>260 ±20</td>
<td>10 ±2</td>
</tr>
<tr>
<td>thiophene-3</td>
<td>25-4</td>
<td>690 ±30</td>
<td>80 ±3</td>
<td>10 ±1</td>
</tr>
<tr>
<td>benzofuran-2 (P-31)</td>
<td>26</td>
<td>580 ±30</td>
<td>70 ±2</td>
<td>10 ±1</td>
</tr>
<tr>
<td>PR-38</td>
<td>27</td>
<td>700 ±101</td>
<td>9 ±2</td>
<td>52 ±9</td>
</tr>
</tbody>
</table>

— Indicates not assessed.
These results supported further exploration as to the effect that incorporation of a fused-heteroaromatic substituent would have at the salvinorin A C(22)-position. This led to the generation of a single analogue incorporating benzofuran-2 at this position (P-3l; Figure 26) (Tidgewell et al., 2008). Compared to the previous aromatic substitutions, including that of the Herkinorin lead, in vitro pharmacological assessment of P-3l revealed an increased binding affinity by this fused-heteroaromatic analogue at all three opioid receptor subtypes (Table 5). Additionally, when assessed utilizing a $[^{35}\text{S}]$ GTP$[\gamma\text{S}]$ binding assay, the P-3l analogue exhibited a functional activity at KOR that was comparable to the Herkinorin lead and at MOR that was comparable to the thiophene-2 analogue (Table 5).

![Figure 26. Structure of 2-O-salvinorin B benzofuran-2-carboxylate (P-3l).](image-url)
Separately, in 2014 while exploring the effect that incorporation of Michael acceptor-type linkers at the C(2)-position of salvinorin A has on resulting aromatic analogues, the Zjawiony group reported the generation of a cinnamoyl analogue designated as PR-38 (Figure 27) (Polepally et al., 2014). This salvinorin A analogue displayed a preferential binding affinity for KOR (Kᵢ of 9 nM) and, interestingly, retained MOR binding affinity (Kᵢ of 52 nM) (Table 5). The high binding dual-affinity at KOR/MOR observed with the PR-38 compound encouraged further in vivo pharmacological assessment.

![Figure 27. Structure of 2-O-cinnamoylsalvinorin B (PR-38).](image)

Utilizing a male BALB/c mouse model of irritable bowel syndrome (IBS) PR-38 (10-20 mg/kg, IP) was found to inhibit gastrointestinal hypermotility (Table 6) and colonic bead expulsion (Salaga et al., 2014). Additionally, PR-38 (10 mg/kg, IP) was found to produce antinociceptive effects in a male BALB/c mouse chemo-nociceptive model (acetic acid-induced
abdominal writhing assay). Of particular interest is that this study utilized, in addition to intraperitoneal administration, oral administration — a route of administration rarely examined, due to the in-activity of the salvinroin A lead, and found PR-38 to be orally active. A subsequent study employing a Swiss-Webster mouse pruritus model reported that PR-38 (10-20 mg/kg) promoted antipruritic activity when administered both subcutaneously and orally (Salaga et al., 2015).

Computational modeling was undertaken to examine the potential ligand-receptor interactions that distinguish PR-38 from the salvinorin A lead (Polepally et al., 2014). Utilizing the then available ‘inactive’ (i.e. antagonist co-crystallized) crystal structure of KOR complexed with JDTic (PDB: 4DJH) the orthosteric binding site for the salvinorin tricyclic core was defined utilizing residues: Asp-138, Gln-115, Thr-111, Val-108, Val-118, Val-134, and Val-135 — these residues were supported by prior mutagenesis studies (Vardy et al., 2013; Wu et al., 2012). This oriented the salvinorin tricyclic core towards TM2, with the C(2)-position interacting with a binding pocket delineated with Asp-138, Met-142, and Tyr-139. What this model showed was that the olefinic chain of the Michael acceptor-type linker is able to effectively fill a Asp-138, Met-142, and Tyr-139 delineated pocket and allow the aromatic substituent to extend beyond into a hydrophobic pocket bounded by TM3 and TM6, displaying key interactions with Met-142 and Ile-294. The researchers concluded that their model indicated that the C(2)-olefin portion of PR-38 serves as hydrophobic linker and likely facilitates the engagement of the aromatic substituent with a critical hydrophobic region within the receptor.
<table>
<thead>
<tr>
<th>Analogue</th>
<th>Model Organism</th>
<th>Test Model</th>
<th>Activity</th>
<th>Dose</th>
<th>Route of Administration</th>
<th>Time-Course</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herkinorin</td>
<td>Male Sprague-Dawley rats</td>
<td>Formalin assay — phase 1 (neurogenic pain)</td>
<td>Antinociceptive</td>
<td>1-10 mg/kg</td>
<td>SC</td>
<td>1 hour</td>
<td>Lamb et al., 2012</td>
</tr>
<tr>
<td>Herkinorin</td>
<td>Male Sprague-Dawley rats</td>
<td>Formalin assay — phase 2 (inflammatory pain)</td>
<td>Antinociceptive</td>
<td>10 mg/kg</td>
<td>SC</td>
<td>1 hour</td>
<td>Lamb et al., 2012</td>
</tr>
<tr>
<td>Herkinorin</td>
<td>Male Sprague-Dawley rats</td>
<td>Tolerance development</td>
<td>No tolerance development</td>
<td>10 mg/kg</td>
<td>SC</td>
<td>5 day</td>
<td>Lamb et al., 2012</td>
</tr>
<tr>
<td>PR-38</td>
<td>Male BALB/c mice</td>
<td>Gastrointestinal hypermotility</td>
<td>Reduced motility</td>
<td>10-20 mg/kg</td>
<td>IP and P.O.</td>
<td>20 min</td>
<td>Salaga et al., 2014</td>
</tr>
<tr>
<td>PR-38</td>
<td>Male BALB/c mice</td>
<td>Colonic bead expulsion test</td>
<td>Inhibited colonic expulsion</td>
<td>10-20 mg/kg</td>
<td>IP</td>
<td>45 min</td>
<td>Salaga et al., 2014</td>
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<tr>
<td>PR-38</td>
<td>Male BALB/c mice</td>
<td>Acetic acid-induced abdominal writhing assay</td>
<td>Antinociceptive</td>
<td>10 mg/kg</td>
<td>IP</td>
<td>5 min</td>
<td>Salaga et al., 2014</td>
</tr>
<tr>
<td>PR-38</td>
<td>Swiss-Webster mice</td>
<td>Pruritus model</td>
<td>Antipruritic</td>
<td>10-20 mg/kg</td>
<td>SC</td>
<td>120 min</td>
<td>Salaga et al., 2015</td>
</tr>
<tr>
<td>PR-38</td>
<td>Swiss-Webster mice</td>
<td>Pruritus model</td>
<td>Antipruritic</td>
<td>10 mg/kg</td>
<td>P.O.</td>
<td>120 min</td>
<td>Salaga et al., 2015</td>
</tr>
</tbody>
</table>
CHAPTER 4: Rationale, Specific Aims, and Expected Outcomes

The field of salvinorin chemistry represents novel and emerging field of opioid research. The novelty of this field is derived from the lead pharmacophore: salvinorin A — a pharmacologically unique compound which represents the first known non-nitrogenous KOR subtype-selective opioid receptor agonist, exhibits a comparatively safe physiological profile with no reports of toxicological effects in clinical trials, and, most importantly, has a steadily growing body of literature indicating potentially useful clinical applications (e.g. antinociceptive, anti-addictive, antipruritic, neuroprotective, etc.) (Aviello et al., 2011; Mowry et al., 2003; Roth et al., 2002; Wang et al., 2005; Xin et al., 2016). The emerging status of this field of research entails both the relatively recent beginnings, arguably founded in 2002 with the identification of the KOR target for salvinorin A by the Roth group, and subsequently with the growing body of salvinorin analogues, exemplified by the recent development of aromatic analogues (Cunningham et al., 2011; Groer et al., 2007; Harding et al., 2005; Polepally et al., 2014; Roth et al., 2002).

The development of salvinorin A aromatic analogues is of particular interest due to the resulting range of pharmacological activity that has been reported. The 2005 report incorporating a benzoyl substituent at the C(2)-position resulted in the first report of a non-nitrogenous high-binding affinity agonist at MOR — a compound subsequently named Herkinorin (Groer et al., 2007; Harding et al., 2005). Ensuing pharmacological characterization
of this aromatic salvinorin A analogue revealed that it did not promote β-arrestin-2 recruitment, receptor internalization, or tolerance development — all undesirable activities commonly seen with classical opioids (Groer et al., 2007; Lamb et al., 2012).

Subsequent incorporation of a cinnamoyl moiety at the C(2)-position of salvinorin A, a compound designated as PR-38, resulted in a molecular probe with high-binding affinity towards KOR/MOR (Table 5). More interestingly, however, PR-38 was uniquely found to be orally active and promote both antinociceptive and antipruritic activities (Table 6) (Polepally et al., 2014; Salaga et al., 2014; Salaga et al., 2015). Along these lines, incorporation of naphthalene and thiophene substituents at the C(22)-position of salvinorin A, which conserve the conjugation observed with the Michael acceptor-type linker found in PR-38, resulted in fused-aromatic and heteroaromatic analogues that display high-binding affinity, with subtype selectivity observed in the case of the naphthalene analogues (Table 5). Lastly, represented by a single molecular probe, the incorporation of a benzofuran-2 moiety (P-3l) at the salvinorin A C(22)-position represents a potentially interesting line of research that has yet to be expanded.

Preliminary in vitro assessment of P-3l (Table 5) reveals that this analogue possesses high-binding affinity, preferential for MOR/KOR, and functional activity as well (as assessed via $^{35}$S GTP[γS] binding). Structurally, it also retains key features of the preceding aromatic analogues: it is a fused-heteroaromatic substituent that merges the fused-bicyclic feature of the naphthalene analogues, the heteroaromaticity of the thiophene analogues, and the aromatic-conjugated system observed with PR-38. This structural template affords a unique opportunity to explore the effect of bioisosteric replacement [e.g. C/N/O/S] at the substituent 1-position and the effect that substituent 2-/3-linkage with salvinorin A has on the ensuing pharmacology to better characterize the opioid system. Due to the importance of this system, this series of
molecular probes will help address the continuing need for new research tools capable of ascertaining potential three-dimensional ligand binding requirements, functional activities, and pharmacological consequences.

Specific Aims:


AIM 2. *In vitro* evaluation of analogues at the opioid receptors.

   2a. Evaluation of ligand binding affinity at δ (Delta), κ (Kappa), and μ (Mu) opioid receptors utilizing a competitive radioligand binding assay.

   2b. Evaluation of ligand induced functional activity at opioid receptors measured by \[^{35}\text{S}]\text{GTP}[\gamma\text{S}]\) binding assay.

AIM 3. *In vivo* antinociceptive evaluation of analogues in mouse models

   3a. Acetic Acid-induced Abdominal Writhing Assay

   3b. Formalin Assay

   3b. Opioid Antagonism Assay

   3c. Hot Plate Assay

Expected Outcomes:


- Elucidation of structural motifs by which KOR/MOR affinity may preferentially be induced.

