THE MU-DELTA OPIOID RECEPTOR HETERODIMER MAY EVOKE SIMILAR
SIGNALLING WITHIN THE STRIATUM, PERIADQUEDUCTAL GRAY, AND BRAINSTEM

By

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Abstract

Opioid drugs like morphine are the gold standard for treating acute and chronic pain, but they induce detrimental side effects such as tolerance and dependence. It has been suggested that the mu-delta opioid receptor heterodimer (MDOR) enhances some of these side effects and that heterodimer targeted drugs could be a solution to weaken these side effects. We have thus created an MDOR selective antagonist called D24M with a ~100-fold in vitro selectivity for the MDOR over the monomers. We then used D24M to examine MDOR physiology in mice. We found that intracerebroventricular (icv) injection of 1 nmol D24M strongly increased oxymorphone antinociception in models of tail flick, paw incision, and chemotherapy-induced neuropathic pain, but had no effects on morphine or buprenorphine antinociception. D24M also strongly decreased morphine withdrawal in acute and chronic dependence models. We then tested ERK activation via western blot analysis with the injection of D24M or vehicle and various opioid drugs. We found that D24M has similar effects on ERK pathway activation for each part of the three tested brain regions (striatum, periaqueductal grey [PAG], and brainstem); further confirming that ERK is a possible pathway for the MDOR to promote kinase activation. This discovery could be a stepping stone for determining the signaling mechanisms of the MDOR.
Introduction

In the 21st century, opioid drugs like morphine are the gold standard for treating acute and chronic pain, but they induce detrimental side effects such as tolerance, dependence, and addiction. In the United States, the number of deaths involving opioid analgesics have increased from 4,041 in 1999 to 14,459 in 2007 [1]. This number is staggering high and correlates to the increased prescription of opioid drugs. Insurers had limited coverage for behavioral pain therapy in the late 1990s, so the pharmaceutical manufacturers took full advantage. The manufacturers marketed the opioid drugs as a way to relieve pain quickly [2]. The opioid crisis is still growing due to the high demand for pain relieving drugs and the severity of people’s pain state. Fentanyl and morphine are indicated in patients that have suffered from a traumatic event and will continue to be used in the hospital setting. Due to the high severity of injuries and the quick analgesic effects of opioids, this is the perfect recipe for a short-term solution that can deliver long-term devastation.

Not only are clinical opioids contributing to the dilemma but also the recreational use of opioids. There is recreational use of opioids such as heroin due to the results of over exposure to morphine or fentanyl in the medical setting. With the constant intake of clinical opiates to control their pain state, people are slowly becoming addicted to opioids due to treatment bedside in a hospital. Once they get out, they need to find another opioid alternative that is more cost efficient or available than morphine or fentanyl. Therefore, they turn to recreational opioids like heroin to meet their addiction. Due to this and other factors, heroin usage was steady for years but has recently spiked, with heroin overdose deaths tripling between 2010 and 2015 [3]. The opioid crisis is a hot topic in the medical field and many researchers are attempting to tackle this dilemma.

Opioids are able to modulate behaviors including pain, reward, and emotional affects with the help of opioid receptors. The main opioid receptors are the μ [MOR], δ [DOR] and κ [KOR]
receptors [4]. Specifically, for the research performed in our lab, we are looking at the interaction of the MOR and DOR. For each receptor, there are different opioid drugs that will bind and evoke the analgesic effects that we see. For example, morphine is a type of opioid drug that binds to the MOR receptor and activates the G-protein pathway to evoke analgesia [5]. DSLET, a DOR agonist, does the same thing for the DOR. These opioid receptors play a crucial role in determining possible solutions and alternatives for opioid use.

