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In vivo effects of the CB1 allosteric modulator LDK1258, a structural analog of ORG-27569

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A dissertation submitted in partial fulfillment of the requirements for the degree of

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by

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List of Abbreviations

2-AG = 2-arachidonoylglycerol

AEA = N-arachidonoylethanolamine; anandamide

CB1 = Cannabinoid receptor type-1

CB2 = Cannabinoid receptor type-2

CCI = chronic constrictive nerve injury

CIPN = chemotherapy-induce peripheral neuropathy

FAAH = fatty acid amide hydrolase

GCPR = G-protein coupled receptor

HPLC-MS = high-pressure liquid chromatography/ mass spectrometry

MAGL = monoacylglycerol lipase

THC = delta-9-tetrahydrocannabinol

Abstract

IN VIVO EFFECTS OF THE CB_1 ALLOSTERIC MODULATOR LDK1258, A STRUTURAL ANALONG OF ORG-27569

Mohammed A. Mustafa, Bachelor of Science

A dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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Advisor: Aron H. Lichtman, PhD, Professor of Pharmacology and Toxicology

Cannabinoid type-1 (CB_1) receptor allosteric modulators are an area of growing interest in the cannabinoid research field as novel experimental tools and a potential therapeutic strategy. While the quantity of publications examining CB_1 allosteric modulators has substantially increased in recent years, most reports describe the cellular mechanisms of these compounds and relatively few published studies have examined the *in vivo* pharmacology of these compounds. ORG-27569 is a first-generation CB_1 allosteric modulator and the most widely studied to date. This compound enhances [^{3}H]CP55,940 binding at the CB_1 receptor *in vitro* (Price et al., 2005), but it's *in vivo* effects are CB_1 receptor independent (Gamage et al., 2014). Subsequent series of CB_1 allosteric modulators have been developed, many of which are structural analogs of the ORG-27569 pharmacophore such as the novel compound LDK1258. The purpose of this

thesis dissertation is to examine LDK1258 in *in vivo* models of neuropathic pain, cannabimimetic side-effects, and feeding behavior.

Cellular studies of LDK1258 report that it shows a concentration dependent inhibition of CP55,940-induced G-protein coupling activity with a K_B value of 89.1 nM in a [^{35}S]GTPγS binding assay (Khurana et al., 2014). This compound was selected as the candidate test compound because of its high binding affinity compared to other structurally similar compounds. In a series of behavioral experiments in mice, we tested LDK1258 in mouse assays sensitive to CB_1 receptor stimulation, which include the tetrad assay (comprised of measures assessing locomotor activity, catalepsy, antinociception, and hypothermia), the drug discrimination paradigm, food consumption, and the chronic constrictive sciatic nerve injury (CCI) model of neuropathic pain. In addition, we tested whether this compound was CNS penetrant and if any active metabolites were detectable following systematic administration in mice. When tested alone in the tetrad assay, LDK1258 produced a significant decrease in locomotor activity and body temperature, but not antinociception, as measured in the tail withdrawal assay, or catalepsy, as assessed in the bar test. LDK1258 decreased locomotor behavior and body temperature to a similar magnitude in CB_1 (-/-) and (+/+) mice, indicating CB_1 receptor independent effects. Moreover, LDK1258 failed to shift the dose-response curves of two orthosteric CB_1 receptor agonists, CP55,490 in C57BL/6J mice and AEA in FAAH (-/-) mice in the tetrad assay. In the mouse drug discrimination assay, LDK1258 failed to substitute or shift the dose-response curve for either of these agonists but dose-dependently suppressed response rates indicating a pharmacologically relevant effect in this assay. Because mice administered the parent

compound ORG-27569 show reduced food intake, we investigated whether LDK1258 affected food consumption and tested CB_1 receptor dependency using CB_1 (-/-) and (+/+) mice. LDK1258 reduced food consumption regardless of genotype, again indicating a CB_1 receptor dispensable effect. In comparison, the CB_1 receptor antagonist rimonabant dose-dependently reduced food intake in CB_1 (+/+) mice, but not in CB_1 (-/-) mice. In the final study, we examined LDK1258 in the CCI model of neuropathic pain. Unexpectedly, it elicited a delayed antinociceptive effect (i.e., beginning at 4 h) that was CB_1 receptor independent. The results of the studies conducted throughout this thesis project demonstrate that LDK1258 decreases locomotor activity, body temperature, and food consumption, as well as elicits a delayed antinociceptive effect in the CCI model of neuropathic pain. However, the CB_1 receptor is not required for these pharmacological effects.

These findings underscore challenges in translating *in vitro* effects of newly developed CB_1 receptor allosteric modulators to the whole animal, as well as emphasize the importance for medicinal chemists, structural biologists, cellular pharmacologists, and behavioral pharmacologists to advance the development of CB_1 receptor allosteric modulators. Additionally, we demonstrated the importance of using a methodology that incorporates series of behavioral tests modeling neuropathic pain, feeding behavior, and THC-like side effects as a model to assess the *in vivo* effects of novel CB_1 allosteric modulators.

Chapter I

General Introduction

A major step in understanding of the endocannabinoid system began when the primary psychoactive constituent of the Cannabis sativa plant, Δ^9-tetrahydrocannabinol (THC) (Gaoni & Mechoulam, 1964) was identified and isolated. Subsequently, medicinal chemists developed synthetic analogs of THC and conducted structure-activity-relationship studies of these novel compounds (Razdan, 1986). One analog, CP55,940 was tritium-labeled to a high specific gravity and used as a tool to facilitate the discovery of the CB_1 receptors (Devane et al., 1988) which were identified and cloned in human brain (Matsuda et al., 1990). In later years, CB_2 receptors were identified in peripheral tissue (Munro et al.,1993). Both receptor types are described as being the primary targets of THC.

CB_1 receptor signal transduction. The CB1 receptor is predominantly expressed on pre-synaptic axon terminals of neurons and is the most abundant GCPR expressed in the central nervous system (CNS) (Alger & Kim, 2011). These receptors are part of the rhodopsin-like family of 7-transmembrane spanning receptors and CB_1 ligands bind within the central core formed by the interaction of the seven transmembrane helices. They are Gi/o-coupled proteins that elicit downstream signaling cascades when activated which allows for release of a G-protein subunit to regulate effector proteins, ultimately dampening pre-synaptic neurotransmitter release (Mackie, 2006). Specifically, release of the G-protein subunit inhibits adenylyl cyclase activity

resulting in an inhibition of cAMP accumulation. Intracellular cAMP and cAMP-dependent protein kinase A (PKA) phosphorylate ion channels in neurons (Howlett & Shim, 2013). When the CB_1 receptor is activated intracellular cAMP concentrations decrease resulting in inhibition of ion channel phosphorylation. This net reduction of ion channel phosphorylation results in a hyperpolarization of axon terminals and a blunted response to depolarizing stimuli (Howlett & Shim, 2013). Studies examining the effects of CB_1 agonists in transgenic animals indicate that CB_1 receptor is responsible for the behavioral effects of THC and other exogenous cannabinoids because these effects are inhibited in CB_1 (-/-) animals but not in (+/+) controls (Grim et al., 2016; Wiley et al., 2005).

General introduction to the endocannabinoid system. Several endogenous ligands that activate the CB_1 receptor have been identified, with the two most predominantly studied being N-arachidonoyl ethanolamine (anandamide; AEA) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). These endogenous cannabinoids (endocannabinoids) function as retrograde messengers that are released on demand from post-synaptic cells to modulate pre-synaptic neurotransmitter release (Mackie, 2006). AEA, 2-AG, and other endocannabinoids are regulated by a distinct set of enzymes that modulate the biosynthesis and degradation of these ligands. It is known that 2-AG is synthesized by diacylglycerol lipases (DAGL-α and DAGL-β) (Bisogno et al, 2003). Alternatively, evidence suggests NAPE-PLD activity regulates AEA biosynthesis but the mechanisms mediating the production of AEA are incompletely understood (Blankman & Cravatt, 2013). Because of the rapid degradation of AEA and 2-AG by their respective metabolic

enzymes, fatty acid amide hydrolase (FAAH) (Cravatt et al, 1996, 2001) and monoacylglycerol lipase (MAGL) (Blankman et al, 2007; Dinh et al, 2002), these endogenous ligands produce a short-lived duration of action.

In the simplest terms, the endocannabinoids in addition to the CB_1 and CB_2 receptors comprise the endogenous cannabinoid system (endocannabinoid system) and are neuromodulatory regulators that affect various physiological processes depending on cell type and location. Processes regulated by the endocannabinoid system include, but are not limited to feeding behavior and energy storage (Wiley et al., 2005), reward (Chen et al.,1991; Gardner et al.,1988; Lepore et al., 1996), stress responses (Patel et al., 2017) and pain and inflammation (Guindon & Hohmann, 2012). Exogenously administered cannabinoids, such as THC, affect these physiological processes as well and can be used for therapeutic application.

Clinical use and side-effects of cannabinoids. Evidence of the therapeutic effects of cannabinoids has been recorded as early as ~2700 BCE in ancient China with the use of herbal cannabis for various ailments (Booth, 2003). In modern medicine, dronabinol (THC) and nabilone (sold under the brand name Cesamet) are FDA-approved as antiemetics to reduce cancer chemotherapy associated nausea and vomiting (Sallan et al., 1975; Poster et al., 1981). Dronabinol is also approved as an appetite stimulator in patients afflicted with AIDS-related cachexia (Gorter et al., 1992).

It is important to note that despite the promise to treat various conditions, the clinical utility of cannabis and other exogenous cannabinoid agonists are limited due to their intoxicating effects, ability to impair cognitive function, and associations with psychiatric conditions such as schizophrenia, acute anxiety, and cannabis use disorder

(Bhattacharyya et al., 2017; Foster et al., 2018; Ramaekers et al., 2009; Renard et al., 2018). CB_1 receptor antagonists, such as rimonabant, have also been developed as therapeutics for weight loss; however, the availability of rimonabant for clinical use in Europe was withdrawn and further evaluation was ceased due to severe side-effects such as depression, anxiety disorder, and suicide ideation (Christensen et al., 2007; Moreira & Crippa, 2009). These side effects of agonists and antagonists that bind the orthosteric (i.e., active) binding site on the CB_1 receptor limit the therapeutic utility of these compounds.

CB_1 allosteric modulators are hypothesized to offer clinical promise with minimal side effects because they modulate and fine-tune the effects of endogenous ligands already on board at the CB_1 receptor site, rather than flooding the system with exogenous ligands (Pertwee, 2005; Ross, 2007). In summary, modulation of the endocannabinoid system which is comprised of CB_1 and CB_2 receptors and biosynthetic and degradative enzymes which metabolize endogenous ligands holds promise as a viable target for therapeutic development with a limited side-effect profile.