- Determination of the pharmacological relevance of designated analogues towards mouse models of antinociceptive activity.
CHAPTER 5: Experimental Methods

Chemistry

Scheme 1. General synthesis procedure. Reagents and conditions: (A) Na₂CO₃, anhyd. MeOH, stirred, r.t. 8 hr.; (B) appropriate carboxylic acid, DCC, DMAP, anhyd. CH₂Cl₂, Ar atmo., stirred, r.t. 12 hr.
**General Synthesis Procedure**

All reagents were purchased from commercial suppliers and, except where indicated, were used without additional purification.

The general procedure used to deacetylate salvinorin A to salvinorin B was as follows: a mixture of salvinorin A (3.5 g, 8.0 mmol, 1 equivalents) and sodium carbonate (3.4 g, 32.2 mmol, 4 equivalents) in absolute methanol (150 mL) was equipped with a stir bar and stirred at room temperature overnight. The methanol was removed under reduced pressure and the residue solubilized in dichloromethane (DCM, 500 mL). This solution was successively washed with 2N hydrochloric acid (HCl, 100 mL), sodium chloride solution (NaCl, 100 mL), and dried with sodium sulfate (Na₂SO₄). The DCM was removed under vacuum and the resulting solid resuspended in methanol, filtered and dried to yield salvinorin B (Tidgewell *et al.* 2004).

The general procedure used to generate this series of analogues outlined (Scheme 1) was as follows: a solution of salvinorin B (0.10 g, 0.26 mmol, 1 equivalents), appropriate carboxylic acid (0.51 mmol, 2-3 equivalents), 1,4-dicyclohexylcarbodiimide (DCC; 0.132 g, 0.61 mmol, 2 equivalents), and catalytic amount of 4-(dimethylamino)pyridine (DMAP) were added to a round bottom flask equipped with stir bar and then flushed with Argon, before being sealed under Argon atmosphere. To the reaction vessel was added anhydrous DCM (5 mL) this was placed in an ice bath (0°C) and while stirring allowed to reach room temperature overnight. After checking with TLC (short/long UV and vanillin stain; Sorbent Technologies, Atlanta, GA; Silica G TLC Plates w/UV254, 200 μm thickness, polyester backed) to ensure completion of reaction, material was partitioned between deionized water and the organic DCM layer dried under reduced pressure to yield the crude reaction material ready for chromatographic purification (Polepally *et al.* 2014; Tidgewell *et al.* 2008).
Chromatography

The general procedure used to purify analogues in this series was as follows: the crude reaction material was first processed via vacuum liquid chromatography (VLC) across silica gel (Sorbent Technologies, Atlanta, GA; Silica Gel, 200g, Porosity: 60 Å, Particle Size: 230 x 400 mesh) using a 10% solvent gradient from 100% n-hexane to 100% ethyl acetate. The collected fractions were checked with TLC (UV and vanillin stain) and combined based on characteristic staining profile. Combined fractions were then dried under vacuum via rotary evaporator and loaded on onto a Sephadex LH-20 gravity column (GE Healthcare Life Sciences, Chicago, IL; 100 g) equilibrated in chloroform:methanol (1:1). The collected fractions were checked with TLC (UV and vanillin stain) and combined based on profile. Combined fractions were then dried under vacuum via rotary evaporator and loaded on onto a C18 reverse phase silica gel column (Acros Organics, NJ; C18 Silica Gel, 200g, 23%C, ca. 1.2 mmol/g, particle size: 40-63 μm) that was equilibrated water:methanol (90:10). This column was processed using a 5% VLC solvent gradient to 100% methanol, with the column stripped with methanol:ethyl acetate (95:5) to yield the target analogue. This general chromatography procedure was validated via a Waters 2487 Analytical HPLC utilizing Phenomenex Hydro-RP C18 column (Phenomenex; Torrance, CA;10x250mm, Synergi Hydro-RP 10um, 80 Å) and a YMC-Polyamine II column (YMC America, Inc.; Allentown, PA;150x10mm) using an elution rate of 3 ml/minute. We processed the analogue utilizing an isocratic acetonitrile:water (70:30) solvent system first across the C18 column (Figure 28) and then the polyamine column (Figure 29). This general chromatography procedure was found to result in material of ≥95% purity by UV detection and a percent yield typically between 30-38% for the resulting analogues.
Structural Elucidation

Nuclear Magnetic Resonance (NMR) spectra were obtained for both carbon ($^{13}$C) and proton ($^1$H) of all compounds measured using a Bruker Avance NMR spectrometer (400 UltraShield, 54 mm standard magnet bore; Billerica, MA) equipped with a 3mm probe with spectra recorded at
101 MHz and 400 MHz, respectively (Appendices A-C). All samples were analyzed using deuterated chloroform as the solvent. High-resolution mass spectrometric (HRMS) data was obtained employing a Bruker MicroTOF-ESI Mass Spectrometer (Billerica, MA) via direct injection with samples (1 mg/ml) solubilized in methanol.

**Pharmacological Characterization**

**In Vitro Methods**

**Opioid Receptor Binding Affinity**

Preliminary and secondary binding affinities were determined utilizing established competitive radioligand binding assays. Briefly, we utilized stably transfected HEK-293 cells expressing human opioid receptor subtypes DOR, KOR, and MOR. Stably transfected cell lines were maintained on Dulbecco’s Modified Eagle Medium (DMEM) nutrient media supplemented with fetal bovine serum (10%), hygromycin B (300 mg/mL), L-glutamine (2mM), and penicillin-streptomycin (0.5%) under a 5% atmosphere of carbon dioxide maintained at a temperature of 37°C. Membranes were prepared by scraping cells in a Tris-HCl buffer, followed by sonication facilitated homogenization and centrifugation (Leon et al., 2013; Polepally et al., 2014).

In the preliminary binding assay the new analogues were assessed at 10 µM test concentrations with competitive incubation with [³H]-Enkephalin, [³H]-U-69,593, or [³H]-DAMGO for DOR, KOR, or MOR, respectively. For controls, unlabeled DPDPE, nor-Binaltorphimine, and DAMGO were used, once more for DOR, KOR, and MOR, respectively. After termination of the incubation cycle and multi-well washing, microplate scintillation values were determined using a TopCount NXT analyzer (Leon et al., 2013). This first assay was used to calculate percent inhibition of the analogue to see if it met the 70% threshold to proceed to
secondary binding affinity evaluation. Analogues meeting the 70% threshold were subsequently evaluated in the secondary bind affinity assay that utilized a 12-step serial dilution from 100 μM to 0.5 nM to calculate the equilibrium dissociation constant (Kᵢ). Analogues possessing a Kᵢ ≤300 nM were evaluated for functional activity utilizing a [³⁵S]GTP[γS] binding assay.

**Opioid Receptor Functional Activity**

Methods similar to those in competitive radioligand binding assays functional assays were used for the [³⁵S]GTP[γS] binding assay. Utilizing [³⁵S]GTP[γS] as a measurable radioligand of GTP we measured the exchange of GDP at the inactive G-protein-coupled receptor (DOR, KOR, or MOR) for [³⁵S]GTP[γS] as a measure of receptor activation (i.e. functional activity) by ligands possessing a Kᵢ ≤300 nM binding affinity in the secondary competitive radioligand binding assays. As before, controls consisted of nor-Binaltorphimine and DAMGO for KOR and MOR, respectively and were assessed via serial dilution to calculate a dose response curve to determine analogue effect (i.e. full agonist, partial agonist, etc.) and to determine effective concentrations (EC₅₀). All *in vitro* assays were performed in triplicate (Leon *et al.*, 2013).

**In Vivo Methods**

**Test animals**

Test animals used in the following *in vivo* assays were male Swiss Webster mice (adults, 21-24g), obtained from Harlan Laboratories. Mice received water and food *ad lib*, while housed in groups of five. Housing facilities were maintained on a lighting schedule of 12 hours (0600 to 1800).
I. Antinociceptive Related Assays

Acetic Acid-induced Abdominal Writhing Test

Groups of mice (n = 10) were treated by gavage (P.O.) with vehicle (10% DMSO, 10 ml/kg, vehicle control), test analogue (1, 3, 10 mg/kg, P.O.), indomethacin 20 mg/kg or morphine 10 mg/kg (positive control for antinociceptive activity), 60 minutes before the administration of acetic acid solution (0.6% v/v; 10 ml/kg, IP) (Koster et al., 1959). The number of writhing movements produced in each group in the 30 minute test period was counted and the results were expressed as mean ± SEM as percentage of control group. A significant reduction in the number of writhing movements was considered to be a positive antinociceptive response.

Formalin Assay

The experimental groups of mice (n = 7) were treated by gavage (P.O.) with vehicle (10% DMSO, 10 mL/kg, vehicle control), test analogue (1, 3, 10 mg/kg, P.O.), indomethacin 20 mg/kg (positive control for antinociceptive activity in the second phase), 60 minutes before, or subcutaneously (SC) with morphine 10 mg/kg (positive control for antinociceptive activity in the first and second phases), 30 minutes before the administration of formalin (20 μl, 3% v/v) in the right hind paw (Melo et al., 2005). After the formalin phlogistic agent injection, the mice were placed into an acrylic test box with a mirror located under the box to enable observation of the formalin-injected paw for 30 minutes. Assessment of pain reaction time (i.e. time until paw licking behavior) was observed for two periods: 0 to 5 minutes — the first phase (neurogenic pain caused by direct stimulation of the nociceptors) and from 15 to 30 minutes — the second phase (inflammatory pain caused by release of inflammatory mediators) (Hunskaar et al., 1985). Results were expressed as mean ± SEM, in seconds.
**Opioid Antagonism Assay**

To examine the involvement of opioids receptors in the observed antinociceptive activity in the formalin-induced pain assay. The experimental group of mice \(n = 7\) were pretreated with saline (0.9 %, 10 ml/kg, IP, negative control) or naloxone 3 mg/kg, IP, opioid antagonist). After 15 minutes the mice were treated with vehicle (20 % DMSO, 10 ml/kg, P.O., vehicle control), test compound (10 mg/kg, P.O.) or morphine (10 mg/kg, P.O., positive control/opioid agonist). Sixty-minutes after oral administration, or 30 minutes after subcutaneous administration, the mice were administrated formalin (20 μl, 3 % v/v) in the right hind paw. The pain reaction time (time until paw licking behavior) was observed in the first phase (neurogenic pain) of formalin assay (Hunskaar et al., 1985).

**Hot Plate Assay**

The hot plate test was performed as previously described by Woolfe and MacDonald (1944). The latency (in seconds) to reaction to the thermal stimulus, expressed by lifting of the hind paws accompanied by licking or flinch was measured using hot plate (Insight®, Ribeirão Preto, São Paulo, Brazil) set at 55.5 ± 0.5 °C. The mice were divided into four experimental groups \(n = 8\): vehicle (10% DMSO, 10 ml/kg, P.O.), test analogue (1, 3, 10 mg/kg, P.O.) or morphine (5 mg/kg, P.O., positive control for antinociceptive activity). The latency baseline was measured 30 minutes before of the treatments (time -30) and immediately after of the treatments (time 0) for each animal. After treatment, the latency to pain reaction was measured at 30, 60, 90, 120, 150 and 180 minutes. A cut-off of 20 seconds was used to prevent the possibility of harm to the test animals. Results were expressed as means ± SEM, in seconds, at the assessed times.
II. Antidepressant Related Assays

Forced Swim Test

Test mice \((n = 10)\) were evaluated by a forced swim test (FST) after oral administration of vehicle (10% DMSO, 10 mL/kg, P.O.), test analogue (1, 3, 10 mg/kg, P.O.) or imipramine (15 mg/kg, P.O.) \((Fajemiroye et al., 2014; Porsolt et al., 1977)\). Treated mice were placed individually in a cylindrical container of 18 cm diameter filled with water to the height of 30 cm in height, total volume of 7.6 L, at 24 ± 2°C. Video from six-minute test sessions was recorded with the swimming and immobility time measured for statistical analysis.