Possible solutions for this epidemic have been in research for a tremendous amount of time, and we still do not have a clean-cut answer. There have been discussions by scientists to experiment with oxytocin and how oxytocin can play a role in anti-nociception, which could relieve pain without the addictive effects of opioids [6]. This topic is a relatively new topic and is being deeply studied. As of right now, a MOR antagonist named naloxone is used to control overdosing on opioid drugs. Naloxone works where naloxone has a higher affinity for opioid receptors than regular opioids, so naloxone will disrupt the binding between the receptor and the opioid [7]. This occurs almost instantly and thus, reduce the chances of overdosing on the specific drug. However, naloxone does cause side effects like withdrawal that once again, feed into the opioid epidemic. Another possible solution for this epidemic is to stray away from opioids entirely and attempt to find an alternative pain killer like oxytocin mentioned above. However, true high efficacy alternatives don’t yet exist, so we cannot move away from opioids yet. We thus need to find a way to take the opioids but reduce the chances of acquiring the side effects, particularly addiction. In our lab, that is exactly what we are looking to achieve. To be able to dive deeper into this dilemma, we explored a relatively new scientific topic called heterodimers.

A heterodimer is defined as two different receptors joining together to provide a unique signaling pathway. What is also interesting is that heterodimers greatly reduce affinities for their
selective ligands to the monomers, implying a unique heterodimer pharmacology [8]. With the decreased affinity for singular selective ligands, the heterodimer is able to bind to heterodimer selective agonists that can induce synergistic effects [8]. With the synergistic effects, these G-protein heterodimers will produce a pathway that could lead to multiple protein kinase activations that will ultimately lead to an alteration in gene expression. One way to achieve heterodimer selectivity is to bind two pharmacophores for individual receptors together by a linker. The linkers can vary in length and enhance heterodimer selectivity thorough avidity, where the binding of one pharmacophore makes the binding of the second linked pharmacophore more likely due to bringing it physically closer to the binding site.

For our studies, we are looking at the mu-delta opioid receptor heterodimer (MDOR). The MDOR is the combination of the MOR and DOR receptors and can produce a unique signaling profile that can contribute to new pharmacological targets and pathways [9]. There have been doubts about the MDOR in vivo due to a lack of selective tools to study this target. However, there are proven compounds for the MDOR such as a compound named CYM51010, and a selective antibody that demonstrates MDOR upregulation after chronic morphine treatment in pain regulatory regions within mice [10]. To be able to test the role of the MDOR, we needed to create an MDOR antagonist that would bind to the heterodimer with the highest affinity, potency, and selectivity.

**Previous Data: Proof of Concept**

**MDOR Antagonists Target the MDOR In Vitro**

To find the highest affinity, potency, and selectivity antagonist for the MDOR, we decided to synthesize multiple heterobivalent ligands that are made up of low affinity MOR and moderate affinity DOR pharmacophores that are physically linked together by a variable length spacer
composed of 15-41 hydrocarbons (Figure 1A-B). The variable length spacers allow us to identify the optimum spacer length that bridges the gap between the binding sites of the heterodimer pair. Looking at Figure 2C, we can see the spacer length of approximately 24 atoms provide the highest selectivity (black line) and highest MDOR potency (IC50HIGH, purple line). The IC50HIGH is the amount of heterodimer receptor antagonists needed to inhibit the DOR by half (50%). Therefore, the lower the IC50 value, the stronger the inhibitor and we see that with the spacer length of 24 atoms with a potency less than 1 nM. We also see that the DOR IC50 line (red) is maintained relatively constant to show the heterobivalent antagonist is binding to the heterodimer instead of the DOR; in other words, the spacer length impacts binding to the MDOR but not the DOR, suggesting heterodimer binding. With those experiments, we concluded that the heterodimer antagonist D24M is the most effective antagonist for the MDOR, with the highest affinity, potency and selectivity (~90 fold) in the series.