Introduction to CB_1 allosteric modulators. Traditionally, the effects of CB_1 receptors are regulated by agonists and antagonists of the primary binding site (orthosteric site) which both endogenous and exogenous cannabinoids bind. The ligands which occupy this site are referred to as orthosteric ligands. Because of adverse side-effects listed above that are associated with orthosteric binding, alternative approaches to targeting the CB_1 receptor are currently being explored. One approach is to utilize CB_1 receptor allosteric modulators, which bind to topographically distinct sites from the orthosteric site on the receptor (Kenakin, 2004; Kenakin & Strachan, 2018;

Khurana et al., 2014; Price et al., 2005; Ross, 2007). These compounds do not directly activate the CB_1 receptor, unlike orthosteric agonists, but rather bind to a secondary (allosteric) site on the receptor, which results in a conformational change of the receptor. Ligands making these conformational changes are conceptualized to enhance (positive allosteric modulator, PAM) or decrease (negative allosteric modulator, NAM) the efficacy and potency of orthosteric ligands that bind to the CB_1 receptor which include endogenous ligands (Kenakin, 2004). It is hypothesized CB_1 allosteric modulators offer therapeutic potential with reduced adverse side-effects compared with orthosteric ligands (Kenakin, 2004) because these molecules may circumvent issues with persistent orthosteric activation and may be more selective based upon the orthosteric endogenous ligand present (Kenakin & Strachan, 2018).

Currently, benzodiazepines, which allosterically modulate GABAa receptors are FDA-approved for the treatment of anxiety. These compounds potentiate the effect of GABA and lack the potentially lethal adverse effects of GABAa agonists, such as respiratory depression. The discovery and clinical success of benzodiazepines demonstrates that allosteric modulation is a viable therapeutic strategy (Wenthur et al., 2014). In comparison, no clinically approved allosteric modulators of CB_1 receptors currently exist. However, efforts have increased to synthesize and evaluate novel CB_1 receptor allosteric modulators.

First generation CB_1 allosteric modulators are defined as compounds with chemical structures (pharmacophores) unlike that of previously reported compounds in this class of drugs. First-generation compounds are either found endogenously such as lipoxin A4 (Pamplona et al., 2012), or are synthesized by medicinal chemists such as

ORG27569 (Price et al., 2005). Other first-generation allosteric modulators of the CB_1 receptor include PSNCBAM-1 and ZCZ011 (B. M. Ignatowska-Jankowska et al., 2015). Second-generation CB_1 allosteric modulators are defined as compounds which are structurally similar to a first-generation compound but have an alteration of one or more functional groups which affects the pharmacodynamic and pharmacokinetic properties of the molecule. Both first- and second-generation CB_1 allosteric modulators either enhance or decrease the binding of orthosteric ligands. In addition, they alter downstream signal transduction to either enhance or decrease orthosteric ligand efficacy. Interestingly, effects on downstream signaling can be independent of enhancement or decrease in binding. Although a growing body of research has characterized the cellular pharmacology of CB_1 receptor allosteric modulators, the relatively few studies investigating the *in vivo* pharmacology of these ligands yielded mixed results.

***In vitro* characterization of first-generation CB_1 allosteric modulators.** ORG-27569 is a first-generation CB_1 allosteric modulator developed by the pharmaceutical company Organon (acquired by Schering-Plough Corporation in 2007) and is the most extensively characterized compound in this class thus far. In initial *in vitro* studies characterizing this compound (Price et al., 2005), ORG-27569 produced a significant, but saturable, increase of orthosteric agonist binding in an equilibrium binding assay using the radioligand probe [^{3}H]CP 55,940. A binding cooperativity factor (α) denotes the allosteric interaction between the orthosteric and allosteric ligands when they both occupy the receptor, i.e., it quantifies the direction of and magnitude by which the affinity of one ligand is changed by the other ligand when both are bound to the receptor to

form the ternary complex (Christopoulos, A. et al., 2004). When α is 1.0, the test modulator does not alter orthosteric ligand binding. If α is less than 1.0, the test modulator reduces orthosteric ligand binding indicating negative allosteric modulation (NAM). A compound with a binding cooperativity factor greater than 1.0, indicates positive allosteric modulation (PAM) (Price et al., 2005). ORG-27569 had a binding cooperativity factor greater than 1.0 when co-administered with [^{3}H]CP 55,940 in mouse brain membranes indicating PAM activity. In contrast, when the CB_1 inverse agonist [^{3}H] SR141716A was co-administered with ORG-27569, the result produced a decrease in the [^{3}H]SR141716A equilibrium binding and binding cooperative value less than 1.0 indicating NAM activity when this probe was used (Price et al., 2005). Additionally, dissociation kinetic experiments showed that ORG-27569 reduced CP55,940 dissociation from the receptor in mouse brain *in vitro* (Price et al., 2005). Whereas CB_1 orthosteric agonists inhibit electrically evoked contractions of isolated mouse vas deferens, ORG-27569 given alone lacked efficacy. However, ORG-27569 ameliorated the actions of the CB_1 receptor agonist WIN 55,212 in this functional CB_1 receptor-mediated assay (Price et al., 2005). Additionally, ORG-27569 produced a rightward shift of the dose response curves of CP55,940 and AEA in stimulation of [^{35}S]GTPγS activity. The effects of ORG-27569 in the isolated mouse vas deferens assay and [^{35}S]GTPγS activity assay are consistent with negative allosteric modulation (Price et al., 2005). Lastly, studies examining the effects of ORG-27569 on luciferase expression by CP55,940 in cloned human CB_1 receptors expressed in Chinese hamster ovary cells showed similar results to studies conducted using mouse CB_1 receptors indicating that

these effects were observable in cells expressing either human- or mouse- CB_1 (Price et al., 2005).

Because ORG-27569 did not produce agonist or inverse agonist properties when tested alone *in vitro*, and modulation of receptor function was observed when the receptor was dually occupied by both the orthosteric and allosteric compounds, these results support the conclusion that ORG-27569 functions as an allosteric modulator of the CB_1 receptor *in vitro* (Price et al., 2005). It is important to note that, despite the enhancement of orthosteric binding, which is consistent with PAM activity, this compound produced a decrease of orthosteric agonist inhibition of electrically evoked contractions in the mouse vas deferens model of measuring effects of CB_1 activation and agonist induced G-protein activity in the [^{35}S]GTPγS binding assay (Price et al., 2005). These findings suggest the functional effect of ORG-27569 in whole animal is consistent with a NAM of the CB_1 receptor, which results in a decrease of efficacy of orthosteric agonists.

Another first-generation compound is the novel CB_1 positive allosteric modulator ZCZ011, which was synthesized at the University of Aberdeen (B. M. Ignatowska-Jankowska et al., 2015). When tested *in vitro*, ZCZ011 produced significant and concentration-dependent increases in the specific equilibrium binding of CB_1 receptor agonists [^{3}H]CP55,940 and [^{3}H]WIN 55,212 with an E_{max} of 207% and 225%, respectively, as demonstrated by an equilibrium binding experiment in mouse brain membranes (B. M. Ignatowska-Jankowska et al., 2015). Additionally, a saturation binding experiment using mouse brain membranes and [^{3}H]CP55,940 as the probe demonstrated that ZCZ011 significantly increases the number of available CB_1 binding

sites for the orthosteric agonist (B. M. Ignatowska-Jankowska et al., 2015). ZCZ011 also enhanced AEA stimulated [^{35}S]GTPγS binding in mouse brain membranes demonstrating an increase of G-protein activity compared to AEA administration alone. Importantly, ZCZ011 did not stimulate [^{35}S]GTPγS binding when administered alone, suggesting that this compound is not acting as an orthosteric agonist at the CB_1 receptor (B. M. Ignatowska-Jankowska et al., 2015). In the PathHunter hCB_1 β-arrestin Recruitment Assay ZCZ011 enhanced β -arrestin recruitment stimulated by AEA. However, when tested alone ZCZ011 also produced an increase in β-arrestin recruitment which was 35.9% that of maximal stimulation (B. M. Ignatowska-Jankowska et al., 2015). Finally, an AlphaScreen Surefire ERK 1/2 phosphorylation assay was utilized and ZCZ011 increased the potency of AEA in activating ERK1/2 phosphorylation in hCB_1 receptor cells (B. M. Ignatowska-Jankowska et al., 2015). All these effects observed *in vitro*, in addition to the *in vivo* effects of ZCZ011 which will be subsequently described in the next chapter section of this thesis, demonstrate that ZCZ011 acts as a positive allosteric modulator of the CB_1 receptor.

A growing body of research has characterized the cellular pharmacology of CB_1 receptor allosteric modulators (Table 1). Comparatively, there are relatively few studies investigating the *in vivo* pharmacology of these ligands yielded mixed results, which will be described in Table 2 and the section below.

***In vivo* characterization of CB_1 allosteric modulators.** Models used to assess the effects of CB_1 orthosteric ligands *in vivo* are also used to evaluate CB_1 allosteric modulation *in vivo*. The tetrad is an assay specifically used to measure cannabimimetic, or THC-like, effects in rodents. It consists of four measures mediated by the CB_1

receptor including locomotor activity, catalepsy, antinociception, and hypothermia (Little et al.,1988). Co-administration of a CB_1 allosteric modulator with an orthosteric agonist is expected to produce an augmentation of the orthosteric dose-response curve in the measures of this assay. However, no effect is expected in the tetrad assay when a CB_1 allosteric modulator is administered alone, indicating CB_1 receptor modulation. Additionally, the mouse drug discrimination paradigm is a measure of discriminative stimuli and is used to assess the subjective effects of CB_1 orthosteric agonists. Similarly, it is expected that co-administration of a CB_1 allosteric modulator in this paradigm results in an augmentation of the subjective effects of the orthosteric agonist while producing no substitution for the CB_1 mediated discriminative stimuli on its own. It also has been demonstrated that CB_1 PAMs produce antinociception in mouse models of neuropathic pain (B. M. Ignatowska-Jankowska et al., 2015; Slivicki et al., 2018) indicating that these models can be used to assess the therapeutic potential of CB_1 allosteric modulators to treat pain and neuropathy. Additionally, antagonism of the CB_1 receptor results in decreases of food intake (Christensen et al., 2007; Wiley et al., 2005), therefore measures of food intake have been used to assess the effects of CB_1 NAMs (Gamage et al., 2014; Horswill et al., 2007).

Behavioral studies demonstrate that ORG-27569 reduces food intake in mice. Because these effects are observed in both wild-type mice and transgenic CB_1 (-/-) mice the reduction in feeding behavior caused by ORG-27569 is CB_1 receptor independent (Gamage et al., 2014). Additionally, the negative allosteric modulator PSNCBAM-1 reduced food intake in rats, indicating actions consistent with CB_1 receptor antagonism (Horswill et al., 2007). However, this study did not assess whether CB_1 receptors

mediated these anorectic effects. ORG-27569 also attenuates both cue- and drug-induced reinstatement of methamphetamine and cocaine seeking behavior (Jing et al., 2014). Again, CB_1 receptor mediation was not assessed in this study by either using transgenic animals that lack the CB_1 receptor or with a pharmacological approach to inhibit the behavioral effect observed, therefore it is unclear if this effect is CB_1-dependant. In addition, ORG-27569 does not augment the cataleptic, antinociceptive, or hypothermic effects of AEA, CP55,940, or THC in the tetrad assay (Gamage et al., 2014). These findings highlight the translational gap between the effects of CB_1 allosteric modulators in cellular assays versus effects in the whole organism.

