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Novel fentanyl-based dual μ/δ -opioid agonists for the treatment of acute and chronic pain

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Abstract

Approximately one third of the adult U.S. population suffers from some type of on-going, chronic pain annually, and many more will have some type of acute pain associated with trauma or surgery. First-line therapies for moderate to severe pain include prescriptions for common μ opioid receptor agonists such as morphine and its various derivatives. The epidemic use, misuse and diversion of prescription opioids has highlighted just one of the adverse effects of μ opioid analgesics. Alternative approaches include novel opioids that target delta or kappa opioid receptors, or compounds that interact with two or more of the opioid receptors.

Aims—Here we report the pharmacology of a newly synthesized bifunctional opioid agonist (RV-Jim-C3) derived from combined structures of fentanyl and enkephalin in rodents. RV-Jim-C3 has high affinity binding to both μ and delta opioid receptors.

Main Methods—Mice and rats were used to test RV-Jim-C3 in a tailflick test with and without opioid selective antagonist for antinociception. RV-Jim-C3 was tested for anti-inflammatory and antihypersensitivity effects in a model of formalin-induced flinching and spinal nerve ligation. To rule out motor impairment, rotarod was tested in rats.

Key findings—RV-Jim-C3 demonstrates potent-efficacious activity in several in vivo pain models including inflammatory pain, antihyperalgesia and antiallodynic with no significant motor impairment.

Significance—This is the first report of a fentanyl-based structure with delta and μ opioid receptor activity that exhibits outstanding antinociceptive efficacy in neuropathic pain, reducing the propensity of unwanted side effects driven by current therapies that are unfunctional μ opioid agonists.

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Keywords

chronic pain; allodynia; hyperalgesia; inflammatory; mice; rat; fentanyl; mu opioid; delta opioid; spinal nerve ligation; formalin flinch; naloxone

Introduction

Over 76 million people currently suffer from chronic pain, becoming the most common reason to seek medical care (Institute of Medicine, 2011; Elliot et al, 1999). The effective management of chronic pain including neuropathic pain is essential in order to reduce the associated personal, social and financial morbidities. Despite the significance of this problem, the current medical treatment modalities only result in a 30% reduction in pain levels (Turk et al., 2011) demonstrating the essential need for novel treatments for the management of chronic pain.

Opioids have fallen out of favor for the treatment of chronic pain due to the wide array of adverse effects associated with long-term use (O'Connor and Dworkin, 2009) resulting in dose-limiting efficacy. In spite of these negative potential outcomes the mechanism of opioid action provides a direct means for inhibiting pain sensation (Vanderah, 2010). The key is to develop a drug with analgesic efficacy but lacks the adverse effects.

The majority of opioids prescribed today are simple modifications of the morphine alkaloid structure. Few studies have investigated the chemical structure of fentanyl, a highly efficacious, μ -selective opioid agonist, with modifications to engage other opioid receptors such as the δ -receptor. Functional association between μ - and δ -opioid receptors was first suggested by studies showing that μ -activity could modulate δ -ligands (Morinville et al, 2004; Decaillot et al, 2008). Yet the true role of δ -ligands remains unrevealed. Some authors have provided evidence that δ -agonists increase antinociceptive responses alone or in combination with μ -receptor agonists (Gendron et al, 2007; Petrillo et al, 2003; Lee et al, 2011; Holdridge et al, 2007) while others suggest that μ -agonist signaling can be equianalgesic by co-treatment with δ -selective antagonists while reducing the unwanted side effects of the μ -receptor agonists (Mosberg et al, 2013; Ananthan et al, 2012). A recent review emphasizes the potential role of δ -agonist as mood stabilizers that would enhance the affective component of chronic pain (Lutz and Kieffer, 2012). Hence, there is a great need to further develop and characterized compounds that can act at both μ - and δ -opioid receptors for human use.

One of the promising new approaches for the questions raised is the creation and investigation of bivalent ligands. Bivalent ligands contain two pharmacophores that are fused together to act at distinct targets resulting in added or synergistic effects (Dietis et al, 2009). Finding the correct pair of ligands with superposition, *in vivo* efficacy and reduced side effects is an attractive yet difficult problem that should be pursued with alternative structures to morphine. The structure of morphine may cause a number of non- μ opioid receptor-related unwanted effects via the TLR4 receptor. (+)Morphine has been shown to activate immune cells, microglia and osteoclasts causing morphine hypersensitivity, antinociceptive tolerance and bone wasting over time (Hutchinson et al, 2010; Franchi et al, 2012). Our preliminary investigations demonstrate a new bivalent ligand with a mixed μ/δ -profile representing combinations of fentanyl and enkephalin-like peptides (Vardanyan et al., submitted to J Med Chem 2013), respectively, with *in vivo* activity in anti-inflammatory and neuropathic pain models.

Material and Methods

Animals

Male ICR (20–25g) mice and male Sprague-Dawley (250–275g) rats were used for all studies. The animals were housed in groups of 5 (mice) or 2–3 (rats) in a temperature controlled room on a light-dark cycle (lights on 7:00). Food and water were available *ad libitum*. All procedures were in accordance with the policies set forth by the Institutional Animal Care and Use Committee of the University of Arizona.

Mouse Injection techniques

Administration of the opioid compound in mice was performed via intrathecal (i.t.) injection using a slightly modified method of the Hylden and Wilcox (1980) technique (Largent-Milnes et al., 2010). This was accomplished using a 10uL Hamilton injector fitted with a 30-gauge needle. All volumes administered were 5uL.