Tail Suspension Test

A modified version of tail suspension test (TST) was employed with test mice \((n = 10)\) administered oral treatment of either vehicle (10% DMSO, 10 mL/kg, P.O.), test analogue (1, 3, 10 mg/kg, P.O.), or imipramine (15 mg/kg, P.O.) \((Steru et al., 1985)\). Treated mice were suspended using adhesive tape placed two centimeters from the tip of the tail one hour after administrartions of selected treatment. Video from six-minute test sessions was recorded and the immobility time scored and analysed.

III. Anxiolytic Related Assay

Open Field Test

A circular open field arena with base area 62 cm\(^2\) and eight equal sectorial-divisions enclosed with a 50 cm high wall was used to evaluate exploratory activity of the mice \((Fajemiroye et al., 2015; Fajemiroye et al., 2014)\). Test mice \((n = 10)\) were treated via oral administration either
vehicle (10% DMSO, 10 mL/kg, P.O.), test analogue (1, 3 or 10 mg/kg, P.O.), diazepam (5 mg/kg, P.O.) one hour prior to the test session (five-minute duration) in the open field. Diazepam was used as a positive control in this model as this dose reliably induced anxiolytic-like effects. Video from five-minute test sessions was recorded and the exploratory activities scored and analysed.

**Computational Modeling**

**Model Design**

We utilized the active-state agonist-bound μ-opioid receptor (MOR) X-ray crystal structure (PDB ID: 5C1M) published in late 2015 to develop an in silico homology model of the κ-opioid receptor (KOR) in its active state (Huang *et al.*, 2015; Vardy *et al.*, 2013). The full sequence of human KOR was downloaded from the Uniprot website ([http://www.uniprot.org/uniprot/P41145](http://www.uniprot.org/uniprot/P41145)) and used to define key residue difference between KOR and MOR. We utilized chain A of the 5C1M crystal structure, in which MOR is bound to the morphinan agonist BU72 as a template for generating multiple homology models. Alignment of the KOR and MOR sequences using ClustalW revealed that KOR and 5C1M possessed a 67% sequence identity and 81% sequence similarity after alignment (Figure 30). Additionally, a critical disulfide bridge located in the extracellular-loop 2 of KOR between Cys-131 and Cys-210 (Cys-140 and Cys-217 in MOR) was included in the model generation (Polepally *et al.*, 2014). We generated ten preliminary models using the knowledge-based method implemented in 2016 Schrödinger suite. Loop regions of these ten models were refined using the Prime module implemented in the Schrödinger suite and the quality of the protein structure models were assessed by Ramachandran plotting. This analysis revealed that only one model expressed
all amino acids in the favored/allowed regions as defined in the Ramachandran plot (Figure 31) — therefore this was the model utilized for subsequent optimization (Schrödinger, 2017).

The crystal structure of active state MOR obtained from the Protein Data Bank (PDB ID: 5C1M) was further optimized for docking by adding hydrogen atoms, removing waters, including structure truncation of side chains for Lys-269, Glu-270, and Arg-273, generating the physiologically appropriate protonation state and hydrogen-bond assignments. Receptor optimization utilized hydrogen-only restrained minimization was performed using the OPLS3 force field (Schrödinger, 2016). Additionally, we defined the putative active-site by incorporating key amino residues previously identified through corroborating point-mutation studies [Gln-115, Tyr-119, Tyr-139, Tyr-312, Tyr-313, Ile-316 and Tyr-320] (Kane et al., 2006; Van et al., 2013; Yan et al., 2005).
Figure 30. Sequence alignment of KOR with the MOR template. Identical residues are in red, while similar residues are in blue. Helical regions are labeled and indicated using yellow background.
Opioid Receptor Docking Studies

Structures of the salvinorin analogues were sketched in Maestro and energy-minimized using the LigPrep module implemented in the Schrödinger software package using the OPLS3 force field (Schrödinger, 2016). A docking receptor grid for the KOR homology model was generated using the centroid of the key residues identified through previous point-mutation studies lining the putative binding site: Gln-115, Tyr-119, Tyr-139, Tyr-312, Tyr-313, Ile-316 and Tyr-320 (Kane et al., 2008; Kane et al, 2006; Yan et al, 2005). Extra-precision docking without constraints was performed utilizing the Glide module of the Schrödinger software (Schrödinger, 2016).
ADME/T Calculations

Considering physiochemical properties such as absorption, distribution, metabolism, excretion, and toxicity (ADME/T) at the early stage of the drug development process is important to avoid downstream drug development issues. Advances in the computational field have steadily improved predictive methods for calculating ADME/T properties (Tao et al., 2015; Wang et al. 2015). As such, pharmaceutically important ADME/T properties of the analogue series were selected (Table 7) for calculated evaluation using the Schrödinger QikProp module implemented in the Maestro software, using LigPrep OPLS3 force field energy-minimized structures for each analogue (Schrödinger, 2016).

Statistical Analyses

The data was analyzed by a two-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test or Bonferroni’s post hoc test. *In vitro* data is expressed as $K_i$ or $EC_{50} \pm$ SEM and *in vivo* data is expressed as mean $\pm$ SEM, with differences being considered statistically significant when P-values were less than 0.05. Statistical analysis was performed with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).
<table>
<thead>
<tr>
<th>Properties</th>
<th>Description</th>
<th>Recommended Range</th>
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</thead>
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<td>Molecular weight of the molecule</td>
<td>130.0 – 725.0</td>
</tr>
<tr>
<td>PSA (Å²)</td>
<td>Van der Waals surface area of polar nitrogen and oxygen atoms</td>
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<tr>
<td>Number of rotatable bonds</td>
<td>Number of non-trivial (not CX3), non-hindered (not alkene, amide, small ring) rotatable bonds</td>
<td>0.0 – 15.0</td>
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<td>QPlogP&lt;sub&gt;oct&lt;/sub&gt; (octanol/water)</td>
<td>Predicted octanol/water partition coefficient</td>
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<td>QPPCaco (nm/s)</td>
<td>Predicted Caco-2 cell permeability in nm/sec [representative for GI-blood barrier]</td>
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</tr>
<tr>
<td>QPPMDCK (nm/s)&lt;sup&gt;Γ&lt;/sup&gt;</td>
<td>Predicted MDCK cell permeability in nm/sec [representative for blood-brain barrier]</td>
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<td>QPlogHERG</td>
<td>Predicted IC50 value for blockage of HERG K+ channels</td>
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<tr>
<td>QPlogK&lt;sub&gt;hsa&lt;/sub&gt;</td>
<td>Prediction of binding to human serum albu</td>
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<tr>
<td>% Human Oral Absorption</td>
<td>Predicted human oral absorption on 0 to 100% scale</td>
<td>&gt;80 is high &lt;25 is poor</td>
</tr>
<tr>
<td>CNS</td>
<td>Predicted central nervous system activity on a –2 (inactive) to +2 (active) scale.</td>
<td>-2.0 – +2.0</td>
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</tbody>
</table>

Data adapted from: Schrödinger, 2016.
Utilizing the general synthesis procedure (outlined in Chapter 5: Experimental Methods) with the appropriate benzofuran-2-carboxylic acid compound 2-O-salvinorin B benzofuran-2-carboxylate (P-31; Figure 32) was synthesized and yielded an amorphous white solid; $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.73 – 7.56 (m, 4H), 7.56 – 7.26 (m, 7H), 6.38 (d, $J = 1.9$ Hz, 1H), 5.48 (ddd, $J = 32.9$, 11.5, 6.7 Hz, 3H), 3.82 – 3.66 (m, 5H), 2.99 – 2.76 (m, 2H), 2.62 – 2.42 (m, 5H), 2.30 (s, 2H), 2.23 – 2.02 (m, 4H), 1.83 (dd, $J = 11.1$, 4.3 Hz, 2H), 1.74 – 1.55 (m, 5H), 1.46 (s, 5H), 1.21 (d, $J = 32.8$ Hz, 5H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 201.34, 171.47, 171.13, 158.41, 156.00, 144.41, 143.70, 139.44, 128.04, 126.83, 125.23, 123.96, 123.00, 115.14, 112.43, 108.41, 75.77, 72.05, 64.02, 53.55, 52.01, 51.33, 43.26, 42.22, 38.19, 35.49, 30.85, 18.17, 16.50,
15.20. HRMS \((m/z)\): \([M+Cl]\) calculated: 569.159, observed: 569.233. Molecular formula: C\(_{30}\)H\(_{30}\)O\(_9\). Molecular weight: 534.19.

![Figure 33. Structure of 2-O-salvinorin B 1H-indole-2-carboxylate (AK-1401).](image)