As a control, we tested D24M and various control compounds in MDOR cells (Figure 2A) and monomer cells (DOR, MOR) (Figure 2B). We also tested D24M, a D15 linker control, and DOR and MOR antagonists against the stimulation of CYM51010 (MDOR agonist) in MDOR cells. Looking at Figure 2A, we see a two-site curve associated with the D24M compound, indicating D24M is binding to the heterodimer at the high site and the DOR monomer at the low site. With the other pharmacophores, we see a one-site low affinity/potency curve indicating that the pharmacophores are binding to the monomer; such as M pharmacophore binding to the MOR. We then tested various concentrations of D24M in different cell lines (MDOR, DOR and MOR) in Figure 2B. Once again, we see a bi-phasic curve for the D24M in the MDOR cell line compared to the other cell lines. We see a low affinity/potency one site curve for the DOR cells and that is
because D24M has a moderate affinity for the DOR compared to the MOR. This control data further suggests that D24M selectively binds to the MDOR.

**D24M Antagonist Selectivity for MDOR *In Vivo***

Our first step to using D24M *in vivo* was to establish the selectivity of D24M for the MDOR. For these experiments, we decided to test various concentrations of D24M with different types of opioids, some of which impact the MDOR and some of which target MOR/DOR monomers. The different types of opioid drugs used for testing were CYM51010, Deltorphin II, DAMGO, DSLET, and vehicle. CYM51010 and Deltorphin II are MDOR agonists and DSLET is a DOR monomer agonist, and DAMGO is a MOR monomer agonist. We then used the tail flick test to evaluate how these drugs interacted with receptors *in vivo*. The tail flick pain model is where mice’s pain tolerance was measured by submerging their tail into a hot water bath of approximately 52°C and measuring the time it takes them to withdraw their tails. Before conducting the experiment, we obtained the mice and placed them in the experimental room for 30 minutes or more. The reason for waiting is to allow the mice to get acclimated to their surroundings. This pain model allows the mice to also express their behavior so when mice are placed in a new environment, they will show abnormal behavior and can disrupt the experiment. After the 30-minute wait, we baselined the mice’s tail flick latency and weighed each mouse. When the baseline latency was obtained, the mice were then prepped for the experiment. Each mouse was placed in a chamber with running isoflurane to start and maintain anesthesia. Once the mouse was sedated, the mouse was injected with 5 μL of D24M of various dosages through intracerebroventricular injection (*icv*). After 5 minutes, we then injected 5 μL of the selected opioid drug through *icv* injection. Once that was complete, we sealed the wound and conducted the hot water tail latency
for a two-hour time course. To eliminate complete variation of tail latency and avoid burning the mouse’s tail, the cut-off time was set to 10 seconds.

Looking at Figure 3A, we tested for CYM51010 with a fixed concentration of 3.2 nmol. Deltorphin II concentration for the experiments was set at 3.2 nmol (Figure 3B), and DAMGO was set at 0.1 nmol concentration (Figure 3C). DSLET (Figure 3D) had a concentration of 1 nmol. Figure 3E is the vehicle and set as our control group. As we can see for the vehicle, all the dosages of D24M had no effect on the tail withdrawal latency and confirms the effects we see are due to the addition of the opioid drugs (Figure 3E). Comparing CYM51010 and Deltorphin II (MDOR agonists) to DAMGO (MOR agonist) and DSLET (DOR agonist), we were able to see antagonism of D24M for the MDOR agonists but not the monomer agonists. When we look at the dose response curves in Figure 3F, we can see the stimulation of CYM51010 and Deltorphin II decrease. This finding suggests that D24M is blocking the two MDOR agonists. When looking at DAMGO and DSLET, we can see there isn’t much change for the DSLET but a slight increase for DAMGO. This suggests that D24M is selective for the MDOR in vivo for doses up to 10 nmol by the icv route.