The endogenous anti-inflammatory mediator Lipoxin A4 represents the first evidence of a CB_1 allosteric modulator producing *in vivo* effects consistent with CB_1 allosteric modulation in whole organisms (Pamplona et al., 2012). Specifically, lipoxin A4 enhanced the pharmacological effects of AEA in both cellular and behavioral assays. It also protected against β-amyloid induced performance deficits in the Morris water maze, an assay indicative of memory and learning. It is known that AEA is endogenously released for one week following β-amyloid treatment. The performance deficit was prevented by co-treatment with lipoxin A4, and this effect was reversed with the CB_1 receptor antagonist rimonabant (Pamplona et al., 2012). This evidence suggests that lipoxin A4 enhances the effect of endogenous AEA and the protection against β-amyloid induced deficits are CB_1 receptor dependent. These results are consistent with the conclusion that lipoxin A4 acts as a CB_1 receptor PAM *in vivo*.

The CB_1 positive allosteric modulator, ZCZ011 was tested for antinociception and cannabimimetic activity *in vivo* (B. M. Ignatowska-Jankowska et al., 2015). ZCZ011

administered alone did not elicit any activity in the tetrad or drug discrimination assays, but when co-administered with AEA or CP55,940 it produced left-wards shifts of the dose-response relationship of the CB_1 agonists, indicating activity consistent with positive allosteric modulation. Additionally, ZCZ011 did not produce conditioned place preference or aversion in mice, suggesting that it lacks rewarding or aversive effects on its own. ZCZ011 crosses the blood-brain-barrier to enter the CNS which was confirmed by HPLC-MS analysis of blood and brain tissue from mice pretreated with ZCZ011 (Poklis et al., 2015). Lastly, ZCZ011 produced antinociception in the chronic-constrictive nerve injury (CCI) model of neuropathic pain and partially reversed carrageenan-induced mechanical allodynia through a CB_1 mechanism of action (B. M. Ignatowska-Jankowska et al., 2015). The observations that ZCZ011 does not produce cannabimimetic side-effects when administered alone, but does produce antinociception suggests it has therapeutic potential to be used as an analgesic in the clinic. Another study of ZCZ011 found that it attenuated somatic signs of THC withdrawal and blocked NSAID-induced gastric hemorrhages in rodents (Trexler et al., 2019) suggesting its therapeutic potential to treat cannabis use disorder and gastric inflammation. These actions of ZCZ011 are consistent with the concept that CB_1 allosteric modulators can produce therapeutic effects without the cannabimimetic side-effects commonly associated with CB_1 receptor agonism.

A compound which is a structurally similar analog of ZCZ011, the CB_1 positive allosteric modulator GAT211 produces *in vivo* effects similar to its parent compound (Slivicki et al., 2018). In the described study, GAT211 produced CB_1 receptor mediated antinociceptive effects in the mouse neuropathic pain model of chemotherapy induced

peripheral neuropathy (CIPN), which did not undergo tolerance or withdrawal following repeated administration, and did not elicit psychotomimetic effects associated with CB_1 receptor allosteric agonists, suggesting *in vivo* actions consistent with CB_1 receptor allosteric modulation (Slivicki et al., 2018). The results of this study provides rational that second-generation compounds derived from first-generation compounds are viable therapeutic candidates that warrant further evaluation.

LDK1258: a second-generation allosteric modulator. We selected the ORG-27569 pharmacophore as the focus of this thesis dissertation since it is the most widely characterized compound in the class of CB_1 allosteric modulators. Several analogs of ORG-27569 have been assessed for binding affinity and allosteric activity (Khurana et al., 2014) but have not been tested for *in vivo* activity. One compound in particular (12f), also known with compound code LDK1258 (M.W. = 401.97 g/mol) has a strong equilibrium disassociation constant value of 89 nM for the allosteric binding site and binding cooperativity factor of 5, defining it as a CB_1 PAM which enhances orthosteric agonist binding affinity (Khurana et al., 2014). In contrast ORG-27569 behaved like a efficacy NAM by decreasing CP55,940-stimulated [^{35}S]GTPγS activity *in vitro* (Khurana et al., 2014). These findings demonstrate that the *in vitro* actions of this novel compound are similar to ORG-27569, however evaluation of this second-generation allosteric modulator has not been conducted *in vivo*.

Table 1.

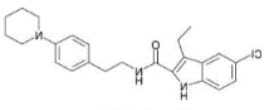
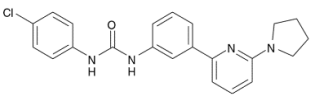
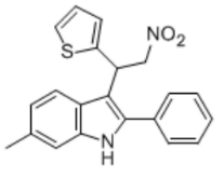
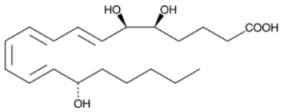
Compound	Molecular structure	*In vitro* effects	References
ORG-27569		- enhances equilibrium binding of CP55,940 - decreases equilibrium binding of SR 141716A - reduces CP55,940 dissociation from receptor - reversed inhibition of electrically evoked contractions of mouse vas deferens by WIN55,212 - inhibits CP55,940 and AEA stimulated [^{35}S]GTPγS activity	Price et al., 2005
PSNCBAM-1		- inhibits stimulation of CB_1 by CP55,940, WIN 55,212, AEA, and 2-AG in yeast reporter assay - reversed binding stimulated by CP55,940 and AEA - reversed AEA-induced inhibition of forskolin-stimulated cyclic AMP accumulation	Horswill et al., 2007
ZCZ011		- enhances equilibrium binding of CP55,940 and WIN 55,212 - increases number of available CB_1 binding sites - enhances AEA stimulated [^{35}S]GTPγS activity - increases β -arrestin recruitment - increases potency of AEA to stimulate ERK1/2phosphorylation	Ignatowska-Jankowska et al., 2015
Lipoxin A4		- enhances equilibrium binding of CP55,940 and WIN 55,212 - increased the potency of AEA in decreasing forskolin (FSK)-induced cAMP levels	Pamplona et al., 2012

Table 2.

Compound	*In vivo* effects	MOA	References
ORG-27569	- reduces food intake - attenuates cue- and drug-induced reinstatement of methamphetamine and cocaine seeking behavior	- Reduction in food intake is CB_1 independent - Not assessed in study of cue- and drug- reinstatement of methamphetamine and cocaine seeking behavior	(Ding et al., 2014; Gamage et al., 2014; Jing et al., 2014)
PSNCBAM-1	- reduces food intake	- Not assessed	Horswill et al., 2007
ZCZ011	- leftward shift of AEA and CP55,940 DR in tetrad and drug discrimination - Antinociception in CCI mouse model - Attenuates THC withdrawal - Attenuates NSAID-induced gastric inflammation	- - CB_1 Dependent	Ignatowska-Jankowska et al., 2015
Lipoxin A4	- produces tetrad effects alone - enhances the effects of AEA in tetrad - protective against β-amyloid induced spatial memory impairment in mice	- Not assessed	Pamplona et al., 2012

Chapter II

In vivo evaluation of the CB_1 allosteric modulator LDK1258 reveals CB_1 receptor independent behavioral effects

Hypothesis. CB_1 allosteric modulators are viable therapeutic tools to treat neuropathic pain or be used as appetite suppressants and produce their effects via a CB_1-mediated mechanism of action without adverse side-effects commonly associated with orthosteric activation or blockade of the CB_1 receptor. The therapeutic potential and side-effect profile of these compounds are reliably evaluated in a series of assays which have been previously used to evaluate CB_1 orthosteric ligands *in vivo*.

Rationale. The purpose of this thesis project is to evaluate a novel CB_1 allosteric modulator *in vivo* in a series of assays to determine whether the selected compound produces pharmacological effects in whole organisms, and whether these effects are CB_1 receptor mediated and consistent with the action of CB_1 allosteric modulators. Novel CB_1 allosteric modulators serve as valuable tools to determine a methodology that evaluates whether cellular effects of CB_1 allosteric modulation translates to whole organisms. In this chapter, the methodology of the assays employed and results from the conducted experiments are reported.

Here, we investigated whether LDK1258 produces *in vivo* effects consistent with those of a CB_1 receptor allosteric modulator. Since previous studies demonstrated that CB_1 allosteric modulators produce antinociceptive effects in CCI or chemotherapy-induced allodynia models of neuropathic pain (B. M. Ignatowska-Jankowska et al.,

2015; Slivicki et al., 2018) as well as reduce food intake (Ding et al., 2014; Gamage et al., 2014; Horswill et al., 2007) in rodents, we tested whether LDK1258 alters either mechanical allodynia in the CCI model of neuropathic pain or food consumption in food-deprived mice. In a subsequent study, we quantified brain and blood levels of LDK1258 following intraperitoneal administration to examine whether it was brain penetrant. In addition, we tested whether LDK1258 substitutes for CP55,940 in C57BL/6J mice or AEA in transgenic mice lacking the primary anandamide degradative enzyme fatty acid amide hydrolase (FAAH; Cravatt et al., 1996; Cravatt et al., 2001) in the drug discrimination assay. Similar to previous studies examining CB_1 receptor allosteric modulators (Gamage et al., 2014; B. M. Ignatowska-Jankowska et al., 2015), we examined whether LDK1258 elicits cannabimimetic effects in the tetrad assay (Little et al., 1988), consisting of measurements of locomotor behavior, thermal nociception, catalepsy, and body temperature. In order to infer whether CB_1 receptors mediate pharmacological effects observed in the assays described above, we employed a genetic approach using CB_1 (-/-) mice or a pharmacological approach using the CB_1 receptor antagonist rimonabant. Finally, because it was reported that ZCZ011 produces leftward shifts of the generalization dose-response curves of AEA and CP55,940 in the drug discriminative assay as well as leftward shifts for thermal antinociception, catalepsy, and hypothermia (B. M. Ignatowska-Jankowska et al., 2015), it can be concluded that these measures are viable for detecting CB_1 allosteric modulation in animal models. Therefore, we tested whether co-administration of LDK1258 alters the pharmacological effects of AEA or CP55,940 in these assays.

Methodology

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, USA) were used in tetrad, and drug discrimination studies. Male FAAH (-/-) mice on a C57BL/6J background (VCU transgenic core, Richmond, Virginia, USA) were used in the tetrad and drug discrimination studies (see below). Male and female CB_1 (-/-) and (+/+) mice backcrossed on a C57BL/6J background (VCU transgenic core, Richmond, Virginia, USA) were used in subsequent feeding, tetrad, and neuropathic pain studies.