Opioid antagonist challenge

The general opioid antagonist naloxone was used to assess opioid activity of the test compounds. Naloxone (1mg/kg) was given intraperitoneally (i.p.) 10 minutes prior to injection of RV-Jim-C3 (i.t.). To determine opioid receptor selectivity, the delta antagonist naltrindole (5ug/5ul) was given by the i.t. route 5 minutes prior to RV-Jim-C3 (10ug/5ul, i.t.). To determine the amount of mu opioid receptor activity, CTAP (5ug/5ul), a selective mu receptor antagonist, was given by the i.t. route 5 minutes prior to RV-Jim-C3 (10ug/5ul, i.t.).

Mouse Tail-Flick Test

The nociceptive stimulus used to initially test for antinociceptive efficacy was warm (52°C) water. The method followed parameters similar to those set out by Janssen *et al.* (1963). In this case, tail flinching, or the thermal tail withdrawal latency, was defined as the time (seconds) from immersion of the tail in water to its withdrawal. Baseline values for all animals were obtained prior to drug administration. Animals were excluded if their tail-flick time was greater than 7 seconds at baseline. Drug was then given i.t. and tail-flick times were then measured at 15, 30, 45, and 60 minutes intervals. A maximal score was assigned to all animals with tailflick times lasting equal to or greater than 15 seconds. Percent activity, or antinociception, was expressed as:

$$\% \text{Antinociception} = 100 * \frac{(\text{test latency after drug treatment}) - (\text{baseline latency})}{(15 \text{ seconds}) - (\text{baseline latency})}$$

Mouse Formalin Flinching/Guarding Test

An acute inflammatory injury model was simulated in the mice using the formalin test similar to that outlined by Zhao *et al.* (2003). Mice were initially allowed to acclimate in a transparent Plexiglas box placed on a rack for 30 minutes prior to injection. Drug or vehicle was injected i.t. and formalin was administered i.paw 10 minutes later by gently restraining each mouse and injecting them with 20uL of 2% formalin subcutaneously into the plantar surface of the left hind paw. Flinching and guarding behavior of the left hindpaw were measured in 5-minute intervals for 40 minutes. Flinches were characterized as vigorous shaking or lifting of the paw and were counted by absolute number during each time interval. Guarding behavior was described as licking or biting and was assessed in terms of overall duration during each time interval (Kayser *et al.*, 2006). First phase responses were seen in the 0–10 min interval whereas the second phase responses were evident in the 10–40

minute intervals. The 40 minute trial time was sufficient to allow the mice to achieve near baseline levels of nociceptive behaviors after injection with formalin.

Rat Intrathecal Catheter Implantation

Each rat was given ketamine/xylazine 100 mg/kg (vol/vol: 80/20, Sigma-Aldrich) i.p. for anesthesia and subsequently placed in a stereotaxic head mount. An incision was made along the posterior aspect of the skull in order to expose the cisterna magna. Once the cisterna magna was exposed, it was then punctured and a 10 cm catheter (PE-10 tubing) was inserted into the intrathecal space following the method described by Yaksh and Rudy (1976). The incision was closed by first suturing the muscle and then the outer skin, which effectively sutured the catheter in place. The exposed catheter was cauterized to seal the open end. Any animals showing signs of neurological defects after 24 hours were not used. All others were allowed to recover for 7 days before any subsequent procedure was performed.

Rat Injection Technique

Compounds to be administered in rats were done using the intrathecal catheter that had been placed as previously described. Preparations were made using a 25 μ L Hamilton syringe and injector connected with tubing. First, 9 μ L of saline was drawn, followed by 1 μ L of air and finally 5 μ L of drug or vehicle. Upon injection, the tip of the cauterized i.t. catheter was clipped and the Hamilton injector was inserted into the catheter. The prepared solution was slowly injected into the catheter with the air bubble serving as a marker for drug/vehicle level and the saline acting as a flush to ensure full delivery of the compound. Following injection, the tip of the catheter was re-cauterized.

Spinal Nerve Ligation

A chronic pain model, which produces allodynia and hyperalgesia, was produced in the rats via spinal nerve ligation surgery. Each rat was first anesthetized using 2.5% isoflurane plus oxygen. The skin was first separated by making an incision along the lumbar region. In order to separate the muscle, a deep incision was made into the muscle, 1cm left from midline. As has been previously reported, once a proper window was established, the left L5 and L6 nerves were exposed, isolated and ligated using 4-0 silk (Vanderah *et al.* 2008). The muscle incision was then sutured closed while the skin was stapled. All animals were allowed to recover for 7 days before any other procedures were performed. Any animals exhibiting motor or sensory defects were immediately euthanized.

Rotarod

Following placement of the intrathecal catheters, the rats were then trained to walk on a rotating rod (8 rev/min; Rotamex 4/8 device) with a maximal cutoff time of 180 seconds (Vanderah *et al.* 2008). Training was initiated by placing the rats on a rotating rod and allowing them to walk on it until they either fell off or 180 seconds was reached. This process was repeated 6 times and the rats were allowed to recover for 24 hours before beginning the treatment session. Prior to treatment, the rats were run once on a moving rod in order to establish a baseline value. Compounds, either vehicle or drug, were administered via the intrathecal catheter as previously mentioned. Assessment consisted of placing the rats on the moving rod and timing until either they fell off or reached a maximum of 180 seconds. This was repeated every 20 minutes for a total of 2 hours.

Tactile Hypersensitivity

Prior to tactile testing, the rats were allowed to acclimate in suspended wire-mesh cages for 30 minutes. The protocol consisted of taking baseline measurements (post-SNL) by

measuring paw withdrawal thresholds (PWT) to calibrated von Frey filaments (0.4 – 15.0g) that were probed perpendicularly to the plantar surface of the left hind paw. Lifting of the paw or licking the paw was considered a positive response. This method was repeated following the up-down method described by Largent-Milnes *et al.* (2008). Drug or vehicle was then administered at $t = 0$ using the i.t. catheter route previously described. Filament probing was then repeated every 20 minutes for 2 hours. Results were then calculated in grams using the Dixon non-parametric test. No testing was performed on the contralateral paw.