**D24M Reduces Morphine Withdrawal**

There have been previous results from the MDAN series of bivalent ligands that the MDOR could regulate dependence and withdrawal [11]. Studies for acute and chronic dependence were thus conducted. For these experiments, we decided to use 1 nmol D24M, well within the selectivity range established above. The acute dependence model was injecting 100 mg/kg morphine into mice through subcutaneous route (sc). For the chronic model, the mice were injected with 10 mg/kg morphine twice daily for the first day and would eventually escalate to 35 mg/kg on the fourth day. On the fourth day, we then intracerebroventricularly (icv) injected the mice with 1 nmol
D24M or vehicle. After five minutes had passed, we then intraperitoneal (ip) injected 10 mg/kg (acute) or 30 mg/kg (chronic) naloxone for precipitation of withdrawal. Withdrawal was observed by placing the mice into a clear tube and measuring the number of jumps performed within 20 minutes. Gastrointestinal (GI) symptoms of withdrawal were also recorded the weight of urine and feces on a filter paper at the end of the 20-minute withdrawal period. With the addition of 1 nmol of D24M, we found that D24M strongly and significantly reduced the jumping behavior in both acute and chronic dependence and withdrawal (p<0.05, p<0.01, respectively; Figure 4A-B). These observations support the idea that the MDOR can promote withdrawal and dependence, which is reversed by our antagonist treatment. However, we did not find significant effects on the GI symptoms between vehicle and D24M with naloxone. This could be due to the fact that we injected D24M through icv injection instead of systemically, which would impact the intestines. The GI tract symptoms are associated with the enteric nervous system and the enteric nervous system is within the intestines [12].

Methods

Male and female CD-1 mice were used for these experiments, 4-5 weeks of age, and obtained from Charles River. The mice were recovered for at least 5 days after shipping before experimenting and kept with no more than 5 mice per cage. The mice had standard chow and water available ad libitum, with a 12 hour light:dark cycle with temperature and humidity control in the University of Arizona AAALAC-accredited vivarium. All experiments were performed in accordance with the NIH Care and Use of Laboratory Animals handbook, and all experiments were approved by the University of Arizona IACUC. The experimenter was blinded to treatment group by the use of coded drug vials, which were decoded after the experiment was complete.
Opioid Anti-Nociception in Different Pain States

To evaluate the opioid anti-nociception, we tested out three different pain models: tail flick, paw incision, and CIPN. Mentioned above, the tail flick pain model is where mice’s pain tolerance was measured by submerging their tail into a hot water bath of approximately 52°C and measuring the time it takes them to withdraw their tails. We baselined the mice’s tail flick latency and weighed each mouse. When the baseline latency was obtained, the mice were then prepped for the experiment. Each mouse was placed in a chamber with running isoflurane to start and maintain anesthesia. Once the mouse was sedated, the mouse was injected with 5 μL of 1 nmol D24M through intracerebroventricular injection (icv). After 5 minutes, we then injected 5 μL of the selected opioid drug through icv injection or sc injection. Once that was complete, we sealed the wound and conducted the hot water tail latency for a two-hour time course. To eliminate complete variation of tail latency and avoid burning the mouse’s tail, the cut-off time was set to 10 seconds.

The paw incision pain state is when we sedate the mice with isoflurane and surgically incise the paw. We allow the mice to recover for 24 hours before using Von Frey filaments to test mechanosensitivity for pain. 0.032 mg/kg of oxymorphone was used for the experimental model and 1 mg/kg of morphine was used. 0.2 mg/kg buprenorphine was also used for the paw incision pain model. After 24 hours, the mice were icv injected with 1 nmol D24M or vehicle five minutes prior to subcutaneous injection of the opioid. After the injection of the opioid, the mice were placed in a Von Frey box and the filaments were recorded for a three-hour time course. The next pain state our lab tested was CIPN.

Chemotherapy-induced peripheral neurotoxicity (CIPN) is a pain state where we ip injected 2 mg/kg paclitaxel in mice on days 1, 3, 5, and 7 with testing on day 8. CIPN is the source of neuropathic pain for mice [15] and D24M was tested to see any effects for opioid analgesia. After
the paclitaxel was injected on the 8th day, D24M was icv injected five minutes prior to the injection of opioids (1 mg/kg morphine, 0.032 mg/kg oxymorphone, or 0.01 mg/kg buprenorphine). Von Frey filaments were used, and mechanical allodynia was measured in a three-hour time course.