Drugs

LDK1258 (compound 12f) and LDK1256 (compound 12d; used as internal standard for UPLC-MS/MS analysis) were synthesized at the Rangel College of Pharmacy Health Science Center at Texas A&M University (Kingsville, Texas, USA) as previously described (Khurana et al., 2014). Anandamide, CP55,940, and rimonabant (SR141716A) were supplied by the National Institute on drug abuse (NIDA) (Rockville, Maryland, USA). All drugs were dissolved in ethanol (Pharmco Products Inc., Brookfield, Connecticut, USA), Alkamuls-620 (Rhodia, Cranbury, New Jersey, USA), and saline (0.73%), in a ratio of 1:1:18. Injections were given via the intraperitoneal (i.p.) route of administration in a volume of 1 ml per 100 g of body mass.

Chronic constrictive injury of the sciatic nerve (CCI) model of neuropathic pain

The surgical procedure for chronic constriction of the sciatic nerve was conducted as described by Bennett & Xie (1988) with modifications (B. M. Ignatowska-Jankowska et al., 2015). Mice were anesthetized with isoflurane and using aseptic procedures the sciatic nerve was isolated and then loosely ligated. Sham surgery was identical, except for ligating the nerve. Mechanical touch was used to assess baseline responses and the development of allodynia after surgery using von Frey monofilaments (North Coast Medical, Morgan Hills, CA), as previously described (Murphy et al., 1999). Mice were unrestrained and placed in a Plexiglas cylinder (8 cm diameter, 15 cm height) on top of a wire mesh screen. Von Frey calibrated microfilaments were applied to each hind paw and the stimulus threshold that induced a response (defined as lifting, licking, or shaking of the paw) was recorded. Following allodynia testing, thermal hypersensitivity was assessed by placing mice on a hot plate analgesia meter (Columbus Instruments, Columbus, OH) and latency to jump, shake, or lick the hind paw was recorded. LDK1258 (30 mg/kg) was injected via the i.p. route of administration and mice were tested for mechanical and thermal allodynia at 0.5, 1, 2, 4, 6, 8, and 24 h in a time-course design. This dose was selected based upon initial pilot studies. Treatments were administered i.p. in a counterbalanced, between-subject design.

Food intake assay

Mice were housed in clear plastic cages with elevated wire mesh floors to allow for food and feces to be out of reach of the mouse. Following a minimum of 72 h acclimation

period, the mice were food deprived for approximately 24 h before receiving LDK1258, rimonabant (control), or vehicle treatments. Following the deprivation period mice were injected 15 min before receiving access to 15 (± 0.1) g of standard rodent chow (Teklad, Madison, Wisconsin, USA). Food consumption was recorded in grams eaten 2 h after food administration during the light-phase of a 12/12 h light/dark cycle. This procedure is like those previously used in examining the consequences of cannabinoid receptor agonists and antagonists on food consumption (Wiley et al., 2005). LDK1258 doses were selected based upon initial pilot studies.

Ultra-performance liquid chromatography tandem mass spectrometer analysis of LDK1258

Mice were given an intraperitoneal (i.p.) injection of vehicle or LDK1258 (30 mg/kg) and were euthanized via cervical dislocation either 30 min or 4 h later. This dose was selected because it produced robust behavioral effects when administered alone in the tetrad, drug discrimination, food intake, and allodynia tests. Blood was collected by breaking the skin with a needle prick from cheek tissue before euthanasia and whole brains were collected immediately after sacrificing the animals. All samples were kept at -80 C until analyzed. On the day of analysis, the brain tissue samples were weighed, diluted 1:5 with water and homogenized. With each analytical analysis seven-point calibration curves at concentrations of 50 - 5000 ng/mL LDK1258 for blood and 50 - 5000 ng/kg LDK1258 for brain tissue homogenate along with a drug free control and a control without internal standard (ISTD) in drug-free mouse blood and brain tissue were prepared. LDK1258 was extracted from blood and brain tissue homogenate using a

liquid/liquid extraction (Poklis et al., 2010). In brief, 200 ng/mL or ng/g of LDK1256, the ISTD, was added to 20 µL aliquots of blood or 100 µL aliquots of brain tissue homogenate of each calibrator, control, or specimen except the negative control. 200 µL of acetonitrile was then added to each of these sample and mixed for 2 min. The samples were then centrifuged at 2054g for 5 min. After centrifuging the top layer containing the acetonitrile was removed via a disposable glass pipette and placed in autosampler vial for analysis.

The ultra-performance liquid chromatography tandem mass spectrometer (UPLC-MS/MS) analysis of was performed on a Sciex 6500+ QTRAP system with an IonDrive Turbo V source for TurbolonSpray® (Sciex, Ontario, Canada) attached to a Shimadzu UPLC system (Kyoto, Japan) controlled by Analyst software (Sciex, Ontario, Canada). Chromatographic separation of LDK1258 and the internal standard, was performed using a Thermo Hypersil Gold column, 50 x 2.1 mm, 3 micron (Waltham, MA). The mobile phase contained water/methanol (10:90, v/v) with 0.1 mM ammonium formate and was delivered at a flow rate of 1 mL/min. The source temperature was set at 650° C, and curtain gas had a flow rate of 30 mL/min. The ionspray voltage was 5500 V, with the ion source gases 1 and 2 having flow rates of 60 mL/min. The declustering potential was 75 eV. The quantification and qualifying transition ions with their collection energies in parenthesis were monitored in positive multiple reaction monitoring (MRM) mode: 426> 192 (35) & 426 > 148 (25) for LDK1258 and 384> 220 (27) & 384> 148 (46) for the ISTD. The total run time for the analytical method was 3 minutes. A linear regression of the peak area of ratios of the quantification transition ions of LDK1258 and the ISTD were used to construct the calibration curves.

Drug discrimination

Separate groups of mice were trained to discriminate either anandamide (6 mg/kg) or CP55,940 (0.1 mg/kg) from vehicle (30 min pretreatment time). One group consisted of C57BL/6J mice trained to discriminate CP55,940, and the second group consisted of FAAH (-/-) mice trained to discriminate anandamide. Training and testing were conducted as previously reported (Ignatowska-Jankowska et al., 2015; Long et al., 2009; Owens et al., 2016; Solinas et al., 2006; Walentiny et al., 2013). Sound-attenuating operant conditioning boxes (MED Associates, St. Albans, VT) were used. Each apparatus contained two nose-poke apertures (left and right) with a receptacle chamber located in the middle of the apertures. A pellet dispenser delivered 14 mg sweetened pellets following every 10th correct response. Nose pokes and food deliveries were recorded using MED-PC IV software (MED Associates). Mice performed on a FR10 schedule of reinforcement during each 15-minute training session. Training sessions were conducted in a double-alternation sequence of drug and vehicle (e.g., vehicle, vehicle, drug, drug). Test sessions were conducted twice per week with mice required to reach criteria to be eligible for testing. Passing criteria were defined as follows: 1) the first 10 consecutive responses on the correct apparatus side, 2) ≥ 80% of responses on the correctly paired aperture, and 3) a response rate ≥ to 10 nose-pokes per min. During test sessions, responses in either aperture resulted in delivery of the sweetened pellet according to the schedule of reinforcement. Substitution tests were conducted by administering LDK1258 (3, 5.6, 10, 30 mg/kg) or vehicle i.p. 30 min prior to the test session when administered alone. In the combination studies, LDK1258 (5.6 mg/kg) or vehicle i.p. was injected 15

min before anandamide or CP55,940. This dose was selected because it was the highest dose tested that did not produce rate-suppressive effects when administered alone. All training drugs were administered subcutaneously. Mice were returned to their home cage after each injection and were placed in the operant chamber immediately before the beginning of the test session.

Tetrad assay

The tetrad assay consists of sequential testing for locomotor activity, catalepsy, thermal antinociception, and body temperature (Little et al., 1988), as described below.

Locomotor activity assessment

The locomotor effects of LDK1258 were assessed by placing the mice in dimly lit Plexiglas chambers (approx. 43 x 21 x 20 cm) for 300 s. The chambers were sound-attenuated and equipped with a LED light source and a fan that provided air circulation and white noise. Locomotor activity was monitored using Anymaze (Stoelting, Wood Dale, IL) software, as described previously (Ignatowska-Jankowska et al., 2015). Distance traveled, time immobile, and mean speed were recorded approximately 0.5 or 4.0 h after LDK1258 or vehicle administration. Recordings were collected using Fire-i™ digital cameras purchased from Unibrain (San Ramon, CA, USA).

Bar test

Catalepsy was measured using the bar test, in which the mouse's front paws were placed on a metal bar 4.5 cm above the platform, and immobility time was measured for a 60 s period. If the mouse removed its forepaws before 60 s elapsed, they were placed back on the bar for a maximum of four tries. The test ended on the fifth attempt or once 60 s elapsed.

Warm-water tail-flick

Thermal nociceptive behavior was measured using the warm-water tail withdrawal assay. The mouse was restrained and approximately 1 cm of the distal portion of the tail was submerged in a 52° C water bath and the tail withdrawal latency was recorded. A 10 s cut-off was employed to prevent tissue damage if the mouse did not remove the tail from the water in this period. In all experiments, tail withdrawal latencies are evaluated prior to injection. Data were expressed as a maximum percent effect (%MPE) using the following formula: %MPE = [(test latency – preinjection latency) / (10 – preinjection latency)] ×100.

Body temperature

Hypothermic effects were assessed by inserting a thermometer probe (Physitemp Instruments, Clifton, NJ) 2 cm into the rectum. For the LDK1258 and CP55,940

cumulative-dose assessment in the triad assay, rectal temperature was measured using an instrument from Traceable Products (Webster, TX). In all experiments, rectal temperature was evaluated prior to injection. Body temperature data was expressed as a change in temperature (° C) from pre-injection values.

Experimental procedure of tetrad assay

Four separate experiments were conducted to evaluate LDK1258 in the tetrad assay. In the first two experiments, C57BL/6J mice were administered LDK1258 (30 mg/kg) or vehicle. In the first experiment, the mice tested for locomotor activity at 20 min and in the second experiment, the mice were assessed for locomotor activity at 4 h. Subjects were tested for locomotor behavior only once in order to avoid acclimation to the chamber. Mice in the first experiment were tested for catalepsy, antinociception, and hypothermia at 0.5, 1, and 2 h. Subjects in the second experiment were tested in these respective measures at 4 and 6 h. The second experiment was conducted because of unexpected delayed antinociceptive effects of LDK1258 in the CCI model. In the third tetrad experiment, we evaluated lower doses of LDK1258 (3 or 10 mg/kg) in a new cohort of mice. Drug- and vehicle-treated mice were evaluated for locomotor activity at 0.5 h. Mice were assessed for each of the other measures at 0.5, 1, and 2 h. The fourth tetrad experiment was conducted to determine whether CB_1 receptors mediate the pharmacological effects of LDK1258 in the tetrad assay. Accordingly, we evaluated vehicle versus LDK1258 (30 mg/kg) in CB_1 (+/+) mice versus CB_1 (-/-) mice. Tetrad testing proceeded identically as described for Experiment 1 above.