Compound 2-O-salvinorin B 1H-indole-2-carboxylate (AK-1401; Figure 33) was synthesized utilizing the general synthesis procedure with indole-2-carboxylic acid and yielded an amorphous white solid; \(^1\)H NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 8.90 (s, 1H), 7.71 (d, \(J = 8.1\) Hz, 1H), 7.51 – 7.31 (m, 5H), 7.17 (t, \(J = 7.6\) Hz, 1H), 6.40 (s, 1H), 5.54 (dd, \(J = 11.8\), 4.9 Hz, 1H), 5.42 (t, \(J = 10.0\) Hz, 1H), 3.76 (s, 3H), 2.89 – 2.79 (m, 1H), 2.53 (dt, \(J = 28.9\), 8.6 Hz, 3H), 2.28 – 2.15 (m, 2H), 2.11 (d, \(J = 10.6\) Hz, 1H), 1.86 (d, \(J = 11.6\) Hz, 1H), 1.68 – 1.57 (m, 3H), 1.48 (s, 2H), 1.20 (s, 2H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 201.73, 171.51, 171.08, 160.57, 143.72, 139.43, 137.12, 127.43, 125.97, 125.90, 125.23, 122.79, 121.03, 111.88, 110.23, 108.39, 75.36, 72.05, 64.20, 53.64, 52.04, 51.43, 43.46, 42.22, 38.22, 35.52, 30.99, 18.17, 16.51, 15.22. HRMS \((m/z)\): \([M-H]\) calculated: 532.572, observed: 532.174. Molecular formula: C\(_{30}\)H\(_{31}\)NO\(_8\). Molecular weight: 533.58.
Compound 2-\(O\)-salvinorin B benzo[\(b\)]thiophene-2-carboxylate (AK-1402; Figure 34) was synthesized utilizing the general synthesis procedure with benzo[\(b\)]thiophene-2-carboxylic acid and yielded an amorphous white solid; \(^1\)H NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 8.16 (s, 1H), 7.93 – 7.85 (m, 2H), 7.53 – 7.38 (m, 4H), 6.40 (dd, \(J = 1.8\), 0.9 Hz, 1H), 5.53 (dd, \(J = 11.7\), 5.2 Hz, 1H), 5.40 (dd, \(J = 11.1\), 8.8 Hz, 1H), 3.76 (s, 3H), 2.91 – 2.78 (m, 1H), 2.61 – 2.45 (m, 4H), 2.30 – 2.07 (m, 4H), 1.89 – 1.78 (m, 2H), 1.76 – 1.56 (m, 5H), 1.41 – 1.24 (m, 2H), 1.19 (s, 4H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 201.44, 171.52, 171.12, 161.72, 143.72, 142.53, 139.47, 138.63, 131.53, 127.27, 125.72, 125.21, 125.05, 122.78, 108.42, 75.87, 72.05, 64.12, 53.61, 52.03, 51.41, 43.39, 42.21, 38.22, 35.51, 30.90, 28.15, 18.17, 16.51, 15.22. HRMS (\(m/z\)): [M+Cl] calculated: 585.589, observed: 585.205. Molecular formula: \(C_{30}H_{30}O_8S\). Molecular weight: 550.62.
Compound 2-O-salvinorin B benzofuran-3-carboxylate (AK-1403; Figure 35) was synthesized utilizing the general synthesis procedure with benzofuran-3-carboxylic acid and yielded an amorphous white solid; $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 8.34 (s, 1H), 8.09 – 7.99 (m, 1H), 7.58 – 7.49 (m, 1H), 7.44 – 7.27 (m, 4H), 6.41 – 6.36 (m, 1H), 5.55 – 5.37 (m, 2H), 3.73 (s, 3H), 2.89 – 2.77 (m, 1H), 2.57 – 2.39 (m, 3H), 2.28 (s, 1H), 2.21 – 2.04 (m, 3H), 1.84 – 1.73 (m, 1H), 1.72 – 1.53 (m, 4H), 1.21 (d, $J = 36.4$ Hz, 5H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 201.86, 171.61, 171.18, 162.09, 155.61, 151.75, 143.68, 139.51, 125.51, 125.25, 124.36, 122.02, 113.63, 111.76, 108.47, 75.08, 72.02, 63.95, 53.50, 51.99, 51.29, 43.28, 42.16, 38.14, 35.46, 30.98, 29.67, 18.16, 16.49, 15.20. HRMS ($m/z$): [M+Cl] calculated: 569.5294, observed: 569.7652. Molecular formula: C$_{30}$H$_{30}$O$_9$. Molecular weight: 534.56.
Compound 2-\textit{O}-salvinorin B benzo[\textit{b}]thiophene-3-carboxylate (AK-1405; Figure 36) was synthesized utilizing the general synthesis procedure with benzo[\textit{b}]thiophene-2-carboxylic acid and yielded an amorphous white solid; \textit{\textsuperscript{1}}H NMR (400 MHz, Chloroform-\textit{d}) \(\delta 8.58 – 8.48 \text{ (m, 2H)}, 7.92 – 7.85 \text{ (m, 1H)}, 7.59 – 7.37 \text{ (m, 5H)}, 6.40 \text{ (dd, } J = 1.9, 0.9 \text{ Hz, 1H}), 5.57 – 5.40 \text{ (m, 3H)}, 3.75 \text{ (s, 4H)}, 2.85 \text{ (dd, } J = 9.2, 7.6 \text{ Hz, 2H}), 2.60 – 2.44 \text{ (m, 5H)}, 2.28 \text{ (s, 1H)}, 2.24 – 2.06 \text{ (m, 3H)}, 1.90 – 1.77 \text{ (m, 2H)}, 1.75 – 1.58 \text{ (m, 5H)}, 1.47 \text{ (s, 4H)}, 1.19 \text{ (s, 4H)}. \textit{\textsuperscript{13}}C NMR (101 MHz, CDCl\textsubscript{3}) \(\delta 201.96, 171.62, 171.14, 161.37, 143.70, 140.02, 139.48, 137.87, 136.55, 125.98, 125.57, 125.25, 125.18, 124.63, 122.56, 108.45, 75.14, 72.05, 64.10, 53.63, 52.01, 51.39, 43.39, 42.21, 38.20, 35.50, 31.05, 18.18, 16.52, 15.23. \) HRMS (\textit{m/z}): [M+Cl] calculated: 585.1394, observed: 585.5306. Molecular formula: C\textsubscript{30}H\textsubscript{30}O\textsubscript{8}S. Molecular weight: 550.17.
Compound 2-\(O\)-salvinorin B 1\(H\)-indene-2-carboxylate (AK-1406; Figure 37) was synthesized utilizing the general synthesis procedure with 1\(H\)-indene-2-carboxylic acid and yielded an amorphous white solid;\(^\text{1}\)H NMR (400 MHz, Chloroform-\(d\)) \(\delta\) 7.89 – 7.83 (m, 1H), 7.58 – 7.49 (m, 2H), 7.45 – 7.31 (m, 4H), 6.39 (dd, \(J = 1.9, 0.9\) Hz, 1H), 5.53 (dd, \(J = 11.6, 5.1\) Hz, 1H), 5.33 (t, \(J = 10.0\) Hz, 1H), 3.75 (d, \(J = 5.2\) Hz, 5H), 2.87 – 2.77 (m, 1H), 2.56 (dd, \(J = 13.5, 5.2\) Hz, 1H), 2.49 – 2.39 (m, 2H), 2.28 – 2.06 (m, 3H), 1.88 – 1.79 (m, 1H), 1.76 – 1.55 (m, 4H), 1.48 (s, 3H), 1.18 (s, 3H). \(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 202.13, 171.62, 171.14, 163.67, 144.96, 143.71, 142.79, 142.49, 139.45, 135.73, 127.94, 126.98, 125.23, 124.30, 123.63, 108.41, 74.98, 72.06, 64.13, 53.67, 51.99, 51.44, 43.43, 42.19, 38.30, 38.23, 35.50, 31.01, 18.18, 16.48, 15.23. HRMS (\(m/z\)): \([M+\text{Cl}]^+\) calculated: 567.1794, observed: 567.4084. Molecular formula: C\(_{31}\)H\(_{32}\)O\(_8\). Molecular weight: 532.21.
**Pharmacological Results**

*In Vitro Results*

Briefly, the generated C(22)-fused-heteroaromatic salvinorin A analogues were pharmacologically characterized utilizing the following *in vitro* scheme: analysis in a primary competitive radioligand binding affinity assay at DOR/KOR/MOR. analogues that met the 70%-threshold inhibition proceeded to the secondary competitive radioligand binding affinity assay to establish $K_i$ values, with analogues exhibiting $K_i \leq 300$ nM proceeding to the $[^{35}S]GTP[\gamma S]$ binding assay as a measure of functional activity to established EC$_{50}$ at the respective receptor.

In summarizing the *in vitro* data (Table 8), it was observed that benzo[b]thiophene-2 and benzo[b]thiophene-3, AK-1402 and AK-1405, respectively, failed to meet the 70%-threshold inhibition in the primary competitive radioligand binding affinity assay at any of the opioid receptors. This is of interest as they represent the fused-heteroaromatic analogues to the previous high-binding affinity heteroaromatic thiphene-2 and thiophene-3 analogues (Table 3).

The next comparable analogue is P-3l, the benzofuran-2 analogue which in our assays displayed increased binding affinity at DOR and KOR (5-fold and 10-fold, respectively), as well as, increased functional activity at both KOR and MOR (14-fold and 8-fold, respectively) — when compared to previous reported values (Table 5). However, this variation may be accounted for by the use of different cell-lines used to express the respective sourced human opioid receptor cDNA, as those used by the Tidgewell group report DOR activity for their salvinorin A control which was not observed in ours (Leon et al., 2013; Tidgewell et al., 2008).

Shifting the ester-type link from the substituent 2-position to the substituent 3-position in AK-1403, our benzofuran-3 analogue, resulted in abolished DOR/KOR binding affinity and strict MOR binding affinity ($K_i$ of 149 nM). However, the functional activity was reduced when
compared to P-3l (MOR EC<sub>50</sub> of 210 nM reduced to 950 nM) — however, the assessed functional activities of both benzofuran-analogues, P-3l and AK-1403, were higher than that previously reported by Tidgewell for P-3l (MOR EC<sub>50</sub> of 1,680 nM) (Tidgewell et al., 2008).

Incorporation of the indene-2 substituent, AK-1406, resulted in binding affinity at all assessed receptors (Table 8), however only DOR and MOR met the secondary binding affinity threshold of K<sub>i</sub> ≤ 300 nM — with a clear preference for MOR (DOR K<sub>i</sub> of 279 and MOR K<sub>i</sub> of 8). Assessment of functional activity by [<sup>35</sup>S]GTP[γS] binding further corroborated this MOR preference with a MOR EC<sub>50</sub> of 85 nM and a DOR EC<sub>50</sub> of 894 nM.

Of interest was the indole-2 substituent, AK-1401, as this analogue incorporates a nitrogen into salvinorin A — a pharmacophore noted as being unique for being non-nitrogenous. This promoted a MOR/KOR dual-binding affinity, with a 100-fold preference for MOR and functional assessment revealed a MOR EC<sub>50</sub> of 187 nM.
Table 8. *In vitro* data summary of C(22)-fused-heteroaromatic salvinorin A analogues.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Delta pKi ±SEM</th>
<th>Delta Ki (nM) ±SEM</th>
<th>Kappa pKi ±SEM</th>
<th>Kappa Ki (nM) ±SEM</th>
<th>Mu pKi ±SEM</th>
<th>Mu Ki (nM) ±SEM</th>
<th>DOR</th>
<th>KOR</th>
<th>MOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPDPE</td>
<td>7.742 ±0.0707</td>
<td>18.12 ±1.71</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2.526 ±0.427</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>U-69,593</td>
<td>—</td>
<td>—</td>
<td>8.964 ±0.0905</td>
<td>1.087 ±0.132</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>25.62 ±4.12</td>
<td>—</td>
</tr>
<tr>
<td>DAMGO</td>
<td>—</td>
<td>—</td>
<td>9.021 ±0.1034</td>
<td>0.9534 ±0.133</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Salvinorin A</td>
<td>—</td>
<td>—</td>
<td>8.748 ±0.0995</td>
<td>1.787 ±0.0239</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>P-31</td>
<td>7.007 ±0.2051</td>
<td>98.36 ±28.1</td>
<td>8.140 ±0.0663</td>
<td>7.242 ±0.642</td>
<td>7.670 ±0.0993</td>
<td>21.36 ±2.85</td>
<td>367.0 ±66.9</td>
<td>80.88 ±8.03</td>
<td>210.7 ±36.5</td>
</tr>
<tr>
<td>AK-1401</td>
<td>—</td>
<td>—</td>
<td>5.998 ±0.1246</td>
<td>1005 ±169</td>
<td>7.979 ±0.1262</td>
<td>10.49 ±1.79</td>
<td>—</td>
<td>—</td>
<td>187.5 ±51.7</td>
</tr>
<tr>
<td>AK-1402</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
<td>AK-1403</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>6.826 ±0.070</td>
<td>149.40 ±14.0</td>
<td>—</td>
<td>—</td>
<td>950.7 ±458.4</td>
<td></td>
</tr>
<tr>
<td>AK-1405</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AK-1406</td>
<td>6.553 ±0.137</td>
<td>279.9 ±51.9</td>
<td>6.266 ±0.086</td>
<td>541.9 ±62.8</td>
<td>8.068 ±0.063</td>
<td>8.553 ±0.715</td>
<td>894.6 ±50.4</td>
<td>—</td>
<td>85.0 ±7.15</td>
</tr>
</tbody>
</table>

—— indicates that 70% inhibition in primary binding affinity assay was not achieved

— indicates that $K_i \leq 300 \text{ nM}$ in secondary binding affinity was not achieved
**In Vivo Results**

I. **Antinociceptive Related Assays**

Based on the preceding *in vitro* results analogues three of the substituent 2-position analogues were selected for *in vivo* characterization focused towards potential antinociceptive effects: P-3l, AK-1401, and AK-1402.