Thermal Hypersensitivity

Prior to thermal testing, the rats were allowed to acclimate in Plexiglas cages for 30 minutes. Baseline values were obtained prior to SNL surgery and again prior to administration of compound. Testing protocol consisted of measuring paw withdrawal latency (PWL) in seconds by placing a mobile infrared heat source directly under the left hind paw as was described by Largent-Milnes *et al.* (2008). A positive response consisted of the animal lifting or licking the paw. A maximum cutoff was set at 30 seconds. Drug or vehicle was administered at Time=0 and PWLs were measured every 20 minutes for 2 hours. The contralateral paw was not tested.

Results

RV-JIM-C3 (i.t.) produces dose dependent antinociception

Mouse baseline tail-flick withdrawal responses were recorded using the warm water 52°C bath apparatus. Mean baseline tail-flick latencies for animals were 3.59 ± 0.21 s ($n=30$). Mice were injected intrathecally (i.t.) in the lumbar region as described (Largent-Milnes *et al.*, 2010) (RV-JIM-C3 10 μ g/5 μ l, 3 μ g/5 μ l, 1 μ g/5 μ l; Vehicle 10% TWEEN, 10% DMSO, 80% saline) and tail withdrawal latency was recorded every 15 min for 60 min. Maximal effect of RV-JIM-C3 occurred 15 min after injection (Figure 1A). The highest dose (10 μ g/5 μ l) resulted in a maximal tail withdrawal latency of 12.3 ± 1.4 s. The 3 μ g/5 μ l and 1 μ g/5 μ l doses produced a tail withdrawal latency of 8.0 ± 1.9 s and 6.1 ± 0.9 s, respectively (Figure 1A). Each dose resulted in significantly higher paw withdrawal latencies as compared to baseline or vehicle treated animals.

Percent antinociception was calculated for each spinal dose of RV-JIM-C3 and a dose response curve was generated. The three doses (1 μ g, 3 μ g, 10 μ g) produced significant dose dependent antinociception in non-injured mice compared to vehicle treated mice (Figure 1B) 15 min after i.t. injection ($20.5 \pm 6.4\%$, $35.6 \pm 15.8\%$, $76.7 \pm 10.7\%$, respectively). The A_{50} dose was calculated to be 3.92 μ g (95% C.I.: 2.21–6.95).

RV-JIM-C3 antinociception is blocked by naloxone, CTAP and Naltrindole

Spinal RV-Jim-C3 was injected following i.p. administered naloxone ($t = -15$ min.) to determine if the antinociceptive effects were in fact due to the opioid pharmacophore. RV-Jim-C3 treated animals at the highest dose (10 μ g/5 μ l, i.t., 12.3 ± 1.38 s) with naloxone (1mg/kg, i.p.) pretreated animals resulted in a significant decrease in tail flick latencies (3.4 ± 0.47 s, $p=0.001$, $n=6$) (Figure 2A,B) and naloxone (1mg/kg) alone did not display any antinociceptive behavior compared to vehicle treated animals (Figure 2).

To test whether there was selective mu and/or delta opioid receptor analgesic activity, spinal administration of either CTAP (mu selective antagonist) or naltrindole (delta selective antagonist) were given spinally 5 minutes prior to spinal RV-Jim-C3. The spinal administration of RV-Jim-C3 (10 μ g/5 μ l, i.t.) 5 minutes after vehicle resulted in a $83.5 \pm 7.2\%$ ($n=6$) antinociceptive effect in the 52°C tail flick test 15 minutes after administration

(Figure 3). CTAP (5 ug/5ul, i.t.) given 5 minutes prior to RV-Jim-C3 (10ug/5ul, i.t.) resulted in a significant inhibition of the antinociceptive effects at $20.9 \pm 13.4\%$ ($p < 0.01$, $n = 6$) antinociception at the 15 minute time point in the 52°C tail flick test (Figure 3). Likewise, Naltrindole (5 ug/5ul, i.t.) given 5 minutes prior to RV-Jim-C3 (10ug/5ul, i.t.) resulted in a significant inhibition of the antinociceptive effects at $30.3 \pm 10.5\%$ ($p < 0.01$, $n = 5$) antinociception at the 15 minute time point in the 52°C tail flick test (Figure 3).

RV-JIM-C3 attenuates formalin induced flinching and guarding

Mice were separated into three groups: (1) vehicle (10% TWEEN/10% DMSO/80% saline), i.t. + 0.9% saline, i.paw, (2) vehicle, i.t. + 0.2% formalin, i.paw, and (3) RV-Jim-C3 (10ug/5ul, i.t.) + 0.2% formalin, i.paw. The group of mice receiving vehicle and formalin had a peak number of flinches 5 min after formalin injection (56.17 ± 7.38 , $n = 6$), which diminished significantly over the course of 40 min (6.83 ± 0.96) (Figure 4a). Mice receiving RV-Jim-C3 (10ug/5ul, i.t., -10min) pretreatment with formalin had significantly fewer flinches (5.16 ± 3.24 , $p = 0.001$, $n = 6$) as compared to those receiving only vehicle and formalin (56.17 ± 7.38 , $n = 6$) at $t = 5$ min. Those animals receiving RV-Jim-C3 and saline were not significantly different from animals receiving vehicle and saline at $t = 5$ min (0.17 ± 0.18 , $n = 6$, and 2.0 ± 0.57 , $n = 6$, Figure 4a).