Evaluation of LDK1258 on the dose-response relationships of CB_1 receptor orthosteric agonists in the triad assay

The *in vivo* cannabimimetic effects of LDK1258 were assessed in combination with the CB_1 orthosteric agonists anandamide in FAAH (-/-) mice or CP55,940 in C57BL/6J mice. Measures of catalepsy, antinociception, and hypothermia were used for the triad assay as previously described (Falenski et al., 2010; Grim et al., 2016). When tested in combination with the cumulative-dose response of orthosteric agonists, LDK1258 (50 mg/kg) was administered 15 min prior to the first administration of anandamide or CP55,940 followed by each subsequent dose every 40 min. Triad was assessed 30 min following each anandamide or CP55,940 injection. Locomotor activity was not measured in the cumulative-dose response assessments due to repeated testing causing habituation effects. Because this assay is less sensitive than others, the highest dose of LDK1258 that can be suspended in solution before reaching max saturation was selected.

Mouse hepatic microsome reaction

In order to investigate and identify metabolite(s), mouse hepatic microsomes were isolated and prepared as previously described (Kessler & Ritter, 1997). Phase I metabolism of LDK1258 and identification of potential p450 metabolites based on a previously employed method (Poklis, Dempsey, et al., 2015). Briefly, 1 mL of medium consisting of consisted of 167 mg total protein of pooled mouse hepatic microsomal preparation, 50 mM Tris–HCl buffer, pH 7.4, 150 mM potassium chloride, 10 mM

magnesium chloride with the addition of 0.4 mM freshly prepared NADPH and with or without 100 µg LDK1258 were prepared. The mixtures were incubated in a 37° C water bath for 60 min. The resultant p450 metabolites were isolated in ultrafiltrates using 30 kDa centrifugal filters (EMD Millipore, Billerica, MA, USA). An ultra-high-performance liquid chromatography tandem mass spectrometer (UHPLC-MS/MS) was used to identify potential metabolites from the exacted brain and blood samples.

Data analysis

All data are represented as mean ± S.E.M or 95% confidence limits (CLs). Data were analyzed using GraphPad Prism 6.0, using either one-way or two-way analysis of variance (ANOVA). Dunnett's test was used for the *post-hoc* analysis. ED_{50} values, potency ratios, and 95% CLs were calculated for the dose-response triad using linear regression analysis (Colquhoun, 1971). Differences were considered significant if $p < 0.05$, or if the upper and lower confidence intervals of the potency ratios did not encompass "1."

Experimental results

LDK1258 produces delayed antinociceptive effects in the chronic constriction injury (CCI) model of neuropathic

As shown in Figure 1a, LDK1258 (30 mg/kg) reversed CCI-induced mechanical allodynia in ipsilateral paw ($F (8, 64) = 12.92$, $P < 0.0001$) and contralateral paws ($F (8, 64) = 17.65$, $P < 0.0001$; data not shown) from 4 to 8 h. Likewise, LDK1258 (30 mg/kg) reversed thermal hyperalgesia in the hot plate test from 2 to 8 h ($F (8, 64) = 18.91$, $P < 0.0001$; Figure 1b). The CB_1 receptor antagonist rimonabant (3 mg/kg) did not block LDK1258-induced antinociception in either assay ($F (5, 18) = 83.43$, $P < 0.0001$; Figure 1a and 1b).

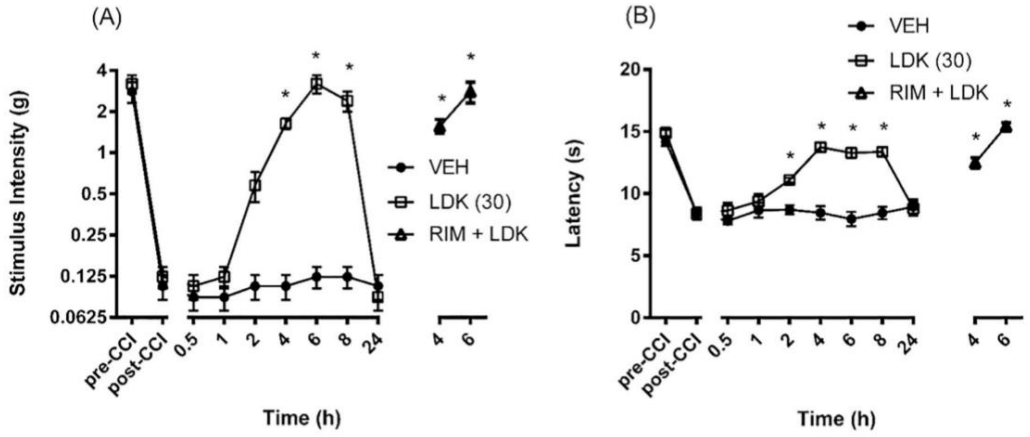


Fig. 1. LDK1258 (LDK) produces a delayed, but sustained, antinociceptive effect in the CCI model of neuropathic pain. (A) LDK1258 (30 mg/kg) significantly reversed mechanical allodynia beginning at the 4 h time point and persisting until the 8 h time point in the ipsilateral paw of C57BL/6J mice that received CCI surgery. (B) LDK1258 (30 mg/kg) reversed thermal hyperalgesia in C57BL/6J mice beginning at the 2 h time point and lasting up to the 8 h time point. The CB_1 antagonist rimonabant did not reverse either of these antinociceptive effects. All treatments were administered *via* i.p. injection. $n = 5$ mice per treatment group. $^*p < 0.05$ *vs.* post-CCI values. Data analyzed using Two-way ANOVA. Antagonism studies analyzed using One-way ANOVA.

LDK1258 reduces food consumption in mice independently of the CB_1 receptor

Subsequent experiments investigated the consequences of LDK1258 on food intake in CB_1 (-/-) and (+/+) mice following a 24 h fast. As CB_1 (-/-) mice consumed less food than CB_1 (+/+) mice ($t = 3.809$, $df = 56$, $P < 0.001$; Figure 2a), drug effects on food intake in each genotype were normalized as % intake of the vehicle-treated mice for each genotype. Two-way ANOVA revealed that LDK1258 significantly reduced food intake to a similar magnitude in both genotypes (LDK1258 main effect: $F(3, 54) = 3.284$, $P < 0.05$; no genotype main effect: $p = 0.54$; no LDK1258 by genotype interaction: $p = 0.30$; Figure 2b). In comparison, rimonabant significantly reduced food intake in CB_1 (+/+) mice, but not in CB_1 (-/-) mice (genotype main effect: $F(1, 26) = 26.42$ $P < 0.0001$; no rimonabant main effect: $p = 0.19$; no rimonabant by genotype interaction: $p = 0.58$; Figure 2c).

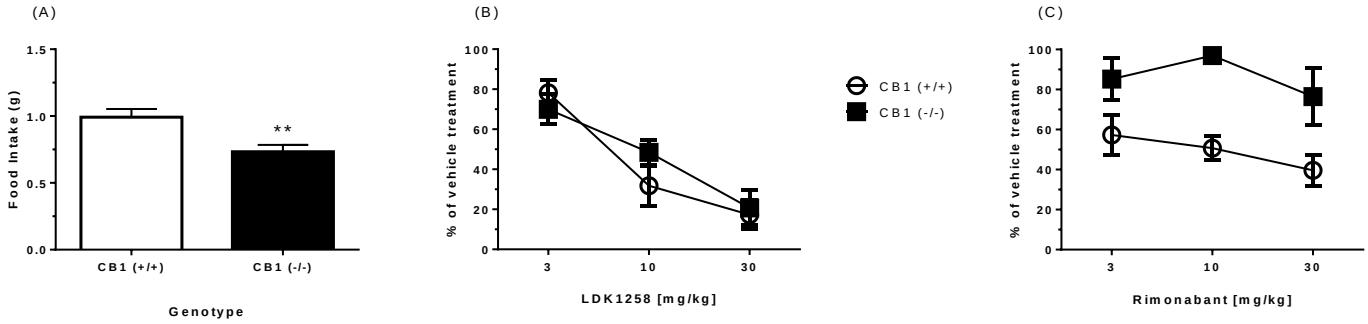


Fig. 2. LDK1258 (LDK) produces CB_1 receptor independent anorectic effects in mice. (A) Food-deprived CB_1 (−/−) mice show a phenotypic reduction in food intake compared with CB_1 (+/+) mice (*$p < 0.05$). Data represent grams of food eaten after vehicle control treatment; (B) LDK1258 (3–30 mg/kg) significantly reduced food intake in both CB_1 (−/−) and CB_1 (+/+) mice; (C) The positive control rimonabant decreased food intake in CB_1 (+/+) mice, but not in CB_1 (−/−) mice. Because of the significant effect of genotype in food consumption, the data were normalized to vehicle control values to examine drug effects in panels B and C. All data were collected 2 h after treatment administration and reported as mean ± SEM. $n = 7$–8 mice per group. *$p < 0.05$ *vs.* CB_1 (−/−), #$p < 0.05$ *vs.* Vehicle. Phenotypic reduction in food intake data were analyzed using Student's *t*-test. All other data were analyzed using two-way ANOVA.

Evaluation of LDK1258 in the mouse drug discrimination paradigm

LDK1258 (3-30 mg/kg) did not substitute for the discriminative stimulus of anandamide (6 mg/kg) in FAAH (-/-) mice or CP55,940 (0.1 mg/kg) in C57BL/6J mice at 30 min post-injection (Figure 3a), but reduced response rates at the highest dose tested in FAAH (-/-) mice ($F (5, 46) = 24.36$, $P < 0.0001$; Figure 3b) and in C57BL/6J mice ($F (5, 52) = 28.07$, $P < 0.0001$; Figure 3b). At 4 h post-administration of LDK1258 (30 mg/kg) no significant effects on the discriminative stimulus or response rates occurred (data not shown). We next evaluated whether LDK1258 would affect the generalization dose-response relationships of anandamide in FAAH (-/-) mice and CP55,940 in C57BL/6J mice. Accordingly, we selected the highest LDK1258 dose (i.e., 5.6 mg/kg) that did not reduce response rates when administered alone. LDK1258 did not alter the generalization dose response curve of either anandamide (1 – 6 mg/kg) or CP55,940 (0.01 – 0.1 mg/kg; Figure 3c) or response rates (Figure 3d).

