Evaluation of Dual-Activity Opioid-NPFF Ligands for Antinociception

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Abstract

Opioids have become a staple in the medical world as an analgesic used to treat acute and chronic pain. However, there are many issues associated with the frequent use of opioids including tolerance, constipation, and opioid-induced hyperalgesia. Fortunately, recent discoveries of neuropeptide FF (NPFF) antagonists, have led to the understanding that tolerance and hyperalgesia caused by opioids can be greatly reduced. This discovery eventually led to the creation of dual ligands with both opioid agonist and NPFF antagonist activity. These dual ligands have been shown to prevent tolerance and opioid-induced hyperalgesia while maintain analgesic qualities in mice. However, these studies have involved injecting the drug directly into the brain due to their inability to cross the blood brain barrier, which makes them unfit to be converted into drugs fit for clinical use. Intravenous or intraperitoneal injection routes can be used but, oral administration would be the best option for therapeutic use. With this goal in mind, we are met with the task of having the drug penetrate the blood-brain barrier so that it may reach the appropriate recepetors. In this study, we had modified the lead small molecule (non-4,5α-epoxymorphinan derived) designated multiple ligands to hopefully have such blood-brain barrier penetration capabilities. We will evaluate the antinociception of these compounds to determine if they have successfully reached their target receptors from peripheral administration. These compounds tested represent only a portion of the compounds created by our lab.

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Introduction

Prescription drugs such as hydrocodone and oxycodone, μ-opioid receptor agonists, are popular opioid analgesics used to treat acute and chronic pain. While serving as excellent analgesics, these opioids have a host of problems associated with their use, particularly when dealing with long-term pain treatment. Prolonged use of opioids may result in a phenomenon known as tolerance. In tolerance, the opioid receptors become acclimated to the constant intake of opioids and the body shuts down its own natural (endogenous) production of opioids in anticipation of receiving more external opioids. As a result, a higher dose of opioids is required to achieve the same desired effect that was previously experienced and the opioid receptors reach a point where they are rendered essentially non-functional.
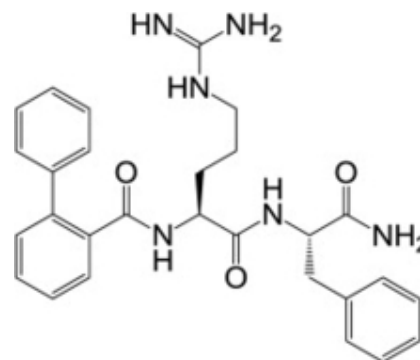
Neuropeptide FF (NPFF) receptors have been identified as an antiopioid or pronociceptive system, essentially working opposite to opioid receptors. Whereas opioids reduce pain, neuropeptide FF causes pain as a protection mechanism when released by itself. If the body is not producing endogenous opioids, it is relying on exogenous opioids to act in its place. However, when we stop taking those exogenous opioids, only neuropeptide FF is being produced. This release of neuropeptide FF without an opioid countermeasure results in increased sensitivity to pain, as a result of not taking exogenous opioids, is known as opioid-induced hyperalgesia. To prevent the release of NPFF in the absence of opioids, it is necessary to have an antagonist acting on the NPFF receptors.

While the entire NPFF system is generally accepted to be an antiopioid system, the two subtypes, NPFF1-R and NPFF2-R, appear to have possibly conflicting effects.The NPFF1-R subtype has been well defined as an antiopioid receptor as shown by in vivo activity of NPFF1-selective dipeptide antagonists (Figure 1-2). But, the NPFF2-R subtype remains doubtful due to a lack of selective ligands specific to it. Tests have shown that NPFF-2R may actually have a pro-opioid effect contrary to the classification of the NPFF system. However, even these claims are speculative because of the weakness of the tests performed. Fortunately, Simonin et al. (2006) discovered that the binding affinity of non-selective dipeptide NPFF antagonist RF9 (Figure 1-1) may be influenced by both subtypes, which suggests that NPFF2-R may also an antiopioid receptor. Even if both subtypes were to be antiopioid in nature, the lack of receptor ligands makes attaining good affinity for NPFF receptors troublesome to obtain. Fortunately, the non-selective binding affinity of RF9 to both subtypes makes it a good candidate to be a potent NPFF antagonist.