Our lab also hoped to find the underlying mechanism for these opioid drugs with respect to D24M. Therefore, looking at the ERK pathway could be the first step in finding the mechanism for D24M and how the MDOR interacts with MOR receptors.

**ERK MAPK Mechanism in the Striatum, Periaqueductal Grey, and Brainstem**

To test morphine and oxymorphone, we looked at the activation levels of ERK MAPK in different parts of the brain. The specific regions of the brain we tested were the striatum, periaqueductal grey (PAG), and brainstem. To keep the experiments consistent, 1 nmol D24M was deemed the correct dosage based on our experiments above. Various combinations of vehicle or D24M and opioids drugs (morphine, oxymorphone, and CYM51010) were tested. Mice were icv injected with either vehicle or 1 nmol D24M. After 5 minutes, the mice were then injected with the opioid drug of choice. DAMGO treated mice were used as a positive control due to the fact that DAMGO is a MOR agonist that stimulates sections of the brain for ERK activity. Once injected with the specific opioid, the mice were then quickly sacrificed, and the three specific regions of the brain were dissected. The dissected regions were then placed in an Eppendorf tube, flash frozen in liquid nitrogen, and stored in the -80°C freezer.

We then analyzed ERK MAPK activation by Western Blot analysis. Western Blots are often used in research to separate specific proteins and locate them by their weight in kDa [16]. The samples were taken from the -80°C freezer and placed in an ice chest. While the samples are in the ice chest, a RIPA-buffer with protein inhibitors solution was created to homogenize our samples in. Once the samples were homogenized, the samples were then spun down to acquire the
cell lysate of the sample (10,000g for 10 minutes at 4°C). We then conducted a protein concentration quantification to determine the amount of protein. Once we figured out the amount of protein, we then proceeded to the western blot protocol in our lab. We created our samples with the cell lysates, dye, and beta-mercaptoethanol. (BME). BME is used as a reducing agent for disulfide bonds to denature the protein’s tertiary and quaternary structures. The sample was then loaded into the gel that was submerged in 1x running buffer. The gel was then ran at 100V until the bands reached the bottom of the gel. Once completed, the gel was transferred (30V for at least 1 hour) onto a membrane and washed to get rid of excess proteins on the membrane. After the washing, the membrane was given a blocking agent (5% BSA in TBST) for 30 minutes. The membrane was washed and then given the primary antibody (1:1000 pERK + tERK antibodies in BSA, from Cell Signaling). The primary antibody was applied overnight and placed in a cold room. The secondary antibody was given the next day with both mouse (680nm IRDye) and rabbit (800 nm IRDye) secondary antibody. The purpose of the secondary antibody is to attach a fluorescent tag onto the primary antibody for detection. The bands were imaged using the Odyssey CLx Infrared imaging system (LiCor Biosciences). The intensity of the band indicates how much protein is present in the sample. Therefore, the thicker the band, the more protein is present for that particular sample. The thickness of the band and amount of protein was recorded for each sample using NIH ImageJ, and analyzed for quantitative expression of ERK MAPK activation in each brain region.