**Acetic Acid-induced Abdominal Writhing Assay**

As outlined in the experimental section (Chapter 5) the the first chemo-nociceptive mode we employed was an acetic acid-induced abdominal writhing assay. The mice (*n* = 10) were treated with vehicle (10ml/kg, P.O.) as a negative control, indomethacin (20 mg/kg, P.O.) or morphine (10 mg/kg, P.O.) as positive controls. The three selected fused-heteroaromatic salvinorin A analogues were administered at three test doses: 1, 3, 10 mg/kg (P.O.). These treatments were 60 minutes prior to the administration of acetic acid solution (IP) and subsequent 30 minute test period in which writhing movements were measured. All three of the assessed analogues produced statistically significant reduction in writhing movements with P-3l (Figures 38) active at 3 and 10 mg/kg, and AK-1401(Figures 39) and AK-1402 (Figures 40) producing statistically significant reductions at all three tested doses. These writhing reductions are interpreted as a measure of antinociceptive effects in this model.
Figure 38. Acetic acid-induced abdominal writhing assay results: P-3l.

Effect of P-3l (1, 3, 10 mg/kg, P.O.) in the number of acetic acid-induced writhes in mice. Indomethacin (20 mg/kg, P.O.) and morphine (10 mg/kg, P.O.) were used as positive control. Vertical bars represent mean ± SEM of cumulated writhing (%) in 30 min for each experimental group. * $P \leq 0.05$ using ANOVA followed by Dunnet test.

Figure 39. Acetic acid-induced abdominal writhing assay results: AK-1401.

Effect of AK-1401 (1, 3, 10 mg/kg, P.O.) in the number of acetic acid-induced writhes in mice. Indomethacin (20 mg/kg, P.O.) and morphine (10 mg/kg, P.O.) were used as positive control. Vertical bars represent mean ± SEM of cumulated writhing (%) in 30 min for each experimental group. * $P \leq 0.05$ using ANOVA followed by Dunnet test.
The second chemo-nociceptive model we employed was a formalin assay which allows us to examine both direct neurogenic pain and subsequent inflammatory pain. As outlined in the experimental section (Chapter 5) the mice \((n = 7)\) were administered vehicle \((10\text{ml/kg, P.O.) as a negative control in both phases, morphine (10 mg/kg, SC) as a positive control in first and second phases, or indomethacin (20 mg/kg, P.O.) as a positive control in the second phase. The three selected fused-heteroaromatic salvinorin A analogues were administered at three test doses: 1, 3, 10 mg/kg (P.O.). All treatments, except for morphine were administered 60 minutes prior to test, with morphine being administered 30 minutes prior. Once administered the formalin phlogistic agent mice were observed for two periods: 0 to 5 minutes — the first phase (direct

**Figure 40.** Acetic acid-induced abdominal writhing assay results: AK-1402.

Effect of AK-1402 \((1, 3, 10 \text{ mg/kg, P.O.)}\) in the number of acetic acid-induced writhes in mice. Indomethacin \((20 \text{ mg/kg, P.O.)}\) and morphine \((10 \text{ mg/kg, P.O.)}\) were used as positive control. Vertical bars represent mean ± SEM of cumulated writhing \((\%\) in 30 min for each experimental group. \(*P \leq 0.05\) using ANOVA followed by Dunnet test.
neurogenic pain) and from 15 to 30 minutes — the second phase (inflammatory pain). Both P-3l (Figure 41) and AK-1401 (Figure 42) produced statistically significant reduction in paw licking behavior at all three doses, in both neurogenic and inflammatory (1st and 2nd) phases. Interestingly, in this model, AK-1402 (Figure 43), failed to promote a reduction of paw licking behavior during the secondary (inflammatory) phase and was only active in the neurogenic phase at the higher doses of 3 and 10 mg/kg.

**Figure 41.** Formalin assay results: P-31.

Effect of P-3l (1, 3, 10 mg/kg, P.O.), indomethacin (20 mg/kg, P.O.) and morphine (10 \( \mu \)mol/kg P.O.) on the licking time of formalin test, in mice, during the first (0-5 min) and second phase (15-30 min). Vertical bars represent mean ± SEM of pain reaction time, in seconds. * \( P \leq 0.05 \) (compared with 1st phase control group) and # \( P \leq 0.05 \) (compared with 2nd phase control group) using ANOVA followed by Dunnet test.
Figure 42. Formalin assay results: AK-1401.

Effect of AK-1401 (1, 3, 10 mg/kg, P.O.), indomethacin (20 mg/kg, P.O.) and morphine (10 µmol/kg P.O.) on the licking time of formalin test, in mice, during the first (0-5 min) and second phase (15-30 min). Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared with 1st phase control group) and # $P \leq 0.05$ (compared with 2nd phase control group) using ANOVA followed by Dunnet test.
Figure 43. Formalin assay results: AK-1402.

Effect of AK-1402 (1, 3, 10 mg/kg P.O.), indomethacin (20 mg/kg P.O.) and morphine (10 µmoL/kg P.O.) on the licking time of formalin test, in mice, during the first (0-5 min) and second phase (15-30 min). Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared with 1st phase control group) and # $P \leq 0.05$ (compared with 2nd phase control group) using ANOVA followed by Dunnet test.
Opioid Antagonism Assay

To evaluate the preceding formalin assay first phase results for opioid pathway involvement we utilized naloxone antagonist model (as outline in the Experimental Methods, Chapter 5). Briefly, we pretreated mice (n = 7) with either saline (10 ml/kg, IP) as a negative control or naloxone (3 mg/kg; IP) as the opioid antagonist. After 15 minutes the mice received one of four treatments: vehicle, or one of the three selected fused-heteroaromatic salvinorin A analogues (10 mg/kg; P.O.) from the previous assay (P-3l, AK-1401, or AK-1402). Pretreatment with naloxone was found to antagonize the reduction previously observed in licking behavior in the first phase of the formalin assay in a statistically significant manner with all three analogues (Figures 44, 45, 45), supporting the involvement of the opioid receptors in mediating the antinocicptive effect in this model.

Figure 44. Opioid antagonism assay results: P-3l.

Effect of pre-treatment with naloxone (3 mg/kg, IP) or saline (10 mL/kg, IP) in the P-3l 10 mg/kg P.O. analgesic activity in the first phase (0-5 min) of formalin test, in mice. Vertical bars represent mean ± SEM of pain reaction time, in seconds. * P ≤ 0.05 (compared control group) and # P ≤ 0.05 (compared with respectively treated group) using ANOVA followed by Dunnet test.
Figure 45. Opioid antagonism assay results: 1401.

Effect of pre-treatment with naloxone (3 mg/kg, IP) or saline (10 mL/kg, IP) in the AK-1401 10 mg/kg P.O. analgesic activity in the first phase (0-5 min) of formalin test, in mice. Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared control group) and # $P \leq 0.05$ (compared with respectively treated group) using ANOVA followed by Dunnet test.
Figure 46. Opioid antagonism assay results: 1402.

Effect of pre-treatment with naloxone (3 mg/kg, IP) or saline (10 mL/kg, IP) in the AK-1402 3 mg/kg P.O. analgesic activity in the first phase (0-5 min) of formalin test, in mice. Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared control group) and # $P \leq 0.05$ (compared with respectively treated group) using ANOVA followed by Dunnet test.
Hot Plate Assay

As our thermo-nociceptive model we utilized a hot plate assay. In this model, mice \( (n = 8) \) were treated either with vehicle control (10 ml/kg, P.O.), morphine (5 mg/kg, P.O.) as a positive control, or one of the three selected fused-heteroaromatic salvinorin A analogues (P-3l, AK-1401, AK-1402) assessed at 1, 3, and 10 mg/kg (P.O.). A 30 minute pretreatment latency baseline was established for each test mouse. After treatment latency response to thermal pain was measured every 30 minutes (0-180 minutes). In this model, both P-3l (Figure 47) and AK-1401 (Figure 48) produced statistically significant increase in latency response to thermal pain at doses 3 and 10 mg/kg for the entire 180 minute test duration. AK-1402 also produced a

Figure 47. Hot plate assay results: P-3l.

Effect of P-3l (1, 3, 10 mg/kg, P.O.) or morphine (10 mg/kg, P.O.- positive control) on the hot plate test, in mice \( (n = 8) \). The values were expressed as mean ± SEM of the latency to nociceptive behavior, in seconds. \( *P \leq 0.05 \) compared to control group, according to two-way ANOVA followed by Bonferroni’s post-hoc test.
statistically significant increase in latency response to thermal pain at 3 and 10 mg/kg for 150 minutes of the test period before returning to baseline by 180 minutes (Figure 49). Additionally, both P-3l and AK-1402 also showed activity at the 1 mg/kg dose from 60 to 150 minutes.

**Figure 48.** Hot plate assay results: AK-1401.

Effect of AK-1401 (1, 3, 10 mg/kg, P.O.) or morphine (10 mg/kg, P.O., positive control) on the hot plate test, in mice (n = 8). The values were expressed as mean ± SEM of the latency to nociceptive behavior, in seconds. *P ≤ 0.05 compared to control group, according to two-way ANOVA followed by Bonferroni’s post-hoc test.
Figure 49. Hot plate assay results: AK-1402.

Effect of AK-1402 (1, 3, 10 mg/kg P.O.) or morphine (10 mg/kg, P.O., positive control) on the hot plate test, in mice (n = 8). The values were expressed as mean ± SEM of the latency to nociceptive behavior, in seconds. *P ≤ 0.05 compared to control group, according to two-way ANOVA followed by Bonferroni’s post-hoc test.
II. Antidepressant Related Assays

Based upon the initial *in vitro* results establishing the preference of AK-1401 for MOR (100-fold more than KOR) and the MOR functional activity at EC$_{50}$ of 187 nM, as well as, the *in vitro* antinociceptive activities established in both chemo-nociceptive and thermo-nociceptive models this analogue was selected for further characterization for potential antidepressant-like activity.

**Forced SwimTest**

Our first model used to assess potential antidepressant-like effects of AK-1401 was the forced swim test that measures effect on swimming time and immobility. Test mice ($n = 10$) were treated with either a vehicle control (10 ml/kg, P.O.), imipramine (IMI, 15 mg/kg, P.O.) or

![Figure 50. Forced swim test, swimming time results: AK-1401.](image)

The effect of oral administration of vehicle, AK-1401 at 1, 3, 10 mg/kg, positive control imipramine (IMI) at 15 mg/kg, and vehicle on the swimming time (B) and immobility time (C) in the forced swimming test. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05, *P < 0.01 or *P < 0.001 versus vehicle treated group (one way ANOVA followed by Dunnett´s test).
AK-1401 at 1, 3, or 10 mg/kg (P.O.) and assessed one hour after for activity. AK-1401 was found to produce statistically significant increases in swimming time (Figure 50) and reduced immobility time (Figure 51) at doses 3 and 10 mg/kg. These effects are interpreted as antidepressant-like activity in this model.