Figure 1.
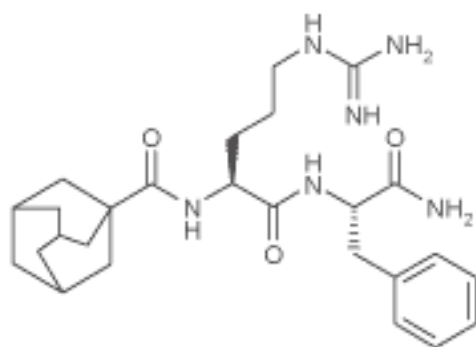
1. RF9, non-selective antagonist   **2. NPFF1-R selective dipeptide antagonist**

Through the co-administration of RF9 and heroin, a μ opioid agonist, the overall drug effects resulted in improved analgesic activity while successfully preventing

tolerance, opioid-induced hyperalgesia, and reducing other related opioid side effects. RF9, as an independent drug (separate from the μ opioid agonist but administered simultaneously), only prevents the development of tolerance and does not cure pre-existing tolerance. It has been hypothesized that a NPFF antagonist with dual opioid agonist activity could resolve this issue (this will be discussed later). Clinically, this is also much more appealing because of reduced patient drug intake, better drug efficacy, and a lower risk of drug-drug interactions.

Returning to RF9 as a NPFF antagonist, RF9 has been found to have good affinity as a NPFF antagonist because of its chemical structure and composition. Good NPFF antagonists have at minimum a phenyl and a guanidine component. Optimally, the chemical compound should contain a C-terminal amide, the guanidine portion of an arginine group, and the aromatic ring (more specifically the benzene group) of a phenylalanine group in order to achieve maximum NPFF receptor affinity. The guanidine group is intended to mimic arginine interaction and the aromatic ring/benzene group is meant to mimic the phenylalanine group. These substitutions are intended to reduce the overall size and molecular weight of the drug so that it may be more bioavailable.

Based on these conditions, Journigan et. al (2014) discovered that (Figure 2-1) makes a good starting template for creating an optimal NPFF antagonist. Not only does this NPFF-binding guanidino-piperidine template retain the desired molecular elements, it has good chemical feasibility and is rather flexible. The piperidine nitrogen component allows for lipophilic substitutions to be made. An additional goal accomplished by creating this template was creating a *non-peptide* NPFF antagonist. Peptides are easily

cleaved by peptidases and they have too short of an action. Because it is a non-peptide small molecule, this template allows for good chemical stability, bioavailability, and duration of action. From this template, a NPFF1-R selective antagonist (Figure 2-2) was also proposed by the group. Tests have shown that it has successfully prevented opioid-induced hyperalgesia.

Figure 2.
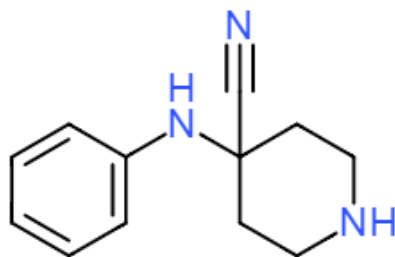
**1. 4-(Phenylamino)piperidine-4-carbonitrile scaffold**
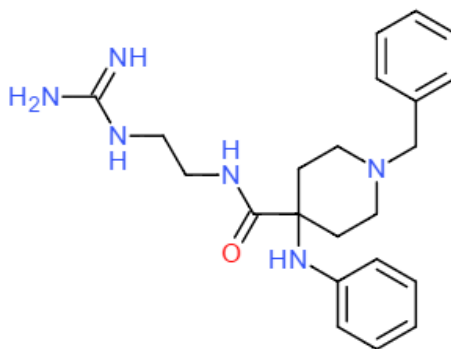

2. NPFF1-R selective antagonist

Now that we have our desired NPFF antagonist, we are now met with the challenge of combining an opioid agonist and a NPFF antagonist into a dual-activity drug (better known as a designed multiple ligand [DML]) while still maintaining both the analgesic effect and the removal of tolerance and hyperalgesia. To achieve this, the drug

must have an affinity for both the opioid receptors and the NPFF receptors. Prior attempts at achieving a dual-activity drug have resulted in DMLs with bioavailability issues because of their high molecular weight and multiple peptide bonds. However, Journigan et. al have described small molecule non-4,5α-epoxymorphinan derived DMLs (Figure 3-3) that act as both an opioid agonist and a NPFF antagonist.