The group of mice receiving vehicle and formalin had a peak hindpaw guarding effect 5 min after formalin injection ($96.83 \pm 13.14\text{s}$, $n = 6$), which diminished significantly over the course of 40 min ($4.50 \pm 3.19\text{s}$) (Figure 4b). Mice receiving RV-Jim-C3 (10ug/5ul, i.t., -10min) pretreatment with formalin had significantly less guarding ($3.17 \pm 2.19\text{s}$, $p = 0.001$, $n = 6$) as compared to those receiving only vehicle and formalin ($96.83 \pm 13.14\text{s}$, $n = 6$) at $t = 5$ min. Those animals receiving RV-Jim-C3 and saline were not significantly different from animals receiving vehicle and saline at $t = 5$ min ($1.50 \pm 1.12\text{s}$, $n = 6$, and $5.83 \pm 3.49\text{s}$, $n = 6$, Figure 4b).

Spinal RV-JIM-C3 reverses nerve injury induced hypersensitivities

Animals undergoing SNL surgeries were tested for pre-injury ipsilateral hindpaw baseline latencies and thresholds using the IR thermal testing and the von Frey mechanical testing setups, respectively. Animals were post-injury baselined on day 7, and thermal latencies and mechanical thresholds recorded. Animals with significant nerve injury-induced reductions in thermal hindpaw latencies and mechanical hindpaw thresholds ipsilateral to the injury were administered an acute bolus of RV-Jim-C3 (10ug/5ul, i.t.) or vehicle (5ul, i.t.). Paw withdrawal latency and paw withdrawal threshold was measured and recorded every 20 min over a 160 min time course.

Mean pre-injury paw withdrawal latency baseline value for all participating animals was $19.8 \pm 0.37\text{ s}$ ($n = 12$). Mean post-injury paw withdrawal latency baseline value was $10.4 \pm 0.36\text{ s}$, indicating the development of thermal hyperalgesia due to spinal nerve ligation (Figure 5a). Administration of RV-Jim-C3 induced a significant reduction of thermal hypersensitivity within 20 min after injection and peaked at 40 min post injection compared to vehicle ($19.57 \pm 3.12\text{ s}$, $p = 0.01$, $n = 6$) (Figure 5a). The paw withdrawal latencies for drug treated animals returned to post-injury baseline levels at 160 min after acute spinal administration ($11.12 \pm 2.64\text{ s}$, $10\mu\text{g}/5\mu\text{l}$, $p = 0.01$, $n = 6$) (Figure 5a). Vehicle (5ul, i.t.) had no significant effect on post-injury paw withdrawal thresholds over a 160 min time course.

Mean pre-injury paw withdrawal threshold baseline value for all animals was $15 \pm 0\text{g}$ ($n = 12$). Mean post-injury ipsilateral paw withdrawal threshold baseline value was $2.19 \pm 0.37\text{g}$, indicating the development of mechanical allodynia due to spinal nerve ligation. Attenuation of mechanical allodynia was evident in RV-Jim-C3 treated animals only 20 min

post spinal injection as compared to vehicle ($7.72 \pm 2.12g$ and $3.86 \pm 1.80g$, respectively, $p=0.01$, $n=6$)(Figure 5b) with the peak effect 100 min post injection ($13.93 \pm 1.12g$, $p=0.001$, $n=6$) and return to post-injury baseline levels at 120 min. Vehicle ($5\mu l$, i.t.) treated animals did not result in any significant increase in hindpaw withdrawal thresholds over post-injury baselines (Figure 5b, $n=6$).

RV-Jim-C3 does not produce motor deficits such as sedation or paralysis in rats

RV-Jim-C3 (i.t.) was evaluated for typical motor deficits found with opioid use including sedation. Rats were trained to walk on a rotating rod with a maximal cutoff time of 180s prior to administration of drug or vehicle. The mean baseline latency for all animals was 170 ± 10.5 s ($n=11$)(Figure 6). Vehicle treated animals remained on the rotarod for an average of 180 ± 0 s over the course of 120 min. Animals treated with $10\mu g/5\mu l$, i.t. of RV-Jim-C3 remained on the rotating rod for a minimum of 160 ± 22.4 s, not significantly different from control (Figure 6).

Discussion

Although there are more analgesics available on the market today than ever in history there is an overwhelming loss in confidence by physicians and the public in prescribing and taking opioid analgesics due to the combination of abuse potential, unwanted side effects and/or lack of analgesic efficacy (Jensen and Finnerup, 2009; Institute of Medicine, 2011; Rowbotham et al, 2003). There has been a lack of success in designing novel pain-relieving drugs over the past 50 years. As humans continue to live longer due to the advances in other areas of medicine including novel antivirals, antibiotics, chemotherapeutics, autoimmune disease therapies, early tests for detection of diseases, advancing surgical and other new procedures, the need to improve medications for acute and chronic pain amplifies desperately.