**Figure 51.** Forced swim test, immobility time results: AK-1401.

The effect of oral administration of vehicle, AK-1401 at 1, 3, 10 mg/kg, positive control imipramine (IMI) at 15 mg/kg, and vehicle on the swimming time (B) and immobility time (C) in the forced swimming test. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05, **P < 0.01 or ***P < 0.001 versus vehicle treated group (one way ANOVA followed by Dunnett´s test).
Tail Suspension Test

Our second model used to assess potential antidepressant-like effects of AK-1401 was a modified version of tail suspension test (TST) that measures immobility. Test mice (n = 10) were treated with either a vehicle control (10 ml/kg, P.O.), imipramine (IMI, 15 mg/kg, P.O.) or AK-1401 at 1, 3, or 10 mg/kg (P.O.) and assessed one hour after for activity. Once more, AK-1401 was found to produce statistically significant activity (i.e. reduction in immobility) at doses 3 and 10 mg/kg (Figure 52) — these results further indicate antidepressant-like activity.

![Figure 52. Tail suspension test results: AK-1401.](image)

The effect of oral administration of AK-1401 at the doses of 1, 3, 10 mg/kg and positive control imipramine (IMI) 15 mg/kg or vehicle on immobility time in the tail suspension test (one-way ANOVA followed by Dunnett’s test).
III. Anxiolytic Related Assay

Open Field Test

Lastly, we utilized a circular open field test (OFT) to assess the potential anxiolytic-like effects of AK-1401 on exploratory activity with the endpoints being: freezing time, total sectorial crossing, rearing number, and center crossing. Test mice (n = 10) were treated with either vehicle (10 ml/kg, P.O.), diazepam (DZP, 5 mg/kg, P.O.), or AK-1401 at 1, 3, or 10 mg/kg (P.O.). However, AK-1401 was largely ineffective at altering these behaviors (Figures 53 and 54), except for center crossing (Figure 54-D) where it significantly increased this exploratory behavior. While these results largely indicate that AK-1401 does not promote anxiolytic-like effects in mice, the effect on exploratory behavior may suggest that further anxiolytic characterization would be advisable.
Figure 53. Open field test results, Freezing time and Total crossing: AK-1401.

The effect of acute oral administration of AK-1401 at 1, 3, 10 mg/kg, positive control diazepam (DZP) 5 mg/kg, or vehicle in the open field exploratory assay on freezing time (A), the total crossing (B), number of rearing events. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 and ***P<0.001 versus vehicle treated group (one way ANOVA followed by Dunnett’s test).
Figure 54. Open field test results, Number of rearing and crossing at the center: AK-1401.

The effect of acute oral administration of AK-1401 at 1, 3, 10 mg/kg, positive control diazepam (DZP) 5 mg/kg, or vehicle in the open field exploratory assay on freezing time (C) and crossing at the center of the open-field (D). Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 and ***P<0.001 versus vehicle treated group (one way ANOVA followed by Dunnett´s test).
Computational Modeling

Opioid Receptor Docking Studies

KOR Docking Studies

The Schrödinger Prime protein-structure prediction program was utilized to calculate the molecular mechanics with generalized born surface area (MM-GBSA) of both salvinorin A and AK-1401 complexed with KOR (Schrödinger, 2017; Vilar et al., 2010). As expected from in vitro binding affinity studies (Table 8; in vitro established salvinorin A KOR $K_i$ of 0.9 nM and AK-1401 KOR $K_i$ of 1.005 nM), our docking calculations indicated a lower binding free energy for the KOR/salvinorin A complex (Binding Energy $= -61.674$ kcal/mol) than for the KOR/AK-1401 complex (Binding Energy $= -53.046$ kcal/mol). Additionally, this optimized KOR model showed that AK-1401 possessed a binding orientation analogous to salvinorin A except at the C(22)-position indole moiety (Figure 55). This predicted orientation of the AK-1401 indole moiety differed from the acetoxy moiety of salvinorin A due to increased solvent exposure, lack of significant hydrophobic and polar interactions — which may account for the lower experimentally derived KOR binding affinity of AK-1401 compared to salvinorin A (Table 8).

The predicted binding pose indicated the fused-tricyclic core of salvinorin A is vertically oriented towards TM2, TM3, and TM6 and surrounded, primarily, by the side chains of residues: Gln-115, Ile-135, Asp-138, Tyr-139, Met-142, Val-230, Ile-294, and Ile-316 (Figure 56). Salvinorin A exhibited strong hydrophobic interactions with Tyr-139, Met-142, Ile-294, and Ile-316. The ketone moiety at the C(1)-position hydrogen-bonded with Gln-115. The C(12)-position furan moiety interacted in a deeper sub-pocket characterized by key interactions with Tyr-320 and Trp-287 through aromatic $\pi$-$\pi$ stacking and exhibited hydrophobic interactions with residues: Val-108, Met-142, Ile-290, and Ile-316. The C(2)-position acetoxy-group was shown
Figure 55. Overlay of AK-1401 (cyan carbons) and salvinorin A (yellow carbons).

to hydrogen-bond with Gln-115 and was delineated by residues: Ile-135, Asp-138, and Cys-210. The C(4)-position carbomethoxy was situated in a small pocket bound by residues: Lys-227, Glu-297, Leu-212 and Tyr-312 (Figure 57). Interestingly, this optimized docking pose was found to correspond well with a previously reported site-directed mutagenesis derived docking pose of salvinorin A (Kane et al., 2006). The primary difference observed between these two
proposed docking poses is that at the C(2)-position the Kane group reported a Tyr-313 hydrophobic interaction while in our model the interaction is calculated to be with Gln-115 (Figure 57).

**Figure 56.** Predicted binding orientation of salvinorin A (yellow carbons) into the active-state KOR model (key active site residues shown with grey carbons).
Figure 57. Predicted key-residue interactions of KOR/salvinroin A complex.
MOR Docking Studies

Similar to the method described for our KOR model, a receptor grid was prepared for MOR centered on the centroid of the co-crystallized agonist (BU72) of PDB: 5C1M. The amino acid terminus (residues Gly-52-Met-65) of the active state MOR forms a lid over the putative orthosteric site. Therefore, we utilized the Induced Fit docking protocol within the Schrödinger software suite to examine the potential MOR/AK-1401 orientation and interactions, chosen due to the activity established in the previous in vitro and in vivo assays. This model was optimized utilizing the OPLS3 force field optimization and extra-precision docking mode of the Glide module in the Schrödinger software suite, without the inclusion of specified docking constraints — to allow for a comprehensive docking range to be examined.

The docking results, and MM-GBSA calculations for each, provided a range of putative binding poses for AK-1401 within the active state MOR model. The best pose based on calculated Glide Gscore value (Glide Gscore = $-9.887$) and binding energy value (Binding Energy = $-96.604$ kcal/mol) revealed that AK-1401 exhibited strong hydrogen-bonding between Gln-124 and the amine on the C(22)-indole, as well as at the ketone moiety of the C(2)-position (Figure 58). In this pose, the C(22)-indole moiety was bounded by key residues (Tyr-128, Trp-318, Phe-320 and Ile-322) forming a hydrophobic-pocket. In addition, the C(22)-indole moiety also participated in aromatic $\pi-\pi$ stacking with His-319 and Trp-318. The C(12)-position furanyl moiety showed hydrogen-bonding with Gly-52 and aromatic $\pi-\pi$ stacking with Phe-221. The C(4)-position carbomethoxy was oriented towards a sub-pocket delineated by residues: Asp-147, Tyr-148 and Met-151. Interestingly, this pose of AK-1401 (Figure 59) did not show direct interactions, such as hydrogen-bonding, with Asp-147 — an interaction which is largely
conserved for MOR classical opioid ligands but not observed with salvinorin A (Manglik et al., 2012; Yuan et al., 2015; Yuan et al., 2013).

Figure 58. Predicted key-residue interactions of MOR/AK-1401 complex.
Figure 59. The putative binding mode of AK-1401 (yellow carbons) into the active-state MOR model (key active site residues shown with grey carbons).
ADME/T Calculations

Lastly, due to the high degree of homology with this bioisosteric C(22)-fused-heteroaromatic salvinorin A analogue series the Schrödinger QikProp module calculated ADME/T properties were similarly homologous (Table 9) and, largely, indicated reasonable drug-like properties for this series (with Schrödinger QikProp recommend range values derived from a dataset comprised of 95% of currently marketed drugs; Schrödinger, 2016). The predicted logP and logS values were in the recommended range to indicate this series would likely possess good aqueous solubility and low lipophilicity. Additionally, except for a single violation (due to high molecular weights) this series also follows Lipinski’s Rule of Five, supporting ease of solubility and oral availability of these compounds. This was further supported by high Percent Human Oral Absorption values (all above 80% which predicts good oral availability) — maintained by our in vivo models by effective use of P.O. administration route. The only calculated property of concern was the QPlogHERG values, which were outside the recommend range — indicating potential hERG potassium channel interaction and possible drug-induced cardiac effects (i.e. cardiac arrhythmia) (Sanguinetti and Tristani-Firouzi, 2006).
<table>
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<tr>
<th>Properties</th>
<th>P-31</th>
<th>AK-1401</th>
<th>AK-1402</th>
<th>AK-1403</th>
<th>AK-1404</th>
<th>AK-1405</th>
<th>AK-1406</th>
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</table>

Physicochemical properties calculated using QikProp. Range is for 95% of known drugs. Recommended range, based on that of 95% of marketed drugs: Molecular weight (130–725 Da); † Van der Waals surface area of polar nitrogen and oxygen atoms (7–200 Å^2); ‡ Predicted octanol/water partition coefficient (−2–6.5); § Predicted aqueous solubility, log S (−6.5–0.5); ¶ Predicted apparent Caco-2 cell permeability (<25 nm/s poor; >500 nm/s great); Γ Predicted apparent MDCK cell permeability (<25 nm/s poor; >500 nm/s great); Δ Predicted IC50 value for blockage of HERG K^+ channels (it is a serious concern if below −5); β Prediction of binding to human serum albumin (−1.5–1.5 acceptable). CNS range: -2 (inactive) to +2 (active).

Schrödinger, 2017.
CHAPTER 7: Conclusions

This study further supports the importance of the continued development of new salvinorin A analogues as essential research tools to ascertain potential three-dimensional ligand binding requirements, functional activities, and pharmacological consequences mediated through the clinically important opioid receptors. This series of pharmacologically evaluated C(22)-fused-heteroaromatic salvinorin A analogues (i.e. P-3l, AK-1401, AK-1402, AK-1403, AK-1405, and AK-1406) revealed a range of activities. Though it would be difficult to conclude a definitive structure-activity-relationship, trends are observed in the generated data that allow for a number of tentative conclusions:

- This study corroborates the effect that aromaticity has on inducing MOR binding affinity in salvinorin A — a lead pharmacophore typified by KOR selectivity.
  
  With one caveat: this trend was not observed with either of the benzo[b]thiophene-analogues (AK-1402 and AK-1405) as neither produced minimum inhibition in the primary competitive radioligand binding assay. However, subsequent in vivo antinociceptive assessment of AK-1402, in both chemo-nociceptive (acetic acid-induced abdominal writhing assay and formalin assay, first-phase) and thermo-nociceptive (hot plate assay) models, revealed statistically significant activity mediated
through the opioid receptors (determined via the opioid antagonism assay). — indicating further assessment would be advisable

○ This study further supports the potential for oral administration of aromatic analogues of salvinorin A — a lead pharmacophore typified by oral inactivity.