In order to create this dual-activity drug, we need to take the important portion of a NPFF antagonist and combine it with the relevant portion of an opioid agonist. Our research group suggested that the dual-activity drug be based on an opioid scaffold with a 1,4-disubstituted piperidine core with at least one phenyl ring at the 4-position. The NPFF1-R antagonist used would be the antagonist created by the group (Figure 3-1) and the opioid agonist (and also the starting point the drug) to be used would be the delta-opioid agonists BW373U86 and SNC80 (Figure 3-2). This agonist was chosen because the previously created guanidine-piperidine template tolerates the phenyl ring on the diarylmethylidene piperidine portion of the agonist. The 4-*N,N*-diethylaminocarbonyl group of the agonists was replaced with a NPFF pharmacophoric guanidine group. This not only gives the drug increased NPFF receptor affinity, it also reduces the delta opioid selectivity of the drug (as we desire μ selectivity). The hydroxyl group was kept because it was hypothesized that it could mimic the tyrosine residue Tyr-Gly-Gly-Phe message sequence present in endogenous opioid peptides. To further increase NPFF receptor affinity, a benzyl group was inserted into the N-1 position. The resulting molecule is shown in Figure 3-3.

Figure 3.
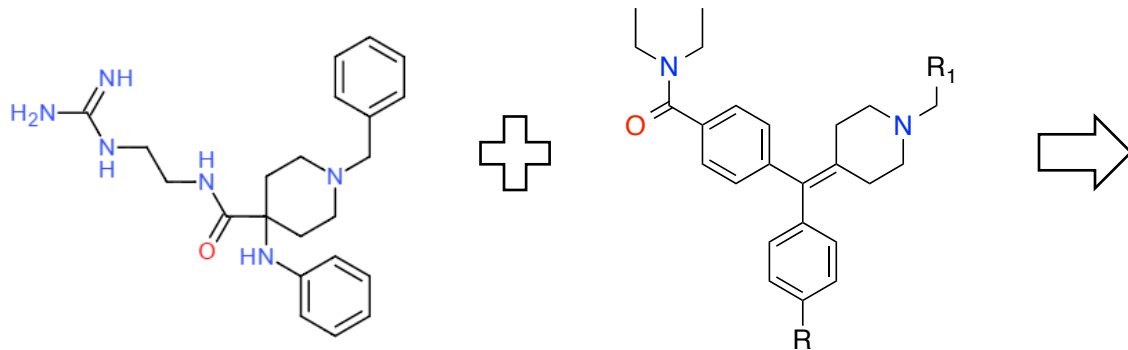
1. NPFF1-R antagonist	2. Delta-opioid agonist
	R = OH, H
	R$_1$ = aliphatic, cyclic, aromatic
	groups
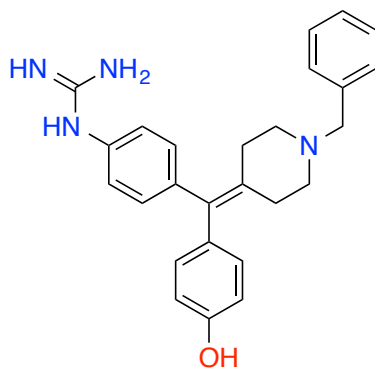
3. Opioid/NPFF Merged DML

In the same study performed to create the DML, the molecules' receptor affinity and functional activity were also evaluated. We are interested in comparing the changes in receptor affinity and activity between the DML (Figure 3-3) and the NPFF1-R antagonist (Figure 3-1). In Figures 4 and 5, the antagonist is compound 30a and the DML is compound 18b. The antagonist starts off with decent NPFF1-R affinity, very strong NPFF2-R binding, and weak opioid binding. The DML maintains its overall

NPFF affinity, except for NPFF2-R which may not be as important, indicating that it may still possess its antagonistic activity. On the other hand, it gains a lot of opioid binding affinity, but it is still comparatively weak compared to the other compounds. However, this is good as the DML maintains NPFF affinity while gaining significant opioid affinity.

Figure 4.