Treatment of chronic pain relies a great deal upon μ opioid analgesics, while δ opioid agonists are known to produce analgesic effects but, are not clinically available. There is evidence to indicate that bifunctional μ - δ opioid compounds with unique biological activity profiles may have therapeutic potential as efficacious analgesics with reduced unwanted side effects (Harvey et al, 2012; Kotlinska et al, 2013; Yamamoto et al, 2011). There has been several novel compounds designed to target both the μ and δ opioid yet, the majority of compounds are based on peptidic structures, non-peptidic structures or utilize the morphine alkaloid structure (Schiller, 2010; Elmagbari et al, 2004; Lowery et al, 2011; Codd, et al, 2009; Ananthan, et al, 2012). Such peptidic structures may result in poor absorption, poor access to the CNS and shorter stability with more frequent dosing (Morphy and Rankovic 2005; Cavalli et al, 2008). Morphine based structures produce off-target activation of Toll-like receptor 4 (TLR4) in a non-stereospecific manner; that is, unnatural enantiomers of alkaloid opioids, which do not activate endogenous opioid receptors, have activity at TLR4. Several studies have provided evidence for the non-stereospecific activation of TLR4 signaling by opioids including morphine *in vivo* and *in vitro* (Hutchinson et al, 2010; Franchi et al, 2012; Loram et al, 2012; Due et al, 2012). TLR-4 activation is an innate immune receptor complex found on myelo-monocytic cells including macrophages, myeloid progenitor cells, and osteoclasts resulting in a number of unwanted effects including activation of the innate inflammatory response, an increase in the production of more inflammatory cells and enhanced bone wasting (Li et al, 2013). Here we present a novel compound that acts at both mu and delta opioid receptors based on a fentanyl structure, reducing the peptidic aspects as well as the off-target of the TLR4 receptor that may hinder a mixed μ - δ opioid compound reaching the clinic. Initial design and synthesis of RV-Jim-C3 demonstrated micromolar binding affinities and *in vitro* efficacy at both μ and δ opioid

receptors (Vardyanan R, in review Journal of Medicinal Chemistry, 2013) with significant analgesic efficacy in several pain models.

RV-Jim-C3 demonstrated significant analgesia using a warm water tail flick test after the spinal administration. These effects were blocked by naloxone suggesting opioid receptor mediated effects. Furthermore, the significant attenuation by the mu selective antagonist CTAP and by the delta selective antagonist naltrindole suggests that the antinociception produced by CV-Jim-C3 is indeed mediated by both the mu and delta opioid receptors.

In order to determine whether RV-Jim-C3 has anti-inflammatory effects it was tested in a murine formalin flinch model. A well-established model for testing acute pain in mice is by using intradermal formalin injections and recording paw flicking and guarding. While certain tests, like the tail-flick model, rely on reflex pain, the formalin model produces pain through direct tissue damage. This is crucial since the pain produced more closely resembles that which occurs in true clinical pain (Abbott et al, 1999; Dubuisson and Dennis, 1977; Murray et al, 1988). The formalin model is highly reproducible with the behavior resulting in two distinct phases that correspond to the specific nociceptive neurons that are activated. The first phase is mediated by direct chemical stimulation of primary myelinated nociceptive afferent fibers (A δ -fiber), the second phase is mediated by activation of central pathways via inflammation and continued unmyelinated (C-fiber) activity (Dubner and Ren, 1999; Hunskaar and Hole, 1987; Puig and Sorkin, 1996; Dickenson and Sullivan 1987). Therefore, using the formalin model is an effective means by which to test the efficacy of novel opioid compounds in the acute inflammatory setting. RV-Jim-C3 resulted in significant inhibition of formalin-induced flinching in both the first and second phase suggesting analgesia by inhibiting both A δ - and C-fiber activity.

Although opioids are often prescribed for acute nociceptive pain, they have limited analgesic efficacy for neuropathic pain at doses that do not produce severe sedation, somnolence and respiratory depression (Rowbotham et al, 2003). In humans, neuropathic pain tends to be a persistent or chronic condition, and thus it is important to utilize a pain model that expands beyond the confines of the acute pain generated by formalin. One popular method, described by Kim and Chung, is to ligate the 5th and 6th lumbar spinal nerves, which are chiefly sensory, distal to the dorsal root ganglia (1992). The key to using this method is that ligation causes injury to both myelinated and unmyelinated fibers (Basbaum et al, 1991). This has the effect of stimulating the A δ - and C-fibers that are activated in neuropathic pain. Furthermore, the nerve ligation involves a more long-term form of injury, unlike the tissue damage that occurs acutely with formalin, effectively producing a chronic pain syndrome. Finally, the nerve ligation produces only a partial injury that allows some afferent input to still be received, facilitating the use of behavioral models in pain testing. Here we measured an animal's ability to withdrawal the nerve injured, hindpaw from non-noxious (mechanical allodynia) and from noxious (thermal hypersensitivity) stimuli in the absence and presence of spinal RV-Jim-C3 administration. Unlike many of the current mu opioid agonists such as morphine that are available for chronic pain, RV-Jim-C3 resulted in significant mechanical antiallodynia and thermal antihypersensitivity in the nerve injured animal. Such studies suggest that a fentanyl based, mixed mu-delta opioid agonist can act to inhibit neuropathic pain. Rotarod experiments were performed in order to demonstrate the lack of RV-Jim-C3-induced motor paralysis and/or signs of sedation since such behavior would mask paw withdrawal results in our pain behavior tests. Do to the added analgesic effects of delta opioid receptor occupation, less mu receptor occupation is required essentially decreasing the dose and unwanted side effects driven by mu opioid receptor agonists. Ultimately, by using a combination of acute and chronic pain models, we demonstrate here the efficacy of the novel, non-peptidic opioid compound RV-Jim-C3 in treating neuropathic pain while lacking sedation and motor incoordination.