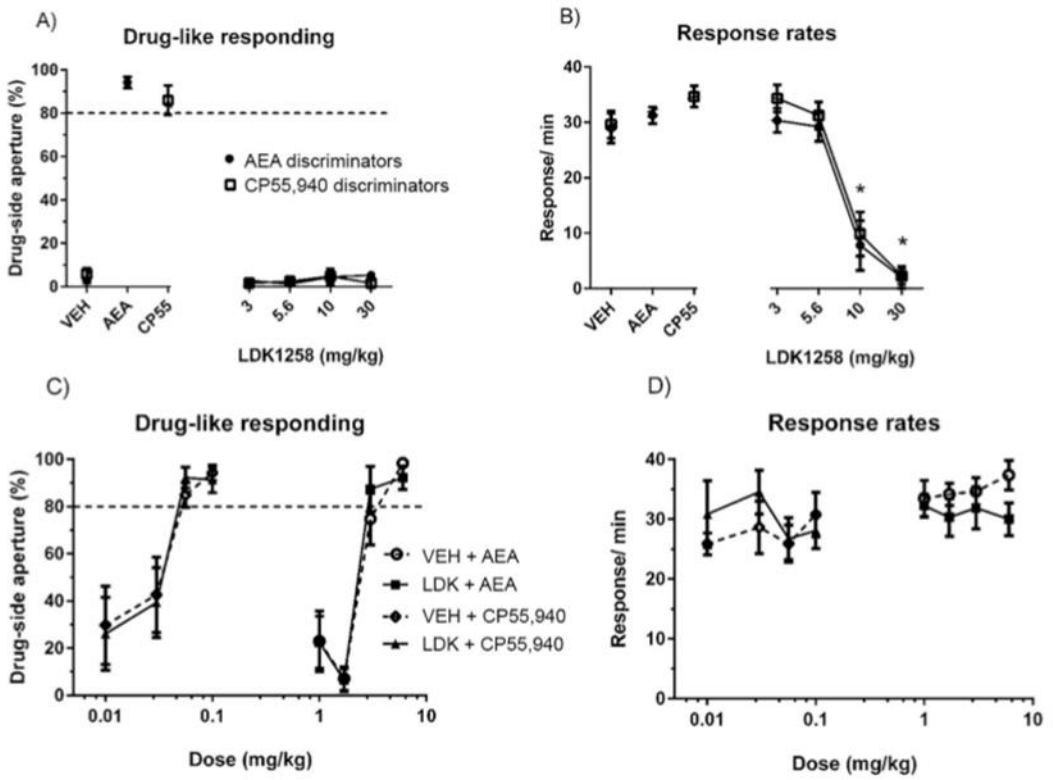


Fig. 3. Evaluation of LDK1258 (LDK) in the mouse drug discrimination paradigm. (A) LDK1258 (3–30 mg/kg) did not substitute for the discriminative stimulus of anandamide (AEA; 6 mg/kg) in FAAH (−/−) mice or for CP55,940 (0.1 mg/kg) in C57BL/6J mice. (B) LDK1258 (3–30 mg/kg) significantly reduced response rates in both FAAH (−/−) mice and C57BL/6J mice. (C) LDK1258 (5.6 mg/kg) did not alter the discriminative stimulus effects of anandamide or CP55,940. (D) LDK1258 (5.6 mg/kg) did not affect response rates when administered prior to anandamide or CP55,940. n = 6–10 mice per treatment group. All data were collected 30–45 min after treatment administration and reported as mean ± SEM. Data were analyzed using two-way ANOVA.

Evaluation of LDK1258 in the tetrad assay

The tetrad assay was conducted in a series of four experiments (see Section 2.7.5 above). Experiments 1 and 2 are shown on the same graphs Figure 4). In the first experiment LDK1258 (30 mg/kg) significantly reduced distance traveled ($t = 4.96$, $df = 14$, $P < 0.001$; Figure 4a) and time immobile ($t = 3.92$, $df = 14$, $P < 0.01$; Figure 4b) at 20 min. In this same experiment, the drug significantly increased catalepsy (LDK1258 main effect: $F(1, 42) = 5.518$; $P < 0.05$) and tail-withdrawal latencies (LDK1258 main effect: $F(1, 42) = 16.40$; $P < 0.001$), though the magnitude of these effects was small (see Figure 4c-d).

LDK1258 also reduced rectal temperature up to 2 h (LDK1258 main effect: $F(1, 42) = 38.35$; $P < 0.0001$; Figure 4e).

The second experiment was conducted in a separate group of mice that were tested 4-6 h after drug administration. LDK1258 (30 mg/kg) did not affect distance traveled, time immobile, catalepsy, or tail-withdrawal latencies at these time points, but did produce hypothermia from 4-6 h (LDK1258 main effect: $F(1, 20) = 9.396$; $P < 0.01$, Time main effect: $F(1, 20) = 5.125$; $P < 0.05$; Figure 4e).

In the third experiment, we examined the effects of 3 and 10 mg/kg LDK1258 in a separate group of mice in the tetrad assay. At these doses, LDK1258 did not affect distance traveled or time immobile (Supplemental Figure 1a-b). In addition, LDK1258 did not affect catalepsy or antinociception at these doses (data not shown), but it significantly decreased body temperature up to 2h (LDK1258 main effect: $F(2, 54) = 10.72$; $P < 0.001$, Supplemental Figure 1c).

We next employed CB_1 (-/-) and (+/+) mice to examine whether CB_1 receptors mediate the locomotor and hypothermic effects of LDK1258. LDK1258 (30 mg/kg) reduced distance traveled (LDK1258 main effect: $F(1, 26) = 28.18$, $P < 0.0001$) as well as time spent immobile (LDK1258 main effect: $F(1, 26) = 16.58$, $P < 0.001$), irrespective of genotype, indicating CB_1 receptor independent effects. The CB_1 (-/-) mice showed a phenotypic decrease in locomotor behavior (main effect of genotype for distance: $F(1, 26) = 5.54$, $P < 0.05$; Figure 5a); main effect of genotype for immobility time: $F(1, 26) = 4.46$; $P < 0.05$; Figure 5b). Significant main effects LDK1258 ($F(1, 26) = 6.870$; $P < 0.05$) and genotype ($F(1, 26) = 4.759$; $P < 0.05$; Figure 5c) were also found for tail withdrawal latencies, though the magnitude of these was very small. Lastly, significant effects of

LDK1258 (F (1, 26) = 55.61; P < 0.0001) and genotype (F (1, 26) = 7.73; P < 0.05; Figure 5d) were found for the hypothermia measure. Again, this drug effect was CB_1 receptor independent. Figure 5 c and 5d depicts the 1 h tail-withdrawal latency and hypothermia data. No significant effects were found in the catalepsy test (data not shown).

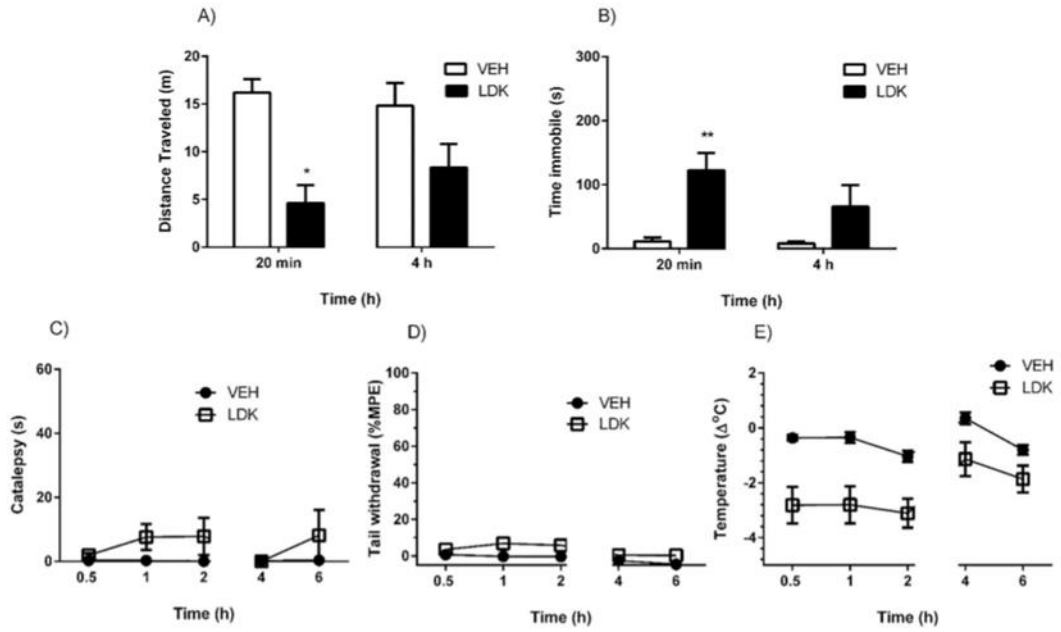


Fig. 4. Evaluation of LDK1258 (LDK; 30 mg/kg) in the mouse tetrad assay. LDK1258 decreased distance traveled and time spent immobile at 20 min, but not at 4 h (Panels A and B). LDK1258 produced small but significant increases in catalepsy (Panel C) and tail-withdrawal latencies up to 2 h (Panels D). The drug also decreased core temperature in C57BL/6J mice for up to 4 h (Panel E). The experiment 1 mean pre-injection tail-withdrawal latencies for vehicle-treated and drug-treated mice were 1.5 and 1.4 s, respectively. The respective mean pre-injection rectal temperatures for this experiment were 37.4 and 37.3 °C. In experiment 2, the respective mean pre-injection tail-withdrawal latencies for vehicle-treated and drug-treated mice were 1.7 and 1.8 s. The respective mean pre-injection rectal temperature means were 37.1 and 37.3 °C. *$p < 0.05$, **$p < 0.01$, n = 6–8 mice per treatment group. All data are reported as mean ± SEM. The student's *t*-test was used data for distance traveled and time immobile were analyzed using. Data for catalepsy, tail-withdrawal, and body temperature were analyzed using two-way ANOVA.