Cmpd.	R	R_1	NPFF$_1$-R	NPFF$_2$-R	μ	δ	κ
				Receptor Binding, K_i (nM)			
17a	3-	3'-OH	311 ± 13	909 ± 73	2.9 ± 0.3	12 ± 2	2.4 ±0.2
18a	3-	4'-OH	570 ± 161	4134 ± 392	13 ± 2	19 ± 2	15 ± 2
17b	4-	3'-OH	1061 ± 94	707 ± 6	16 ± 3	7.2 ± 0.9	3.2 ± 0.3
18b	4-	4'-OH	935 ± 46	1251 ± 110	116 ± 23	76 ± 19	3.3 ± 0.4
29a	3-	H	314	534	4.7 ± 0.6	38 ± 5	23 ± 12
30a	4-	H	976	106	340 ± 40	230 ± 30	140 ± 10

NPFF and Opioid Receptor Binding Affinity of the Compounds

As for the functional activity, we see that all of the compounds possess significant NPFF antagonist activity. The NPFF1-R antagonist also possesses a lot of opioid agonist activity, but it is not as μ-selective as we would like it to be. Fortunately, the DML continues to possess both NPFF antagonist and opioid agonist activity as well as focusing its opioid activity on the μ receptor. Overall, the changes made to create the DML resulted in relatively good receptor binding to both NPFF and opioids receptors. As a result, the DML also exhibited good NPFF activity and good μ-opioid activity.

Figure 5.

	NPFF1	NPFF2	μ	δ	κ
	Functional Activity				
	EC_{50}	EC_{50}	EC_{50}	EC_{50}	EC_{50}
17a	>10K	>10K	7.6	4.5	36.3
18a	>10K	>10K	79.1	79.1	134
17b	>10K	>10K	147	1.08	42.7
18b	>10K	>10K	770	33.9	7.63
29a	>10K	>10K	31.4	130	158
30a	>10K	>10K	882	194	524

**NPFF and Opioid Functional Activity of the Compounds**

While studies have demonstrated that dual-activity opioid-NPFF ligands successfully block tolerance and opioid-induced hyperalgesia while maintaining analgesic effects, the synthesized drugs used have only been directly injected into the brain. Clinically, it would be unrealistic to directly inject a drug into a patient's brain on a consistent basis. Optimally, we would want for the drug to be administered through intravenous or intraperitoneal injection or even through oral administration. The key issue is modifying the drug so that it will be able to permeate through the blood-brain barrier to reach the appropriate receptors. In this experiment, we are not as concerned with preventing tolerance or hyperalgesia as we are with observing antinociception to ensure that the drug has reached the brain.

We initially avoided the use of peptides by adding a guanidine group to our drug to make it more stable and to help it last longer. The problem now is that guanidine has a positive charge associated with it which makes it very basic in nature. Consequently, guanidine does not cross membranes, like the blood-brain barrier, very well. However to work effectively, the drug requires at least two nitrogen groups (guanidine provides three

of them); so the drug must be modified around guanidine rather than removing it completely.

The concept of optimizing brain penetration is centered on the 5-Hydroxytryptamine (5-HT) (particularly the 5-HT$_{5A}$R subtype) receptor, lipophilicity, and pH. Selective 5-HT$_{5A}$R receptor antagonists have shown to have good antipsychotic-like potential and have become a target of interest for drugs. Tests with a guanidine-type 5-HT$_{5A}$R antagonist (Figure 6-1) shows high affinity to the receptor as well as a modest brain penetration. Brain penetration is optimized when the drug is highly lipophilic (high logD) and when basicity is low. Peters et. al (2007) found that fluoro-ethyl substituents substantially improve lipophilicity and reduce basicity of the guanidine. Further experiments determined that difluoroethyl is the superior flouro-ethyl substituents as its insertion as a side chain into the compound led to high activity, reduced basicity, and improved lipophilicity with minimal reduction in 5-HT$_{5A}$R receptor affinity (Figure 6-2).

Figure 6.
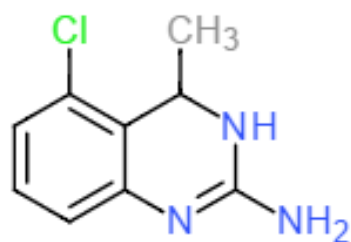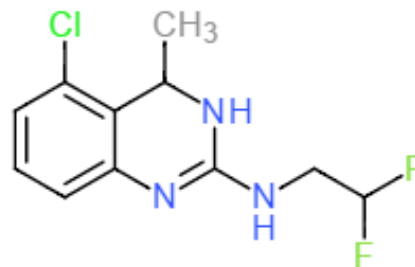