Recent decisions by the FDA in eliminating acetaminophen from mu opioid agonists (i.e., vicodin® and percocet®) due to liver toxicity have resulted in an urgent need to produce better opioids for chronic pain without unwanted side effects (FDA Options Paper, 2009; Chen et al, 2002). In light of this, it is widely accepted that biological activity at a single receptor is often insufficient with recent research focusing on innovative compounds that have more than one site of action (Morphy and Rankovic 2005; Cavalli et al, 2008). Bifunctional drugs may have improved efficacy due to synergistic effects with added benefits of reducing side effects. Bifunctional drugs as compared with individual drug combinations result in more predictable pharmacokinetic (PK) and pharmacodynamic (PD) relationship. There is often unpredictable variability between patients in drug mixtures versus a bifunctional compound due to variability in relative rates of metabolism between patients. Finally, bifunctional drugs may result in improved patient compliance and a lower risk of drug-drug interactions compared to individual drug combinations (Edwards and Aronson, 2000).

Conclusions

There is a great need to develop novel pharmaceuticals for chronic pain. Current therapies are dose limiting due to the unwanted side effects and lack of efficacy. Studies here demonstrate a novel dual acting fentanyl-based structure that contains both mu and delta opioid receptor agonist activity resulting in high efficacy in an anti-inflammatory and neuropathic pain model with the potential of reduced unwanted side effects. A mu/delta opioid agonist, non-morphine based structure like RV-Jim-C3 is less likely to produce TLR4 receptor mediated hyperalgesia, resulting in the propensity of long-lasting pain relief.

References

- Abbott FV, Ocvirk R, Najafee R, Franklin KB. Improving the efficiency of the formalin test. *Pain*. 1999; 83:561–569. [PubMed: 10568865]
- Ananthan S, Saini SK, Dersch CM, Xu H, McGlinchey N, Giuvelis D, et al. 14-Alkoxy- and 14-acyloxy-pyridomorphinans: μ agonist/ δ antagonist opioid analgesics with diminished tolerance and dependence side effects. *J Med Chem*. 2012; 55:8350–8363. [PubMed: 23016952]
- Basbaum AI, Gautron M, Jazat F, Mayes M, Guilbaud G. The spectrum of fiber loss in a model of neuropathic pain in the rat: an electron microscopic study. *Pain*. 1991; 47:359–367. [PubMed: 1664510]
- Cavalli A, Bolognesi ML, Minarini A, Rosini M, Tumiatti V, Recanatini M, Melchiorre C. Multi-target-directed ligands to combat neurodegenerative diseases. *J Med Chem*. 2008; 51(3):347–372. [PubMed: 18181565]
- Chen L, Schneider S, Wax P. Knowledge about acetaminophen toxicity among emergency department visitors. *Vet Hum Toxicol*. 2002; 44(6):370–373. [PubMed: 12458645]
- Codd EE, Carson JR, Colburn RW, Stone DJ, Van Besien CR, Zhang SP, et al. JNJ-20788560 [9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide], a selective delta opioid receptor agonist, is a potent and efficacious antihyperalgesic agent that does not produce respiratory depression, pharmacologic tolerance, or physical dependence. *J Pharmacol Exp Ther*. 2009; 329(1):241–251. [PubMed: 19151246]
- Decaillot FM, Rozenfeld R, Gupta A, Devi LA. Cell surface targeting of mu-delta opioid receptor heterodimers by RTP4. *Proc Natl Acad Sci USA*. 2008; 105:16045–16050. [PubMed: 18836069]
- Dickenson AH, Sullivan AF. Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. *Neurosci Lett*. 1987; 83:207–211. [PubMed: 3441298]
- Dietis N, Guerrini R, Calo G, Salvadori S, Rowbotham DJ, Lambert DG. Simultaneous targeting of multiple opioid receptors: a strategy to improve side-effect profile. *Br. J. Anaesth*. 2009; 103(1):38–49. [PubMed: 19474215]
- Dubner R, Ren K. Endogenous mechanisms of sensory modulation. *Pain*. 1999; (Suppl. 6):S45–S53. [PubMed: 10491972]

- Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain*. 1977; 4:161–174. [PubMed: 564014]
- Due MR, Piekarz AD, Wilson N, Feldman P, Ripsch MS, Chavez S, et al. Neuroexcitatory effects of morphine-3-glucuronide are dependent on Toll-like receptor 4 signaling. *Journal of Neuroinflammation*. 2012; 9:200:1–12.
- Edwards IR, Aronson JK. Adverse drug reactions: definitions, diagnosis, and management. *Lancet*. 2000; 356(9237):1255–1259. [PubMed: 11072960]
- Elliott AM, Smith BH, Penny KI, Smith WC, Chambers WA. The epidemiology of chronic pain in the community. *Lancet*. 1999; 354:1248–1252. [PubMed: 10520633]
- Elmagbari NO, Egletton RD, Palian MM, Lowery JJ, Schmid WR, Davis P, Navratilova E, et al. Antinociceptive structure-activity studies with enkephalin-based opioid glycopeptides. *J Pharmacol Exp Ther*. 2004; 311:290–297. [PubMed: 15166257]
- FDA Options Paper. Acetaminophen Overdose and Liver Injury — Background and Options for Reducing Injury. 2009
- Franchi S, Moretti S, Castelli M, Lattuada D, Scavullo C, Panerai AE, Sacerdote P. Mu opioid receptor activation modulates Toll like receptor 4 in murine macrophages. *Brain, behavior, and immunity*. 2012; 26:480–488.
- Gendron L, Pintar JE, Chavkin C. Essential role of mu opioid receptor in the regulation of delta opioid receptor-mediated antihyperalgesia. *Neuroscience*. 2007; 150:807–817. [PubMed: 17997230]
- Harvey JH, Long DH, England PM, Whistler JL. Tuned-Affinity Bivalent Ligands for the Characterization of Opioid Receptor Heteromers. *ACS Med Chem Lett*. 2012; 3(8):640–644. [PubMed: 23585918]
- Holdridge SV, Cahill CM. Spinal administration of a delta opioid receptor agonist attenuates hyperalgesia and allodynia in a rat model of neuropathic pain. *Eur J Pain*. 2007; 11:685–693. [PubMed: 17175187]
- Hunskar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain*. 1987; 30:103–114. [PubMed: 3614974]
- Hutchinson MR, Lewis SS, Coats BD, Rezvani N, Zhang Y, Wieseler JL, et al. Possible involvement of toll-like receptor 4/myeloid differentiation factor-2 activity of opioid inactive isomers causes spinal proinflammation and related behavioral consequences. *Neuroscience*. 2010; 167:880–893. [PubMed: 20178837]
- Hylden JLK, Wilcox GL. Intrathecal morphine in mice: a new technique. *Eur. J. Pharmacol*. 1980; 67:313–316. [PubMed: 6893963]
- Institute of Medicine Report from the Committee on Advancing Pain Research, Care, and Education. *Relieving Pain in America, A Blueprint for Transforming Prevention, Care, Education and Research*. Washington, DC: The National Academy Press; 2011.
- Janssen PAJ, Niemegeers CJE, Dorg JGH. The inhibitory effects of fentanyl and other morphine-like analgesics on the warm-water-induced tail-withdrawal reflex in rats. *Arzneim Forsch*. 1963; 13:502–505. [PubMed: 13957426]
- Jensen TS, Finnerup NB. Neuropathic pain treatment: a further step forward. *Lancet*. 2009; 374:1218–1219. [PubMed: 19796803]
- Kayser V, Elfassi IE, Aubel B, Melfort M, Julius D, Gingrich JA, Hamon M, Bourgoin S. Mechanical, thermal and formalin-induced nociception is differentially altered in 5-HT1A^{-/-}, 5-HT1B^{-/-}, 5-HT2A^{-/-}, 5-HT3A^{-/-} and 5-HTT^{-/-} knock-out male mice. *Pain*. 2007; 130:235–248. [PubMed: 17250964]
- Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain*. 1992; 50:355–363. [PubMed: 1333581]
- Kotlinska JH, Gibula-Bruzda E, Witkowska E, Chung NN, Schiller PW, Izdebski J. Peptides. Antinociceptive effects of two deltorphins analogs in the tail-immersion test in rats. *Peptides*. 2013; 39:103–110. [PubMed: 23183627]
- Largent-Milnes TM, Guo W, Wang HY, Burns LH, Vanderah TW. Oxycodone plus ultra-low-dose naltrexone attenuates neuropathic pain and associated mu-opioid receptor-Gs coupling. *J Pain*. 2008; 9:700–713. [PubMed: 18468954]

- Largent-Milnes TM, Yamamoto T, Nair P, Hruby VJ, Lai J, Porreca F, Vanderah TW. Spinal or systemic TY005, a peptidic opioid agonist/neurokinin 1 antagonist, attenuates pain with reduced tolerance. *British J Pharmacol.* 2010; 161(5):968–1001.
- Lee Y-S, Kulkarni V, Cowell SM, Ma S-W, Davis P, Hanlon KE, et al. Development of Potent μ and δ Opioid Agonists with High Lipophilicity. *J. Med. Chem.* 2011; 54(1):382–386. [PubMed: 21128594]
- Li J, Wang X, Zhang F, Yin H. Toll-like receptors as therapeutic targets for autoimmune connective tissue diseases. *Pharmacol Ther.* 2013; 138(3):441–451. [PubMed: 23531543]
- Loram LC, Grace PM, Strand KA, Taylor FR, Ellis A, Berkelhammer D, et al. Prior exposure to repeated morphine potentiates mechanical allodynia induced by peripheral inflammation and neuropathy. *Brain, behavior, and immunity.* 2012; 26:1256–1264.
- Lowery JJ, Raymond TJ, Giuvelis D, Bidlack JM, Polt R, Bilsky EJ. In vivo characterization of MMP-2200, a mixed δ/μ opioid agonist, in mice. *J Pharmacol Exp Ther.* 2011; 336:767–778. [PubMed: 21118955]
- Lutz PE, Kieffer BL. Opioid receptors: distinct roles in mood disorders. *Trends Neurosci.* 2012; 12:S0166–S2236. [PubMed: 23219016]
- Morinville A, Cahill CM, Kieffer B, Collier B, Beaudet A. μ -opioid receptor knockout prevents changes in delta-opioid receptor trafficking induced by chronic inflammatory pain. *Pain.* 2004; 109:266–273. [PubMed: 15157687]
- Morphy R, Rankovic Z. Designed multiple ligands. An emerging drug discovery paradigm. *J Med Chem.* 2005; 48(21):6523–6543. [PubMed: 16220969]
- Mosberg HI, Yeomans L, Harland AA, Bender AM, Sobczyk-Kojiro K, Anand JP, et al. Opioid Peptidomimetics: Leads for the Design of Bioavailable Mixed Efficacy μ Opioid Receptor (MOR) Agonist/ δ Opioid Receptor (DOR) Antagonist Ligands. *J Med Chem.* 2013; 56:2139–2149. [PubMed: 23419026]
- Murray CW, Porreca F, Cowan A. Methodological refinements to the mouse paw formalin test. An animal model of tonic pain. *J. Pharmacol.* 1988; 20:175–186.
- O'Connor AB, Dworkin RH. Treatment of neuropathic pain: an overview of recent guidelines. *Am J Med.* 2009; 122:S22–S32. [PubMed: 19801049]
- Petrillo P, Angelici O, Bingham S, Ficalora G, Garnier M, Zaratini PF, et al. Evidence for a selective role of the delta-opioid agonist [8R-(4bS*, 8aalpha, 8abeta, 12bbeta)] 7,10-Dimethyl-1-methoxy-11-(2-methylpropyl) oxycarbonyl 5,6,7,8,12,12b-hexahydro-(9H)-4,8-methanobenzofuro (3,2-e) pyrrolo (2,3-g) isoquinoline hydrochloride (SB-235863) in blocking hyperalgesia associated with inflammatory and neuropathic pain responses. *J Pharmacol Exp Ther.* 2003; 307:1079–1089. [PubMed: 14551288]
- Puig S, Sorkin LS. Formalin-evoked activity in identified primary afferent fibers: systemic lidocaine suppresses phase-2 activity. *Pain.* 1996; 64:345–355. [PubMed: 8740613]
- Rowbotham MC, Twilling L, Davies PS, Reisner L, Taylor K, Mohr D. Oral opioid therapy for chronic peripheral and central neuropathic pain. *New Eng. J. Med.* 2003; 348:1223–1232. [PubMed: 12660386]
- Schiller PW. Bi- or multifunctional opioid peptide drugs. *Life Sci.* 2010; 86:598–603. [PubMed: 19285088]
- Turk DC, Wilson HD, Cahana A. Treatment of chronic non-cancer pain. *Lancet.* 2011; 377:2226–2235. [PubMed: 21704872]
- Vanderah TW, Largent-Milnes T, Lai J, Porreca F, Houghten RA, Menzaghi F, et al. Novel D-amino acid tetrapeptides produce potent antinociception by selectively acting at peripheral kappa-opioid receptors. *Eur J Pharmacol.* 2008; 583:62–72. [PubMed: 18282565]
- Vanderah TW. Delta and kappa opioid receptors as suitable drug targets for pain. *Clin J Pain.* 2010; 26:S10–S15. [PubMed: 20026960]
- Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. *Physiol Behav.* 1976; 17:1031–1036. [PubMed: 14677603]
- Yamamoto T, Nair P, Largent-Milnes TM, Jacobsen NE, Davis P, Ma SW, et al. Discovery of a potent and efficacious peptide derivative for δ/μ opioid agonist/neurokinin 1 antagonist activity with a 2',