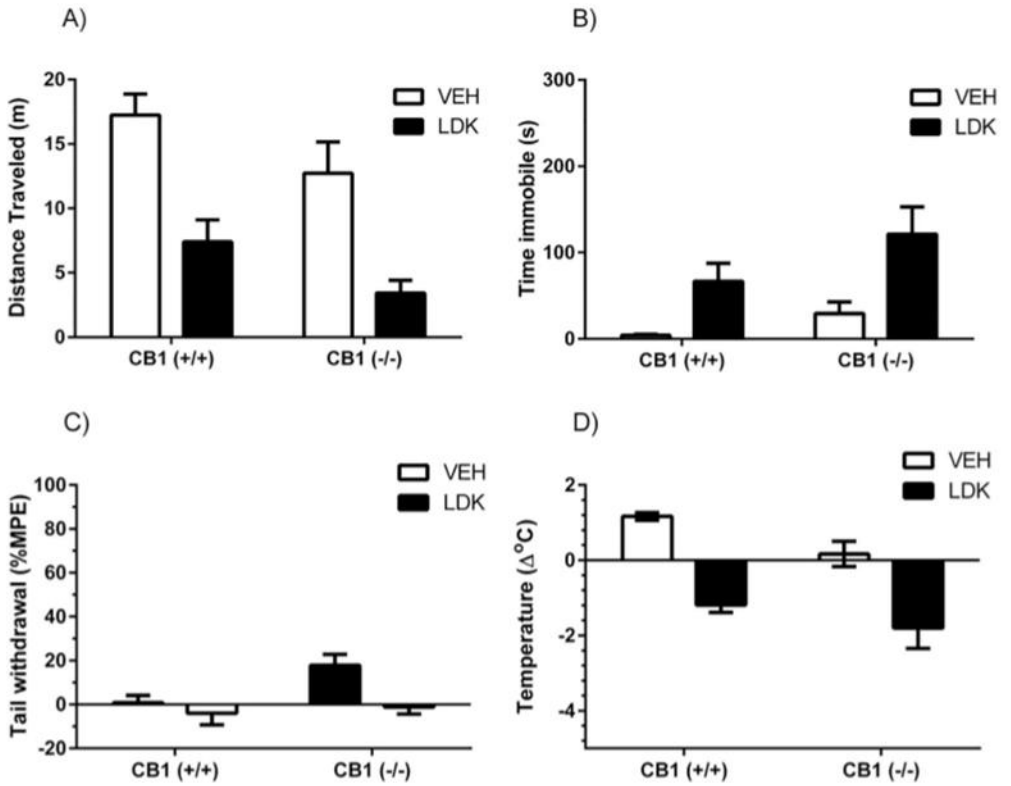


Fig. 5. LDK1258 (LDK; 30 mg/kg) produces a subset of tetrad effects in CB_1 (+/+) and CB_1 (−/−) mice. LDK1258 decreased distance traveled and time spent immobile at 20 min (Panels A–B). LDK1258 also produced small but significant effects on tail-withdrawal latencies and hypothermia up to 2 h (1 h data shown only; Panels C–D). Pre-injection tail-withdrawal means for vehicle-treated CB_1 (+/+) and CB_1 (−/−) mice were 2.5 and 2.1 s, respectively, and 2.6 and 2.1 s for drug-treated mice. For CB_1 (+/+) and CB_1 (−/−) mice, the respective pre-injection mean body temperatures for the vehicle condition were 36.6 and 36.3 °C, and were 36.4 and 36.1 °C for the drug condition. n = 6–9 mice per treatment group. All data are reported as mean ± SEM. Data were analyzed using two-way ANOVA.

Evaluation of whether LDK1258 alters the dose-response relationships of orthosteric agonists in the triad assay

In the final series of experiments, we investigated whether LDK1258 (50 mg/kg) would alter the cumulative dose-response relationship of anandamide (2.5 – 50 mg/kg) in FAAH (-/-) mice or CP55,940 (0.1 – 1 mg/kg) in C57BL/6J mice in measures of catalepsy, antinociception, and rectal temperature. As shown in Figure 6, LDK1258 failed to affect the dose-response relationships of each orthosteric CB_1 receptor agonist. The respective

ED_{50} values of anandamide or CP55,940 did not differ between groups pretreated with LDK1258 or vehicle, and potency ratio calculations verified the lack of an LDK1258 effect on the potency of each orthosteric agonist (Table 3).

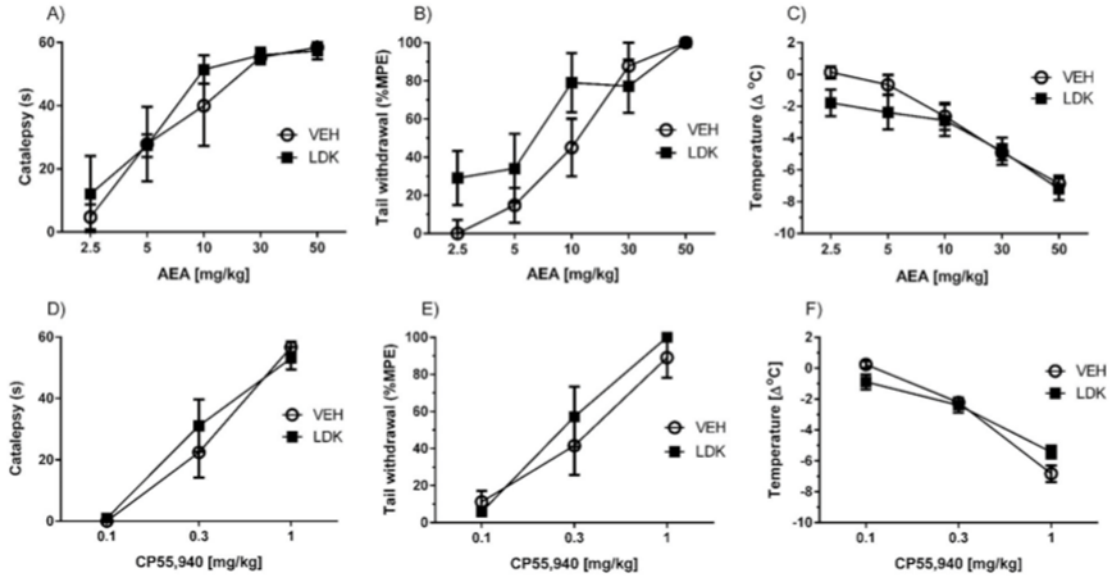


Fig. 6. LDK1258 (LDK; 50 mg/kg, i.p.) does not alter the dose-response relationships of either anandamide or CP55,940 in the triad assay. LDK1258 did not affect the dose-response relationships of anandamide (AEA) in measures of catalepsy (A), antinociception (B), and hypothermia (C). LDK1258 did not affect the dose-response relationships of CP55,940 in measures of catalepsy (D), antinociception (E), and hypothermia (F). For the anandamide experiment, respective pre-injection tail-withdrawal latency averages for vehicle-treated and drug-treated mice were 3.5 and 3.0 s, respectively and respective body temperature pre-injection means were 36.0 and 35.9 °C. For the CP55,940 experiment, the vehicle-treated and drug-treated mice had respective pre-injection tail withdrawal means of 1.9 and 1.6 s, and pre-injection body temperature means of 36.1 and 35.7 °C, respectively. n = 5 mice per group for anandamide studies and 7–8 mice per group for CP55,940 studies. All data reported as mean ± SEM.

Table 3.

ED50 (CL 95%) anandamide (mg/kg)			
Measure	Vehicle	LDK1258	Potency ratio
Catalepsy	7.45 (4.87–11.39)	5.54 (3.64–8.43)	1.25 (0.70–2.30)
Antinociception	11.79 (9.07–15.32)	6.22 (3.52–11.01)	1.67 (0.98–3.06)
Hypothermia	16.96 (13.51–21.31)	12.96 (7.89–21.28)	1.43 (0.88–2.42)
ED50 (CL 95%) CP55,940 (mg/kg)			
Measure	Vehicle	LDK1258	Potency ratio
Catalepsy	0.36 (0.28–0.45)	0.33 (0.25–0.44)	1.09 (0.75–1.59)
Antinociception	0.34 (0.22–0.51)	0.28 (0.21–0.37)	1.21 (0.75–1.99)
Hypothermia	0.43 (0.37–0.51)	0.54 (0.41–0.70)	0.96 (0.72–1.28)

Detection of LDK1258 in mouse blood and brain tissue

To determine whether LDK1258 brain and blood levels, we quantified drug levels in mouse blood and brain tissue at 0.5 and 4 h after administration. LDK1258 (30 mg/kg; i.p.) resulted in brain and blood levels detectable at both time points (Table 4). No drug was detected in the brains or blood of vehicle-treated mice. Statistical analysis releveled no significant changes in LDK1258 levels between these time points in each respective tissue (n = 5-6 mice per group).

Table 4.

Time (h)	Whole Brain (ng/g) mean +/- SEM	Blood (ng/mL) mean +/- SEM
0.5	35.5 (± 7.2)	352.5 (± 104.3)
4	43.2 (± 1.8)	174.4 (± 63.3)

Evaluation of LDK1258 metabolites in a mouse hepatic microsomal reaction

As the delayed effects of the LDK1258 may have resulted from the formation of an active metabolite or metabolites, we used UHPLC-MS/MS on blood and brain tissue described above to screen and identify potential metabolites. No P450 metabolites were detected in the samples from either the 0.5 h or 4 h collection time-point (data not shown).

Chapter III

Conclusions and Discussion

The results presented in this thesis dissertation represent the first study to investigate LDK1258, a novel analog of the well-characterized CB_1 allosteric modulator ORG27569, in established *in vivo* assays highly sensitive to CB_1 receptor activity. Although several other structurally similar analogs of ORG-27569 have been developed in recent years (Khurana et al., 2014), few studies have evaluated whether the effects of these analogs translate from *in vitro* assays to the whole animal. CB_1 allosteric modulators developed in recent years utilize the pharmacophores of first-generation compounds and have similar cellular and behavioral effects to their parent compounds (Gamage et al., 2017; Slivicki et al., 2018). For the purpose of this thesis study we elected to focus on one candidate compound and evaluate LDK1258 because of its high binding cooperativity factor and strong equilibrium disassociation constant compared with other structurally similar compounds (Khurana et al., 2014).

Summary of results. We report that HP-LCMS analysis of blood and brain tissue of mice pretreated with LDK1258 reveals that this compound crosses the blood-brain barrier to enter the CNS. A battery of *in vivo* experiments used in our methodology reveal that LDK1258 produces behavioral effects, including hypolocomotion, hypothermia, decreases in food intake, and delayed anti-allodynia in mice. These pharmacological effects persisted in wild type mice administered the CB_1 receptor antagonist rimonabant or CB_1 (-/-) mice, indicating actions inconsistent with that of CB_1 receptor allosteric modulation. Surprisingly, LDK1258 produced a delayed antinociceptive effect in the CCI

model beginning at 4 h post-administration. This delayed response is different from the onset of action of other CB_1 positive allosteric modulators. For example, the antinociceptive effects of the CB_1 PAM ZCZ011 emerged within 30 min (B. M. Ignatowska-Jankowska et al., 2015). Although the reason for the delayed onset of action found here remains unknown, it is noteworthy that LDK1258 was detected in whole brain at similar concentrations at 0.5 and 4 h. While a delayed onset of behavioral effects has not been reported in studies examining other CB_1 allosteric modulators, inhibitors of biosynthetic and degradative endocannabinoid enzymes similarly display peak antinociceptive effects at 1-3 hrs post-administration (Ignatowska-Jankowska et al., 2015; Wilkerson et al., 2016). It is possible that LDK1258 has off-target effects, such as acting on an enzyme or being metabolized into an active metabolite which are mediating the delayed onset of antinociceptive effects. To determine whether metabolites were present following LDK1258 administration we used a mouse hepatic microsomal assay which modeled the P450 pathway. Although no metabolites were detected in mouse brain and blood samples treated with LDK1258, this lack of finding does not rule out the possibility that the delayed activity resulted from unidentified metabolites from a non-P450 metabolic pathway. Lastly, the observation that the CB_1 receptor antagonist rimonabant did not block the delayed antinociceptive response suggests a CB_1 receptor independent mechanism.