**Results**

We sought to measure the impact of D24M and the MDOR on opioid anti-nociception in acute and chronic pain models. We chose to test the clinical opioids morphine, buprenorphine, and
oxymorphone. Comparing morphine and oxymorphone, there are many similarities between the two. The most significant information we needed for our experiments is that both morphine and oxymorphone are MOR agonists; buprenorphine is also a MOR partial agonist but also has activities at the KOR, nociception receptor, and potentially other targets [5, 13]. Both morphine and oxymorphone should theoretically have the same results in analgesia and possibly behavior due to activating the MOR selectively, while buprenorphine could have other impacts due to the other receptors it targets. These experiments thus determine how the MDOR regulates typical MOR-mediated anti-nociception and other potential targets like the KOR. Looking at Figures 5 and 6, we found that D24M increases anti-nociception for various pain models for oxymorphone but does not have an effect for morphine or buprenorphine. This was particularly odd because oxymorphone and morphine are both MOR opioid agonists and should theoretically perform similarly. We first found this result in the tail flick pain model (Figure 5A, Figure 6A). For the post-surgical paw incision model, we discovered that the morphine induced mice and buprenorphine injected mice had no effects with D24M (p>0.05) [Figure 5B, 5E]. However we found that D24M enhanced oxymorphone anti-nociception (p<0.05) [Figure 6B]. CIPN is a unique pain model where the drug-induced neurotoxicity can cause the peripheral nervous system to be vulnerable and disrupt nerve fibers [14]. For CIPN, paclitaxel was used. Paclitaxel is a type of chemotherapy drug that can aid in stopping the growth of cancer cells but has side effects such as inducing pain or disrupting the peripheral nervous system. With our results, we see an increase in anti-nociception for oxymorphone treated mice and no effects for the morphine and buprenorphine treated mice in the CIPN model (Figure 6C, 5C, 5F). With these pain model results, we have successfully showed that D24M increases anti-nociception for oxymorphone in various pain models and D24M does not have an effect for morphine and buprenorphine.
We next sought to determine the signaling mechanism underlying this increase in antinociception, beginning with testing ERK MAPK activation by Western Blot as described in the Methods. The images were taken and the amount of protein that are within the western blot bands were analyzed using ImageJ. Each protein amount was taken and plotted into the graphs in Prizm. The graphs have the first letter initials for the types of treatments the samples enclosed with. The first sets of letters V and D represent the injection of either vehicle or 1 nmol D24M. The second set of letters represent the type of opioid drug the mice received (M = morphine, O = oxymorphone, CYM = CYM51010). Lastly, the last set of letters represents the region of the brain the sample represents (Str. = striatum, PAG = periaqueductal gray, BS = brainstem).

Looking at Figures 7A, 7C, and 7E, the amount of protein was detailed by dividing activated phospho-ERK (p-ERK) with total ERK protein (t-ERK) to find the ratio of activated ERK. DAMGO, the positive control group, did not seem to show significantly higher amount compared to the control for all three regions of the brain. The control group in figure 7C had tremendous amount of ERK activation compared to the other groups in the periaqueductal gray area. There were no significant changes between the different samples for all three parts of the brain.

Looking at Figures 7B, 7D, and 7F, the p-ERK/t-ERK results were then normalized to the vehicle/vehicle amount of protein. Once again, there were no significant changes for the normalized data for all three regions of the brain. There was a slight decrease in the normalized data for the D24M/CYM51010 sample in the PAG, however, it was not significant (figure 7D). With all these combined results, we can say we successfully determined the anti-nociception differences between oxymorphone and morphine but we could not make a clean conclusion on the mechanistic approach with ERK MAPK.
Discussion

Heterodimers are a unique concept and is a new topic in the scientific community. In the previous data, we were able to support this new concept and developed ways to test the role of the MDOR heterodimer in vivo, which did not have a selective antagonist before we made D24M. We found that D24M increased opioid anti-nociception in multiple pain states for oxymorphone but had no effects with morphine. Both of these opioids are MOR agonists so it was quite fascinating to see that oxymorphone created data opposite of morphine. There is the possibility that oxymorphone was able to produce anti-nociception with D24M because oxymorphone has a higher affinity for the MOR compared to morphine. The binding of the MOR alongside D24M could stimulate the receptor more efficiently than morphine; causing anti-nociception. With that, we also found D24M reducing morphine withdrawal, by extension suggesting that the MDOR acts as a negative feedback loop to suppress analgesia and enhance side effects.