1. 5-HT$_{5A}$R antagonist   **2.Difluoroethyl substituent**

Based on these findings, we can modify the compound that Journigan et. al synthesized (Figure 7-1) and optimize its brain penetration. One way to do this is to replace one of guanidine's nitrogen groups with a difluoroethyl group (Figure 7-3). This

produces a similar effect by reducing the basicity of guanidine enough to allow it to pass

through the blood-brain barrier. Another less effective method is to replace guanidine

with amidine (Figure 7-2), which is similar in structure to guanidine minus one nitrogen

group. While still basic in nature, the removal of one nitrogen group has been proven to

reduce the drug's basicity enough for it to cross the blood-brain barrier.

Figure 7.
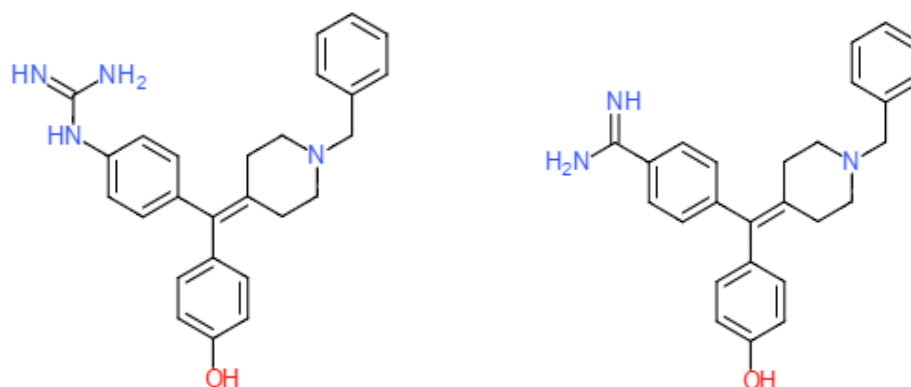
1. Opioid/NPFF Merged DML   **2. DML w/amidine**
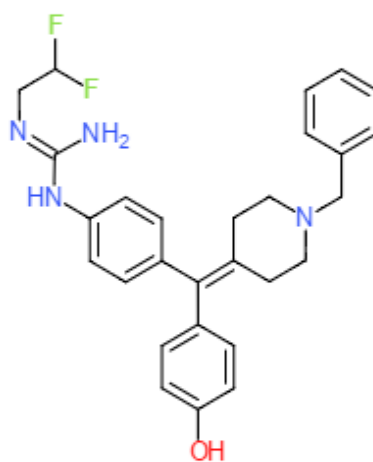

3. DML w/ difluoroethyl substituent

Materials and Methods

For this experiment, black C57BL6 mice from Harlan Laboratories were used as test subjects; mice were tested on in groups of eight. In order to measure antinociception, the mice were put through an organized regimen of trials and injection. The amount of drug given to each mouse was dependent on their weight in grams. For every one gram the mouse weighed, 0.1 mL of drug was injected into the mouse. The drug was injected intraperitoneally to determine if the drug could travel to the brain by permeating through the blood-brain barrier.

The three drugs tested in vivo were CNK404 (Figure 8-1), CNK406 (Figure 8-2), and CNK408 (Figure 8-3). A vehicle drug, a mixture of ethanol, cremophor, and saline (1:1:18), was used for baseline measurements and as the base for dissolving the drugs to be tested. Morphine was also tested as a positive control. Each drug was tested three times in varying doses of 5 mg/kg, 10 mg/kg, and 20 mg/kg, except for morphine which was only tested in a 10 mg/kg dose. It is important to note that the biding affinities for NPFF and opioids receptors have not yet been obtained in these three drugs.

Figure 8.