Previous studies demonstrated that ORG27569 and PSNCBAM-1 decreased food intake in rodents; however, the effects were either CB_1-receptor independent or receptor mechanism of action was not examined (Ding et al., 2014; Gamage et al., 2014; Horswill et al., 2007). Similar to these first-generation CB_1 allosteric modulators, LDK1258

reduced food consumption in food-deprived mice. In order to provide a positive control for the reduction in feeding behavior it was necessary to test a compound that is known to suppress food intake via CB_1. Therefore, we also replicated the finding that rimonabant reduces food intake in CB_1 (+/+) mice, but not in CB_1 (-/-) mice (Gamage et al., 2014; Wiley et al., 2005). However, LDK1258 produced anorectic effects regardless of genotype indicating a CB_1 receptor dispensable effect. Similarly, ORG-27569 reduced food intake in CB_1 (+/+) and (-/-) mice (Gamage et al., 2014). Thus, the anorectic effects of these structurally related ligands occur through a CB_1 receptor independent mechanism. On the other hand, human subjects smoking cannabis in a laboratory setting increased snack food consumption (Foltin et al., 1988). Moreover, as gavage administration of THC in rats led to increased consumption of palatable food (Williams et al., 1998), it would be worthwhile to assess effects of LDK1258 or CB_1 allosteric modulators on consumption of palatable food. However, this model of assessing food intake of standard chow in rodents has been established to be sensitive to CB_1 orthosteric ligands

In the tetrad assay, LDK1258 reduced locomotor activity and body temperature, and produced small, but significant effects on catalepsy and thermal antinociception measures. CB_1 (-/-) mice showed a similar pattern of pharmacological effects indicating a CB_1 receptor independent mechanism of action. Similar to ORG-27569 (Gamage et al., 2014), LDK1258 failed to alter the dose response curves of the high-efficacy CB_1 receptor agonist CP55,940 and the low-efficacy CB_1 receptor agonist AEA in producing thermal antinociception, catalepsy, and hypothermia. Prior studies have demonstrated the utility of the mouse drug discrimination paradigm in detecting *in vivo* pharmacological effects of the CB_1 PAM ZCZ011 (B. M. Ignatowska-Jankowska et al., 2015). Here, LDK1258 did

not alter the generalization dose-response relationships of AEA or CP55,940 in the drug discrimination paradigm, though it reduced response rates (i.e. number of nose pokes per min). Similarly, ORG-27569 did not alter the dose-response relationships of CB_1 receptor orthosteric agonists in the drug discrimination paradigm (Gamage et al., 2014). Thus, assays previously shown to detect *in vivo* pharmacological effects of ZCZ011 (B. M. Ignatowska-Jankowska et al., 2015) were negative for ORG-27568 (Gamage et al., 2014) and LDK1258 (present results).

Limitations. A limitation of the current study is that we did not evaluate the mechanism of action of effects that were shown to be CB_1 receptor independent. For example, it was not tested whether non-CB_1 targets, such as CB2 or mu-opioid receptors, mediate the delayed antinociceptive effect of LDK1258. Additionally, we did not evaluate whether non-CB_1 mechanisms of action were responsible for the reductions in food intake, locomotor activity, and body temperature; effects that are consistent with the actions of serotonergic drugs. Therefore, this study was limited in determining the mechanism of action of these effects by not testing drugs that target CB2, mu-opioid, 5-HT receptors, or other possible off-target sites. Effects on endocannabinoid tone was not evaluated in this study, presenting another limitation and leaving undetermined the possibility that LDK1258 is either decreasing or enhancing endocannabinoid tone via inhibition of biosynthetic or degradative enzymes.

It is worthwhile to note that although previous studies have examined the dose-response relationship of CB_1 agonists when co-administered with a CB_1 allosteric modulator in the tetrad and drug discrimination assays (Gamage et al., 2014; B. M. Ignatowska-Jankowska et al., 2015; Pamplona et al., 2012), an effect in these models

has only been demonstrated for positive allosteric modulation of the CB_1 receptor (B. M. Ignatowska-Jankowska et al., 2015; Pamplona et al., 2012). Comparatively, no significant effect was detected in studies evaluating ORG-27569 (Gamage et al., 2014) or its analog LDK1258 in the tetrad or drug discrimination assays, which were expected to cause rightward shifts of the dose-response relationship of CB_1 agonists due to their inhibition of G-protein signal transduction pathways that mediate cAMP accumulation and ion channel phosphorylation. This inability to alter the effects of orthosteric ligands in these models for compounds expected to have CB_1 NAM activity raises the possibility that the tetrad assay and drug discrimination paradigm may be limited to detecting the effects of CB_1 positive allosteric modulation but not CB_1 negative allosteric modulation.

Studies within this thesis examining food consumption may be limited in their interpretation due to the fact that mice were food-deprived for 24 h prior to testing. Determining the baseline food consumption of free-fed mice following drug administration would have greater therapeutic and translational relevance since human subjects are not normally food-deprived for long periods of time. It has been reported that cannabinoid receptors modulate the consumption of palatable food in rodents (Amancio-Belmont et al., 2017), therefore the use of standard rodent chow rather than palatable food is another limitation of the present study.

Additionally, sham animals were not used for the CCI studies which presents a limitation in the proper controls. It is possible that LDK1258 acts differently in animals who receive CCI surgery versus sham animals that are not in an injured state. Using a pharmacological approach to determine CB_1 receptor mediation of the delayed antinociceptive effects in the CCI model presents another limitation of the present study.

It is important to note that previous observations that ZCZ011 significantly increases specific binding of CP55,940 and WIN55,212-2, but reduces rimonabant CB_1 receptor binding (B. M. Ignatowska-Jankowska et al., 2015) questions the utility of employing CB_1 orthosteric antagonists to infer receptor mediation of CB_1 allosteric modulators. To address this limitation, transgenic animals lacking the CB_1 receptor should be used to assess CB_1 receptor mediation. Nonetheless, rimonabant blocked the antinociceptive effects of ZCZ011 in the CCI model of neuropathic pain (B. M. Ignatowska-Jankowska et al., 2015). Moreover, the CB_1 receptor antagonist AM251 blocked the antinociceptive effects of GAT211 in the paclitaxel model of neuropathic pain (Slivicki et al., 2018). Another limitation is that we used a mouse hepatic model of the P450 pathway only. It is possible that LDK1258 is metabolized by a different pathway which may produce active metabolites. Lastly, it is unclear the extent to which LDK1258 may act as an ago-allosteric modulator or a probe-dependent allosteric modulator that requires the orthosteric site to be occupied (Ahn et al., 2012).

Future directions. The results indicating LDK1258 has antinociceptive and anorectic properties provides rational for further investigation of LDK1258 to determine whether this compound holds therapeutic promise to treat neuropathic pain or as an appetite suppressant in models not utilized in the present study such as the chemotherapy-induced peripheral neuropathy (CIPN) mouse model and operant behavior assays which utilize palatable food. The observation that this compound decreases locomotion, hypothermia, and food intake via a non-CB_1 receptor mechanism of action raises the need for future studies to examine the receptors and/or neurotransmitter systems involved in mediating these effects. One approach to answer

this question would be to utilize a pharmacological approach by testing various antagonists of receptor systems that also mediate locomotion, body temperature, and feeding behavior. For example, these physiological processes are also affected by serotonergic compounds. Therefore, examination of a non-selective 5-HT receptor antagonist may result in an inhibition of these LDK1258 effects. An alternative method to identify the mechanism of action of the off-target effects of LDK1258 is to utilize a Basic Alignment Search Tool (BLAST) protocol which is an algorithmic program that can screen for binding selectivity of novel compounds against various GCPRs. In addition, effects on biosynthetic or degradative enzymes should be assessed. Future studies that examine brain levels of AEA, 2-AG, and other endogenous ligands are necessary to determine if LDK1258 treatment has any effect on endocannabinoid levels in the CNS.

Future studies should also consider the limitation presented in this thesis by using a pharmacological approach, rather than a genetic approach, to test CB_1 receptor mediation of the delayed antinociceptive effects in the CCI mouse model. This limitation is presented by the observation that ORG-27569 and ZCZ011 decreases the binding of the CB_1 receptor inverse agonist rimonabant *in vitro* (B. M. Ignatowska-Jankowska et al., 2015; Price et al., 2005), therefore it is possible that co-administration results in a decrease of rimonabant binding *in vivo.* Subsequent studies of the antinociceptive effects of LDK1258 should utilize transgenic CB_1 (-/-) mice to confirm that the delayed antinociceptive effect are indeed CB_1 receptor independent. In addition, a full dose-response of LDK1258 in addition to including a sham surgery control group should be considered in any future assessments of this compound in the CCI model.

Lastly, other non-P450 hepatic metabolic pathways may be contributing to the metabolism of LDK1258 and should be investigated. Further collaboration with medicinal chemists may result in the isolation and resynthesis of active metabolites which may hold therapeutic potential.

Final conclusions. The ORG-27569 pharmacophore represents one of the most widely used by medicinal chemists in the development of novel CB_1 allosteric modulators. Interestingly, ORG-27569 increases CB_1 receptor binding of CP55,940, while dampening functional receptor responses (Price et al., 2005). Increased rates of CB_1 desensitization that concomitantly cause cAMP levels and hyperpolarization states to return to baseline more rapidly than in the absence of the modulator may contribute to this paradoxical response (Cawston et al., 2013). In comparison, LDK1258 displays actions similar to ORG-27569 by enhancing agonist binging in radioligand binding assays and decreasing functional responses *in vitro* (Khurana et al., 2014). Although the *in vitro* effects of ORG-27569 and LDK1258 are consistent with CB_1 allosteric modulation, these actions do not translate to their *in vivo* actions, which are CB_1 receptor independent.

Collectively, the studies conducted within this dissertation demonstrate a translational gap from the cellular level to the whole organism in the development of CB_1 receptor allosteric modulators. In comparison, studies examining the structurally related CB_1 PAMs, ZCZ011 and GAT211, in *in vivo* assays show CB_1 receptor dependent pharmacological effects (B. M. Ignatowska-Jankowska et al., 2015; Slivicki et al., 2018). ZCZ011 produces leftward shifts of the dose-response relationships of AEA and CP55,940 in the triad and drug discrimination assays consistent with the action of a CB_1

PAM; thus, demonstrating the utility of these assays in evaluating CB_1 allosteric modulators.

The results of the present dissertation demonstrate that the novel CB_1 allosteric modulator, LDK1258, produces effects inconsistent with CB_1 receptor allosteric modulation and produces CB_1 independent effects *in vivo*. A major contribution of this study is that the methodology employed provides an efficient screen to evaluate the behavioral effects of novel purported CB_1 allosteric modulators. These results along with studies evaluating the *in vivo* effects of ORG-27569 (Gamage et al., 2014), ZCZ011 (B. M. Ignatowska-Jankowska et al., 2015), and their analogs (Slivicki et al., 2018; present study) underscore the importance of screening purported CB_1 allosteric modulators in rodent models. Future development of the next generation of CB_1 allosteric modulators will require the combination of medicinal chemistry, cellular pharmacology, and behavioral pharmacology and close collaboration between these fields.

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Vita

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