We then sought to find the mechanism of the MDOR for these effects using D24M. The Ras/Raf/MEK/ERK pathway was used for the experiment. The ERK pathway is home to multiple signaling networks that aid in cell proliferation, differentiation, and cell survival [17]. This specific pathway is able to accomplish these cell functions by phosphorylating protein kinases that will eventually lead to change in gene expression. Specifically, the MOR can play a role in opioid tolerance by the ability to promote G protein-coupled receptor kinases to cause phosphorylation of the MOR [18]. There are many ways to get MOR phosphorylation such as GRK2 and β-arrestin so the ERK pathway is definitely a possible mechanism for the heterodimer. Based on the results, we did not see significant differences for ERK stimulation for the striatum, PAG, and brainstem. This finding may be due to the fact that all samples in these three regions have incorporated the ERK pathway in similar amounts. Because ERK is a well-known pathway and we know the MOR
phosphorylation pathways that are within ERK, it isn’t surprising to see each sample have some sort of stimulation. There is also the limitation of the DAMGO sample where the activation of ERK for DAMGO is not as high as we hoped. The primary antibodies may not have attached the membrane as efficiently and could’ve caused a decrease amount of protein on the membrane. With the decreased amount, the bands will be thinner and the activation of ERK will be decreased. There is also the possibility that the injection of DAMGO did not hit the mice as quickly and did not allow the drug to spread into the respective regions. Further testing will be needed for this mechanistic approach and other alternative pathways will need to be evaluated. One possible approach would be to use phosphoproteomics to broadly test signaling changes in an unbiased way; these experiments are in progress.

**Future Studies**

Our current data suggest that we successfully generated a MDOR targeting antagonist that could be a promising tool for the pharmacological investigation of the MDOR heterodimer, which might play an important role in the regulation of opioid tolerance and dependence. With our findings for this experiment, there are multiple directions we can shift towards. One of our future studies we would like to examine is looking at different g-protein pathways for the same three parts of the brain (striatum, PAG, and brainstem). While we looked at the ERK signaling pathway, we can look at other signaling pathways that also causes multiple protein kinases to become activated and produce phosphorylation and subsequent alteration in gene expression. With exploring more pathways, we can develop a better understanding of the MDOR mechanism and possibly aid in the heterodimer pathways in general. Once we develop a better understanding of the mechanism, we hope to further strive in improving opioid therapy. Another future study we would like to look at is the physiology of the MDOR. The development for the physiology of the
heterodimer will come back to solidifying our understanding of the mechanism. Within our hydrophobic hydrocarbon space linker, there is a carboxylic acid functional group. With the carboxylic acid, we are able to fluorescently tag or attach any tag for D24M to aid in our understanding of the possible mechanism for this unique compound.
Figure 1: Design, synthesis, and evaluation of novel heterobivalent linked MDOR antagonists. A) Conceptual design of linked bivalent compounds with antagonist pharmacophores that bridge the length gap between 2 receptors in the MDOR pair. The created compound can then be used to investigate MDOR-specific opioid physiology like anti-nociception, tolerance, and dependence. B) The structures of the heterobivalent series, with linker lengths from 15 to 41 atoms.
Figure 2: A) $^{35}$S-GTPγS coupling vs. 1 μM CYM51010 in MDOR-expressing cells. Only the linked bivalent compound D24M and not the pharmacophores or D15 controls produces a 2 site inhibition curve, suggesting targeting of the MDOR and the monomers. B) $^{35}$S-GTPγS analysis of D24M vs. 1 μM CYM51010 in MDOR, DOR, and MOR expressing cells. D24M only produces a 2-site curve in MDOR cells, not MOR or DOR. C) Analysis of the entire series for potency and selectivity vs. linker length in MDOR and DOR cells. The compounds produce a classic U-shaped curve in MDOR but not DOR cells, with D24M producing the highest potency and selectivity in MDOR cells.
A. **Tail Flick - D24M + CYM51010**