1. CNK404

2. CNK406

3. CNK408
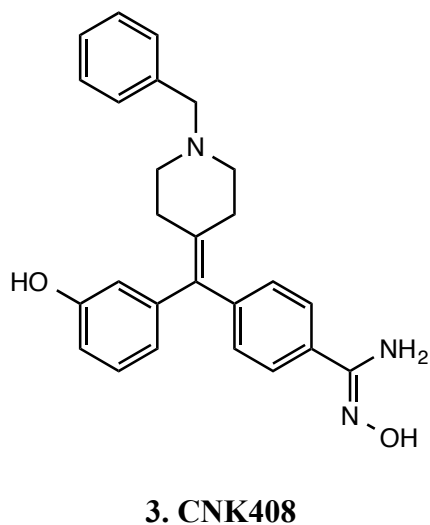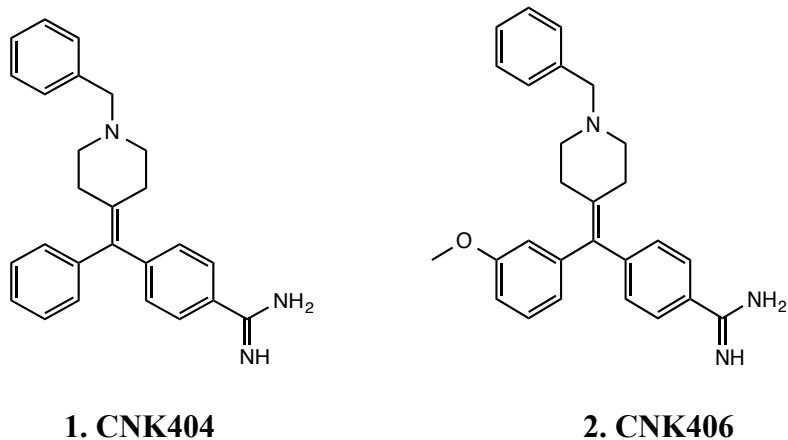
There were two different assays that were used in determining the impact of the drugs on the mice. One was the hotplate assay and the other was the tail-flick assay. These assays were designed to test how quickly (in seconds) the mice will react to pain stimulation (in this case intense heat), thus testing the analgesic effects of the drugs. The hotplate assay is designed to demonstrate a conscious awareness of pain, similar to stepping barefoot onto hot asphalt. The tail-flick assay is designed to measure a reflex action to pain, similar to touching a flame or an extremely hot burner. These two assays represent measurements of supraspinal (hotplate) and spinal (tail-flick) analgesia.

Before experimentation, a baseline reading for antinociception was taken for future comparison. After a baseline assessment using the hotplate and tail-flick assay, the mice were immediately injected with the designated drug. The mice were allowed to sit for 30 minutes so that the drug would have a chance to travel to its target location, the receptors in the brain. After 30 minutes, the mice were assessed again using the same hotplate and tail-flick assay.

The hotplate assay is used to examine the effects of the drug on thermal nociception. This assay utilizes a hotplate machine that maintains a constant heat level and a plexiglass cylinder to ensure that the mouse is continuously exposed to the heat and is able to be clearly observed. A timer is manually started once all four paws of the mouse make contact with the hotplate. The timer is stopped when a reaction to the heat is noticed on the hindpaws including jumping, toe curling, rapid tapping, and paw-licking. To prevent tissue damage, we have established cut-off periods in which we stop the trial even if we do not see a reaction. The overall cut-off period is 45 seconds. Only one trial of the hotplate assay is performed each time.

The tail-flick assay also examines thermal nociception using a special apparatus. The apparatus works by shining a concentrated beam of light at the mouse's tail and then recording how long it takes for the mouse to react in response to pain. To prevent unnecessary movements, the mouse's body is confined within a restrainer with only the tail exposed and free to move. The tail is positioned over a groove on the apparatus where the thermal stimulus is to be applied. The thermal stimulus is simultaneously activated along with an automated response timer. When the mouse flicks his tail, the beam of light is broken and both the thermal stimulus and the timer are stopped. Two

trials are taken approximately 20 to 30 seconds apart from each other. The cut-off period before drug injection is 5 seconds. If the mouse does not react to 5 second thermal stimulus twice, that mouse is unable to be utilized for that experiment. The cut-off period after drug injection is 15 seconds. If the mouse exceeds 10 seconds on the first of two trials, it is not necessary to run a second trial.

Results and Discussion

The hotplate and tail-flick data for the three drugs and morphine were compared to the vehicle drug primarily through mean value +/- SEM (standard error of the mean or standard deviation) of % MPE (percent maximum effect).