This route of administration was previously employed for PR-38 in models of gastrointestinal hypermotility and antipruritic activity (Table 6) and was the elected route of effective administration (i.e. active) in our study for P-3l, AK-1401, and AK-1402 in respective antinociceptive and antidepressant-like assays.

○ This study further bolsters the development of salvinorin A analogues towards potential clinical applications, specifically, antinociception and depression, with increased durations of activity when compared to other salvinorin compounds.

This line of development has a steadily growing body of literature indicating potentially useful clinical applications (e.g. antinociceptive, anti-addictive, antipruritic, neuroprotective, etc.) of not only the lead, salvinorin A, but an increasing number of analogues, such as Herkinorin (avoids β-arrestin-2 recruitment, receptor internalization, and tolerance development), PR-38 (orally active, promotes both antinociceptive and antipruritic activities) and, in this study, P-3l, AK-1401, and AK-1402 (respectively, promoting statistically significant antinociceptive and antidepressant-like effects).
Lastly, the computationally derived ADME/T properties of this study indicated potential hERG potassium channel interaction by this series of salvinorin A analogues as all predicted values were outside of the recommended safety range.

While this pharmacological contradiction has not been reported in the salvinorin class, due to the potential for drug-induced cardiac effects (i.e. cardiac arrhythmia) mediated through hERG potassium channel interaction, assessment would be advisable prior to further drug development considerations.
LIST OF REFERENCES


Schrödinger Release 2016-1: LigPrep, Schrödinger, LLC, New York, NY.

Schrödinger Release 2016-1: Maestro, Schrödinger, LLC, New York, NY.

Schrödinger Release 2016-1: QikProp, Schrödinger, LLC, New York, NY.


APPENDICES
APPENDIX A:

$^1$H NMR Assignments
Calculated $^1$H NMR of Salvinorin A

Calculated $^1$H NMR of P-3l
Calculated $^1H$ NMR of AK-1401

Calculated $^1H$ NMR of AK-1402
Calculated $^1$H NMR of AK-1403

Calculated $^1$H NMR of AK-1404
Calculated $^1$H NMR of AK-1405

Calculated $^1$H NMR of AK-1406
Calculated $^1$H NMR of AK-1407
APPENDIX B:

\(^{13}\)C NMR Assignments
Calculated $^{13}$C NMR of Salvinorin A

Calculated $^{13}$C NMR of P-3l
Calculated $^{13}$C NMR of AK-1401

Calculated $^{13}$C NMR of AK-1402
Calculated $^{13}$C NMR of AK-1403

Calculated $^{13}$C NMR of AK-1404
Calculated $^{13}$C NMR of AK-1405

Calculated $^{13}$C NMR of AK-1406
Calculated $^{13}$C NMR of AK-1407
APPENDIX C:

Measured $^1\text{H}$ and $^{13}\text{C}$ NMR Spectra
Measured $^1$H NMR of Salvinorin A
Measured $^{13}$C NMR of Salvinorin A
Measured $^1$H NMR of P-3l
Measured $^{13}$C NMR of P-3l
Measured $^1$H NMR of AK-1401
Measured $^{13}$C NMR of AK-1401
Measured $^1$H NMR of AK-1402
Measured $^{13}$C NMR of AK-1402
Measured $^1$H NMR of AK-1403
Measured $^{13}\text{C}$ NMR of AK-1403
Measured $^1$H NMR of AK-1405
Measured $^{13}$C NMR of AK-1405
Measured $^1$H NMR of AK-1406
Measured $^{13}$C NMR of AK-1406
APPENDIX D:

In Vitro Datasets
Binding Affinity of Salvinorin A

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kappa pKi +/- SEM</th>
<th>Kappa Ki (nM) +/- SEM</th>
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</thead>
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<tr>
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<td>9.021±0.1034</td>
<td>0.9534±0.133</td>
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<tr>
<td>Naloxone HCl</td>
<td>8.964±0.0905</td>
<td>1.087±0.132</td>
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![Graph showing binding affinity](image)

<table>
<thead>
<tr>
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<th>Salvinorin A</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>IC50</td>
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<tr>
<td>Ki</td>
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Binding Affinity of P-3I

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<tr>
<th>Compound</th>
<th>Delta pKi +/- SEM</th>
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<td>7.007±0.2051</td>
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<td>Naloxone HCl</td>
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- **IC50**: 1.967e-007 (P-3I) 3.624e-008 (Control)
- **Ki**: 9.836e-008 (P-3I) 1.812e-008 (Control)
### Binding Affinity of P-3l

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<td>Naloxone HCl</td>
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#### IC50 and Ki Values

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<td>Ki</td>
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Binding Affinity of P-3l

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<th>Compound</th>
<th>Mu pKi +/- SEM</th>
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<tr>
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<td>Naloxone HCl</td>
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![Graph showing binding affinity of P-3l and control](image-url)

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**[^35S]GTP[γS] Opioid Functional Assessment for P-3l**

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<tr>
<th>Compound</th>
<th>Type</th>
<th>Delta EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Type</th>
<th>Kappa EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<th>Mu EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tr>
<td>P-3l</td>
<td>Full Agonist</td>
<td>367.0 ± 66.9</td>
<td>Full Agonist</td>
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<td>Full Agonist</td>
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<tr>
<td>Control</td>
<td>DPDPE</td>
<td>2.526 ± 0.427</td>
<td>U69,693</td>
<td>25.62 ± 4.12</td>
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**GTPγS Delta Agonist Functional Assay**

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<td>-8.598</td>
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**GTPγS Kappa Agonist Functional Assay**

- **LogEC50**: -7.092, -7.591
- **EC50**: 8.088e-008, 2.562e-008

**GTPγS Mu Agonist Functional Assay**

- **LogEC50**: -6.676, -8.142
- **EC50**: 2.107e-007, 7.204e-009
Binding Affinity of AK-1401

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![Graph showing binding affinity](image)

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<td>1.066e-009</td>
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</tbody>
</table>
The table displays the results of the GTPγS Opioid Functional Assessment for AK-1401. The table includes the following columns: Compound, Type, Delta EC50 (nM), Kappa EC50 (nM), Mu EC50 (nM), and Type of agonist.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type</th>
<th>Delta EC50 (nM)</th>
<th>Kappa EC50 (nM)</th>
<th>Type</th>
<th>Mu EC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-1401</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Full Agonist</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>DAMGO</td>
</tr>
</tbody>
</table>

![Graph showing the GTPγS Mu Agonist Functional Assay](image-url)

The graph illustrates the percent over basal response to various concentrations of the compound and control. The log concentration values are as follows:

<table>
<thead>
<tr>
<th>LogEC50</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>-6.727</td>
<td>1.875e-007</td>
</tr>
<tr>
<td>-8.378</td>
<td>4.185e-009</td>
</tr>
</tbody>
</table>
Binding Affinity of AK-1403

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mu pKi +/- SEM</th>
<th>Mu Ki (nM) +/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-1403</td>
<td>6.826 ±0.070</td>
<td>149.4 ±14.0</td>
</tr>
<tr>
<td>Naloxone HCl</td>
<td>8.718 ±0.071</td>
<td>1.914 ±0.182</td>
</tr>
</tbody>
</table>

**Binding Assay - Mu**

<table>
<thead>
<tr>
<th></th>
<th>AK-1403</th>
<th>Naloxone - Mu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki</td>
<td>1.494e-007</td>
<td>1.755e-009</td>
</tr>
<tr>
<td>IC50</td>
<td>2.988e-007</td>
<td>3.511e-009</td>
</tr>
</tbody>
</table>
[\textsuperscript{35}S]GTP[\gamma S] Opioid Functional Assessment for AK-1403

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type</th>
<th>Delta EC\textsubscript{50} (nM)</th>
<th>Type</th>
<th>Kappa EC\textsubscript{50} (nM)</th>
<th>Type</th>
<th>Mu EC\textsubscript{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-1403</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Partial agonist 950.7 ± 458.4</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>DAMGO 22.71 ± 2.96</td>
</tr>
</tbody>
</table>

GTP\textsubscript{\gamma}S Mu Agonist Functional Assay

<table>
<thead>
<tr>
<th></th>
<th>AK-1403</th>
<th>DAMGO</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC\textsubscript{50}</td>
<td>9.507e-007</td>
<td>3.963e-008</td>
</tr>
</tbody>
</table>
## Binding Affinity of AK-1406

<table>
<thead>
<tr>
<th>Compound</th>
<th>Delta pKi +/- SEM</th>
<th>Delta Ki (nM) +/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-1406</td>
<td>6.553 ±0.137</td>
<td>279.9 ±51.9</td>
</tr>
<tr>
<td>Naloxone HCl</td>
<td>7.522 ±0.059</td>
<td>30.03 ±2.36</td>
</tr>
</tbody>
</table>

### Binding Assay - Delta

![Binding Assay - Delta](image)

<table>
<thead>
<tr>
<th></th>
<th>AK-1406</th>
<th>Naloxone - Delta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki</td>
<td>2.799e-007</td>
<td>3.094e-008</td>
</tr>
<tr>
<td>IC50</td>
<td>5.599e-007</td>
<td>6.188e-008</td>
</tr>
</tbody>
</table>
## Binding Affinity of AK-1406

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kappa pKi +/- SEM</th>
<th>Kappa Ki (nM) +/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-1406</td>
<td>6.266 ±0.086</td>
<td>541.9 ±62.8</td>
</tr>
<tr>
<td>Naloxone HCl</td>
<td>8.648 ±0.071</td>
<td>2.249 ±0.212</td>
</tr>
</tbody>
</table>

### Binding Assay - Kappa

![Graph of binding assay](#)

<table>
<thead>
<tr>
<th>Compound</th>
<th>AK-1406</th>
<th>Naloxone - Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki</td>
<td>5.419e-007</td>
<td>2.572e-009</td>
</tr>
<tr>
<td>IC50</td>
<td>1.084e-006</td>
<td>5.143e-009</td>
</tr>
</tbody>
</table>
## Binding Affinity of AK-1406

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mu pKi +/- SEM</th>
<th>Mu Ki (nM) +/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-1406</td>
<td>8.068 ±0.063</td>
<td>8.553 ±0.715</td>
</tr>
<tr>
<td>Naloxone HCl</td>
<td>8.718 ±0.071</td>
<td>1.914 ±0.182</td>
</tr>
</tbody>
</table>

### Binding Assay - Mu

![Graph showing binding assay data for AK-1406 and Naloxone - Mu](image)

<table>
<thead>
<tr>
<th></th>
<th>AK-1406</th>
<th>Naloxone - Mu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki</td>
<td>8.553e-009</td>
<td>1.858e-009</td>
</tr>
<tr>
<td>IC50</td>
<td>1.711e-008</td>
<td>3.716e-009</td>
</tr>
</tbody>
</table>
GTPγS Delta Agonist Functional Assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type</th>
<th>Delta EC₅₀ (nM)</th>
<th>Type</th>
<th>Kappa EC₅₀ (nM)</th>
<th>Type</th>
<th>Mu EC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-1406</td>
<td>Full agonist</td>
<td>894.5 ± 50.4</td>
<td>—</td>
<td>—</td>
<td>Full agonist</td>
<td>82.0 ± 7.15</td>
</tr>
<tr>
<td>Control</td>
<td>DPDPE</td>
<td>1.121 ± 0.093</td>
<td>—</td>
<td>—</td>
<td>DAMGO</td>
<td>22.71 ± 2.96</td>
</tr>
</tbody>
</table>