B. **Tail Flick - D24M + Deltorphin II**

C. **Tail Flick - D24M + DAMGO**

D. **Tail Flick - D24M + DSLET**

E. **Tail Flick - D24M + Vehicle**

F. **D24M Dose-Response Curves**

**Figure 3:** D24M targets the MDOR selectively *in vivo*. CD-1 male mice used in 52°C hot water tail flick assay, 10 sec cutoff. All mice *icv* injected with D24M or vehicle, as noted, 5 minutes, followed by *icv* equi-efficacious agonist and time course. **A)** CYM51010, 3.2 nmol (MDOR-prefering agonist). **B)** Deltorphin II, 3.2 nmol (MDOR interacting agonist). **C)** DAMGO, 0.1 nmol (MOR monomer agonist). **D)** DSLET, 1 nmol (DOR monomer agonist). **E)** Vehicle post-injection, no agonist; D24M has no effect on its own. **F)** Baseline-subtracted peak calculated and normalized to vehicle (100%) for DRCs. Mean ± SEM, sample size of mice/group noted below each legend. D24M blocks the MDOR agonists CYM51010 and Deltorphin II without affecting the monomer agonists DAMGO and DSLET.
Figure 4: D24M reduces morphine withdrawal. CD-1 male mice had morphine dependence induced. 1 nmol D24M or Vehicle icv injected 5 minutes before naloxone precipitation of withdrawal. Behaviors scored for 20 min, reported as mean ± SEM. A) Acute dependence. 100 mg/kg morphine, 4 hrs, then 10 mg/kg naloxone ip. N = 18-19 mice/group. *** = P < 0.001 vs. D24M, No Naloxone group; # = P < 0.05 vs. Vehicle group; by 1 Way ANOVA with Fisher’s LSD post-hoc test. B) Chronic dependence. 4 day escalating protocol, 2x/day, 10, 20, 35 mg/kg morphine, 30 mg/kg naloxone ip. N = 10 mice/group. ** = P < 0.01 vs. Vehicle control by unpaired 2-tailed t test.
Figure 5: D24M has no effect on morphine and buprenorphine anti-nociception. Mice were icv injected with 1 nmol D24M or Veh 5 min. prior to sc injection of opioid, using the same pain states established in Figure 4. D24M had no effect on morphine and buprenorphine in any pain state (P > 0.05). A-C) Morphine in tail flick (A, 3.2 mg/kg), paw incision (B, 1 mg/kg), and CIPN (C, 1 mg/kg) pain. D-F) Buprenorphine in tail flick (D, 0.2 mg/kg), paw incision (E, 0.01 mg/kg), and CIPN (F, 0.01 mg/kg) pain.
**Figure 6:** D24M enhances oxymorphone anti-nociception in multiple pain states. Male CD-1 mice were injected icv with Vehicle, 1 nmol D24M, or CTAP/NTI as noted below for 5 minutes, followed by sc oxymorphone as noted in each model. Reported as mean ± SEM, sample sizes of mice/group noted in legends. *, **, ***, **** = P < 0.05, 0.01, 0.001, 0.0001 vs. same time point Vehicle group by 2 Way ANOVA with Sidak’s post-hoc test. **A)** Tail flick (all at 52°C, 10 sec. cutoff). 0.3 mg/kg oxymorphone. AUC increase of 52.3% with D24M. **B)** Post-surgical paw incision model, 24 hrs after surgery, measured with Von Frey filaments. 0.032 mg/kg oxymorphone. AUC increase of 628.0%. **C)** CIPN model, 2 mg/kg paclitaxel ip on days 1, 3, 5, 7, test on day 8 with Von Frey filaments. 0.032 mg/kg oxymorphone. AUC increase of 249.6%.
Figure 7: Western Signaling in CD-1 Female Mice with Vehicle and Various Treatments All female mice were treated with different treatments of vehicle, DAMGO, Morphine, Oxymorphone, and CYM51010 along with vehicle (saline) or D24M. There were no significant differences between the samples (P > 0.05) A) and B): Striatum samples were tested with pERK and tERK antibodies. B) was normalized using the vehicle C) and D): Periaqueductal gray samples were tested with pERK and tERK antibodies. D) was normalized using the vehicle. E) and F): Brainstem samples were tested with pERK and tERK antibodies. F) was normalized using the vehicle.
References


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