The %MPE equation for the tail-flick assay is:

$$\% \, MPE = \frac{postdrug \, latency \, (s) - baseline \, latency(s)}{15 \, seconds - baseline \, latency \, (s)} * 100\%$$

The %MPE equation for the hotplate assay is:

$$\%MPE = \frac{postdrug \, patency \, (s) - baseline \, latency(s)}{cut \, off \, time \, (45 \, s) - baseline \, patency \, (s)} * 100\%$$

The %MPE obtained from the experiments was analyzed using GraphPad Prism and used to calculate the mean value +/- SEM for each drug and dose. Using a one-way ANOVA, preceded by the Dunnett's post hoc test, the mean values +/- SEMs for the three drugs and morphine were analyzed for statistical significance against the vehicle control at $p < 0.05$. Separate analyses were performed for the hotplate and the tail-flick assays.

Figure 9 below shows the average %MPE for the vehicle drug, morphine, and three drugs tested for the tail-flick assay. From the statistical analysis, none of the three drugs were found to be statistically significant from the vehicle drug at all three doses.

Morphine, on the other hand, was found to have high statistical significance. Increasing the dose may have a small effect on increasing the antinociception of the CNK404 drug but it has no apparent effect on the other two drugs. The antinociception of the drugs in regards to the tail-flick assay are effectively absent in comparison to both the vehicle drug and morphine.

Figure 9.
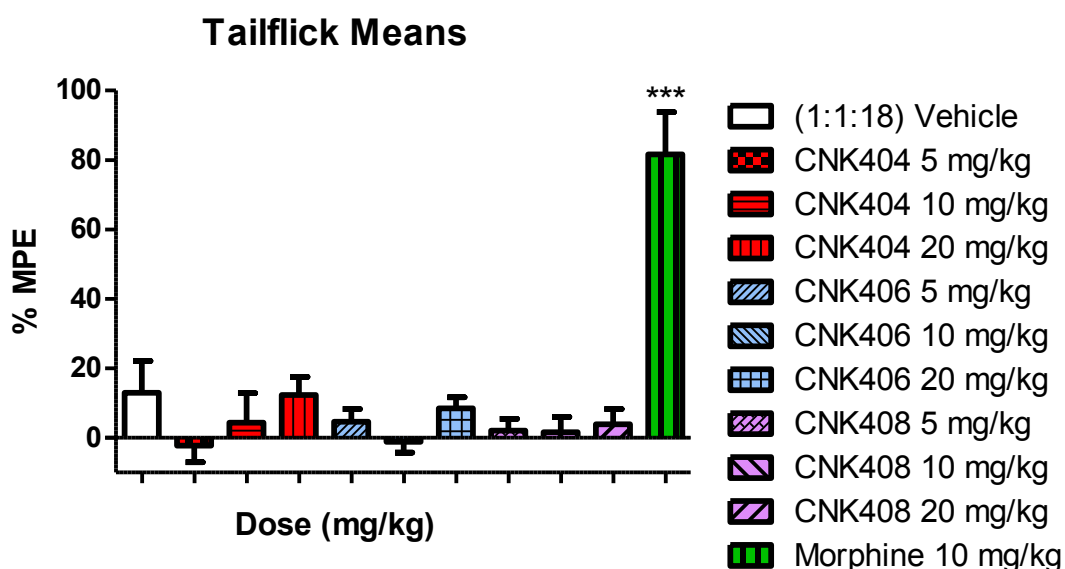
Comparison of tail-flick %MPE between CNK drugs, morphine, and vehicle drug

Figure 10 below shows the average %MPE for the vehicle drug, morphine, and the three drugs tested for the hotplate assay. The CNK404 drug looked promising at first glance as it exceeded the vehicle drug %MPE, but further analysis showed that it was statistically insignificant. However, the increasing dose and antinociception correlation is still present in CNK404 but not in the other two drugs. Just like in the tail-flick assay, none of the three drugs showed statistical significance while morphine showed high

statistical significance. Unfortunately, this means that antinociception remains absent in regards to the hotplate assay.