GTPγS Delta Agonist Functional Assay

% over basal

<table>
<thead>
<tr>
<th></th>
<th>AK-1406</th>
<th>DPDPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀</td>
<td>8.946e-007</td>
<td>1.204e-009</td>
</tr>
</tbody>
</table>
GTP\gamma S Mu Agonist Functional Assay

% over basal

\( \text{Log(M)} \)

<table>
<thead>
<tr>
<th></th>
<th>AK-1406</th>
<th>DAMGO</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>8.200e-008</td>
<td>2.343e-008</td>
</tr>
</tbody>
</table>
## Opioid Binding Assays

<table>
<thead>
<tr>
<th></th>
<th>DOR</th>
<th>KOR</th>
<th>MOR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay Buffer:</strong></td>
<td>50 mM Tris-HCl, pH 7.4 Buffer</td>
<td>50 mM Tris-HCl, pH 7.4 Buffer</td>
<td>50 mM Tris-HCl, pH 7.4 Buffer</td>
</tr>
<tr>
<td><strong>Assay Volume:</strong></td>
<td>0.2 mL</td>
<td>0.2 mL</td>
<td>0.2 mL</td>
</tr>
<tr>
<td><strong>Radioligand:</strong></td>
<td>Enkephalin(DPDPE), [Tyrosyl-3,5-3H(N)]</td>
<td>U-69,593, [Phenyl-3,4-3H]</td>
<td>DAMGO, [Tyrosyl-3,5-3H(N)]</td>
</tr>
<tr>
<td><strong>Radioligand</strong></td>
<td>Perkin Elmer, Cat# NET922</td>
<td>Perkin Elmer, Cat# NET952</td>
<td>Perkin Elmer, Cat# NET902</td>
</tr>
<tr>
<td><strong>Radioligand</strong></td>
<td>1.615 nM</td>
<td>1.197 nM</td>
<td>2.295 nM</td>
</tr>
<tr>
<td><strong>Receptor Membrane:</strong></td>
<td>HEKhDOR P7 1/20/17</td>
<td>HEKhKOR P10 1/26/17</td>
<td>HEKhMOR P15 5/3/16</td>
</tr>
<tr>
<td><strong>Membrane Concentration:</strong></td>
<td>7 µg/well</td>
<td>3 µg/well</td>
<td>25 µg/well</td>
</tr>
<tr>
<td><strong>Nonspecific Binding Control (NSB):</strong></td>
<td>DPDPE (Tocris Bioscience, Cat# 1431)</td>
<td>U69,593 (Tocris Bioscience, Cat# 1171)</td>
<td>DAMGO (Tocris Bioscience, Cat# 1171)</td>
</tr>
<tr>
<td><strong>NSB Concentration:</strong></td>
<td>10 µM</td>
<td>10 µM</td>
<td>10 µM</td>
</tr>
<tr>
<td><strong>Assay Incubation:</strong></td>
<td>60 min @ room temperature</td>
<td>60 min @ room temperature</td>
<td>60 min @ room temperature</td>
</tr>
<tr>
<td><strong>Filter Plate:</strong></td>
<td>UniFilter-96 GF/B, pre-treated w/0.3% BSA</td>
<td>UniFilter-96 GF/B, pre-treated w/0.3% BSA</td>
<td>UniFilter-96 GF/B, pre-treated w/0.3% BSA</td>
</tr>
<tr>
<td><strong>Range of compound dilutions:</strong></td>
<td>100 µM nM – 560 pM</td>
<td>100 µM nM – 560 pM</td>
<td>100 µM nM – 560 pM</td>
</tr>
<tr>
<td><strong>Range of control dilutions:</strong></td>
<td>3 µM – 17 pM</td>
<td>3 µM – 17 pM</td>
<td>3 µM – 17 pM</td>
</tr>
<tr>
<td><strong>Fold increment for serial dilution:</strong></td>
<td>3-fold</td>
<td>3-fold</td>
<td>3-fold</td>
</tr>
</tbody>
</table>
APPENDIX E:

In Vivo Datasets
I. Antinociceptive related assays

Acetic Acid-induced Abdominal Writhing Assay

Effect of P-31 (1, 3, 10 mg/kg, P.O.) in the number of acetic acid-induced writhes in mice. Indomethacin (20 mg/kg P.O.) and morphine (10 mg/kg P.O.) were used as positive control. Vertical bars represent mean ± SEM of cumulated writhing (%) in 30 min for each experimental group. * $P \leq 0.05$ using ANOVA followed by Dunnet test.
Effect of AK-1401 (1, 3, 10 mg/kg, P.O.) in the number of acetic acid-induced writhes in mice. Indomethacin (20 mg/kg P.O.) and morphine (10 mg/kg P.O.) were used as positive control. Vertical bars represent mean ± SEM of cumulated writhing (%) in 30 min for each experimental group. * $P \leq 0.05$ using ANOVA followed by Dunnet test.
Effect of AK-1402 (1, 3, 10 mg/kg, P.O.) in the number of acetic acid-induced writhes in mice. Indomethacin (20 mg/kg P.O.) and morphine (10 mg/kg P.O.) were used as positive control. Vertical bars represent mean ± SEM of cumulated writhing (%) in 30 min for each experimental group. *$P \leq 0.05$ using ANOVA followed by Dunnet test.
Formalin Assay

Effect of P-31 (1, 3, 10 mg/kg, P.O.), indomethacin (20 mg/kg, P.O.) and morphine (10 µmoL/kg P.O.) on the licking time of formalin test, in mice, during the first (0-5 min) and second phase (15-30 min). Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared with 1st phase control group) and $^\# P \leq 0.05$ (compared with 2nd phase control group) using ANOVA followed by Dunnet test.
Effect of AK-1401 (1, 3, 10 mg/kg, P.O.), indomethacin (20 mg/kg, P.O.) and morphine (10 μmol/kg P.O.) on the licking time of formalin test, in mice, during the first (0-5 min) and second phase (15-30 min). Vertical bars represent mean ± SEM of pain reaction time, in seconds. *$P \leq 0.05$ (compared with 1st phase control group) and #$P \leq 0.05$ (compared with 2nd phase control group) using ANOVA followed by Dunnet test.
Effect of AK-1402 (1, 3, 10 mg/kg P.O.), indomethacin (20 mg/kg P.O.) and morphine (10 µmol/kg P.O.) on the licking time of formalin test, in mice, during the first (0-5 min) and second phase (15-30 min). Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared with 1st phase control group) and # $P \leq 0.05$ (compared with 2nd phase control group) using ANOVA followed by Dunnet test.
Opioid Antagonism Assay

Effect of pre-treatment with naloxone (3 mg/kg, IP) or saline (10 mL/kg, IP) in the P-31 10 mg/kg P.O. analgesic activity in the first phase (0-5 min) of formalin test, in mice. Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared control group) and # $P \leq 0.05$ (compared with respectively treated group) using ANOVA followed by Dunnet test.
Effect of pre-treatment with naloxone (3 mg/kg, IP) or saline (10 mL/kg, IP) in the AK-1401 10 mg/kg P.O. analgesic activity in the first phase (0-5 min) of formalin test, in mice. Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared control group) and # $P \leq 0.05$ (compared with respectively treated group) using ANOVA followed by Dunnet test.
Effect of pre-treatment with naloxone (3 mg/kg, IP) or saline (10 mL/kg, IP) in the AK-1402 3 mg/kg P.O. analgesic activity in the first phase (0-5 min) of formalin test, in mice. Vertical bars represent mean ± SEM of pain reaction time, in seconds. * $P \leq 0.05$ (compared control group) and # $P \leq 0.05$ (compared with respectively treated group) using ANOVA followed by Dunnet test.
Effect of P-31 (1, 3, 10 mg/kg, P.O.) or morphine (10 mg/kg, P.O.- positive control) on the hot plate test, in mice (n = 8). The values were expressed as mean ± SEM of the latency to nociceptive behavior, in seconds. *P ≤ 0.05 compared to control group, according to two-way ANOVA followed by Bonferroni’s post-hoc test.
Effect of AK-1401 (1, 3, 10 mg/kg, P.O.) or morphine (10 mg/kg, P.O., positive control) on the hot plate test, in mice ($n = 8$). The values were expressed as mean ± SEM of the latency to nociceptive behavior, in seconds. *$P \leq 0.05$ compared to control group, according to two-way ANOVA followed by Bonferroni’s post-hoc test.
Effect of AK-1402 (1, 3, 10 mg/kg P.O.) or morphine (10 mg/kg, P.O., positive control) on the hot plate test, in mice (n = 8). The values were expressed as mean ± SEM of the latency to nociceptive behavior, in seconds. *P ≤ 0.05 compared to control group, according to two-way ANOVA followed by Bonferroni’s post-hoc test.
II. Antidepressant related assays

Forced swimming test

The effect of oral administration of vehicle, AK-1401 at 1, 3, 10 mg/kg, positive control imipramine (IMI) at 15 mg/kg, and vehicle on the swimming time (B) and immobility time (C) in the forced swimming test. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05, *P < 0.01 or *P < 0.001 vs vehicle treated group (one way ANOVA followed by Dunnett’s test).
Tail suspension test

The effect of oral administration of AK-1401 at the doses of 1, 3, 10 mg/kg and positive control imipramine (IMI) 15 mg/kg or vehicle on immobility time in the tail suspension test (one-way ANOVA followed by Dunnett’s test).
Open Field Test

Open field test results, Freezing time and Total crossing: AK-1401.

The effect of acute oral administration of AK-1401 at 1, 3, 10 mg/kg, positive control diazepam (DZP) 5 mg/kg, or vehicle in the open field exploratory assay on freezing time (A), the total crossing (B), number of rearing events. Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 and ***P<0.001 versus vehicle treated group (one way ANOVA followed by Dunnett´s test).
Open field test results, Number of rearing and crossing at the center: AK-1401.

The effect of acute oral administration of AK-1401 at 1, 3, 10 mg/kg, positive control diazepam (DZP) 5 mg/kg, or vehicle in the open field exploratory assay on freezing time (C) and crossing at the center of the open-field (D). Each column represents the mean ± S.E.M. of 10 animals. *P < 0.05 and ***P<0.001 versus vehicle treated group (one way ANOVA followed by Dunnett´s test).
APPENDIX F:

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Figure 4. “Salvia_divinorum_Herba_de_Maria”
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  o https://upload.wikimedia.org/wikipedia/commons/3/3b/Opium_pod_cut_to_demonstrate_fluid_extraction1.jpg

Figure 15. Public Domain image obtained from Drug Enforcement Agency. 2017. Image of dried opium.
  o https://www.dea.gov/pr/multimedia-library/image-gallery/opium/opium3.jpg

Figure 17. Public Domain image provided by Mpv_51. 2017. Bayer Heroin bottle.
  o https://upload.wikimedia.org/wikipedia/commons/f/ff/Bayer_Heroin_bottle.jpg

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Figure 24. Reprinted by permission from ASPET: Copyright 2007.
Curriculum Vitae

January 2013 — May 2017
University of Mississippi
School of Pharmacy
Department of Biomolecular Sciences
Doctor of Philosophy: Pharmaceutical Sciences
Specialization: Pharmacognosy