- 6'-dimethyl-L-tyrosine: in vitro, in vivo, and NMR-based structural studies. *J Med Chem.* 2011; 54(7):2029–2038. [PubMed: 21366266]
- Zhao CS, Tao YX, Tall JM, Donovan DM, Meyer RA, Raja SN. Role of micro-opioid receptors in formalin-induced pain behavior in mice. *Exp Neurol.* 2003; 184:839–845. [PubMed: 14769376]

A

B

Figure 1.

A) RV-Jim-C3 results in a dose- and time-related antinociceptive activity after spinal administration. Warm water (52°C) tail flick test using mice to demonstrate that Rv-Jim-C3 produces a maximal effect 15 minutes after intrathecal administration with recovery to baseline latencies by 60 mins (N=10 per dose). Intrathecal vehicle had no significant effect on tail flick latencies. **B)** The dose response curve for RV-Jim-C3 in the tail flick test resulted in an A_{50} of 3.9 ug/5ul (95% C.I. = 2.2–6.9 with R^2 value of 0.95) at the 15 minute time point.

Figure 2.

A) Systemic naloxone blocks the antinociceptive effects of RV-Jim-C3. RV-Jim-C3 when administered by the i.t. route results in a significant increase in tailflick latencies with a maximum effect at 10µg/5µl. The RV-Jim-C3 increase in tail flick latencies was a significantly reduced by a 10 min pretreatment with 1 mg/kg naloxone. **B)** Demonstrates the percent antinociceptive activity of RV-Jim-C3 in the absence and presence of systemic naloxone. Naloxone alone had no effect. (n= 10, *p<0.05, **p<0.001).

Figure 3.

The 5 minute intrathecal pretreatment with either Naltrindole (5µg/5µl), or CTAP (5 µg/5µl) significantly attenuated the antinociceptive activity of intrathecal RV-Jim-C3. (n= 6, *p<0.05).

Figure 4.

A) Formalin (2%/20ul, i.paw) in mice results in two phases of paw flinching characterized as phase I, 0–10 minutes and phase II, from 10 minutes to 40 minutes. RV-Jim-C3 (10ug/5ul) when given intrathecally 10 minutes prior to formalin resulted in a significant decrease in flinching in both the first and second phase at all time points (n=6). **B)** Likewise, formalin (2%/20ul, i.paw) in mice results in two phase of paw guarding. RV-Jim-C3 (10ug/5ul, i.t.) 10 mins prior to formalin significantly reduced paw guarding in both phases (n=6). Vehicle has no effect on formalin flinching or guarding (n=6).

Figure 5.

The spinal administration of RV-Jim-C3 (10µg/5µl) resulted in a significant inhibition of spinal nerve ligation (SNL)-induced thermal and mechanical hypersensitivity in rats. **A)** Paw withdrawal latencies resulted in a significant decrease post-SNL that was significantly reversed at the 20 and 40 minute time points as well as later time points with a return to post-injury baselines at 160 minutes (n=6, *p<0.05). **B)** Paw withdrawal thresholds resulted in a significant decrease post-SNL that was significantly reversed at the 20 through til the 100 minute time point with a return to post-injury baselines at 140 minutes (n=6, *p<0.05). Vehicle had no effect on mechanical or thermal latencies.

Figure 6.

Rats were tested for sedation and/or motor impairment using a rotating rod. Animals are trained on a rotating rod for 3 consecutive trials for a total of 120 seconds. The spinal administration of RV-Jim-C3 or vehicle did not result in any significant changes in an animals' ability to walk on a rotating rod when compared to naive baselines.