Figure 10.
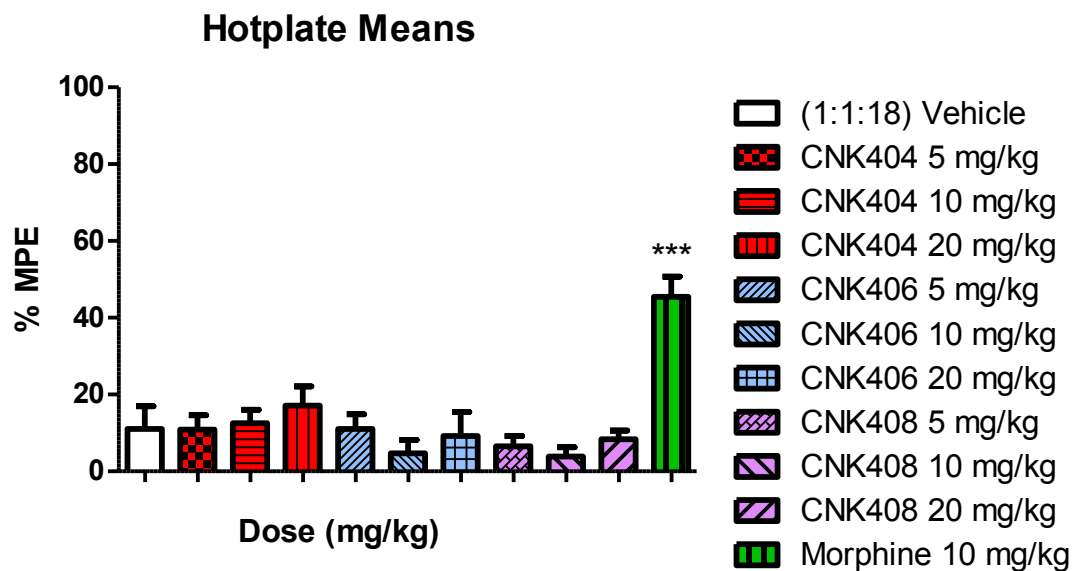
Hotplate Means

Comparison of hotplate %MPE between CNK drugs, morphine, and vehicle drug

Conclusion

Based on the results gathered from the experiment, all three CNK drugs are not active at these dosage levels. Using morphine as a comparison, we are still far from achieving the antinociception that we desire from these drugs. The CNK404 drug looked promising because of the subsequent increase in %MPE with increasing dose, but more research must be conducted to determine the strength and accuracy of that perceived correlation. The remaining two drugs have no consistency nor patterns that come from increasing the dose.

Several things could have happened for the drug to not work. While it is fairly certain that the drug did not penetrate the brain, we cannot definitively say that until we further examine the pharmacodynamics of the drugs. Assuming that the compounds did not penetrate the brain, the modifications to the original small molecule non-4,5α-epoxymorphinan derived DMLs (Figure 3-3) structure did not actually improve brain penetration enough or at all. On the other hand, if the drug did in fact penetrate the brain, more possibilities arise. The drug dose may not have been high enough for the drug to show analgesic effects, the drug may not have bound to the proper receptors (or bound to the proper receptors too weakly), or perhaps the drug failed to bind to a receptor at all.

Also, we should consider the possible pharmacokinetics of the drug. Two parameters that may present issues are half-life and maximum concentration (C_{max}). It typically takes about five half-lives for a drug to reach its steady-state concentration so

the timing between pre- and post-injection evaluations is critical in finding that concentration. If we are too early, the drug will not have any efficacy yet; if we are too late, the drug will have been eliminated from circulation. C_{max} is also important because we need to find the proper concentration between toxic and therapeutic levels. It is important to find a proper balance so that the drug will be as effective as possible.

Conclusions drawn from this experiment are limited by the nature of the experiment. The experiment was only designed to test whether or not the drug has antinociception potential (and to what degree) and did not explore the chemistry of the compounds. The only thing that we can confidently conclude from this experiment is that the CNK drugs are not active at these particular doses. Until we further explore the chemistry of the drugs, the other conclusions are mere speculation as to why the compounds failed. However based on previous findings, the most likely explanation remains: the drugs did not penetrate the brain and need to be further optimized in order to be active as an analgesic.

There are still many things that can be done to possibly improve the brain penetration of the dual-activity ligands. The simplest way is to change the dosage of the drugs and see if there is a correlation between dose and brain penetration at varying doses as well as helping us to discover an optimal C_{max}. We can also adjust the wait time in-between pre- and post-injection evaluations to see if we can discover the half-life of the drug and consequently discover the amount of time it takes for the take to exhibit full efficacy. We can also continue to optimize the drug by further manipulating the original compound, though there are no specific considerations at this time. We could also improve our current assays or expand upon our repertoire of assays in order to obtain

more accurate measures of antinociception. Our group is working on creating more

compounds and we continue to be optimistic about the antinociception potential of these

drugs.

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