Novel peptide ligands with dual acting pharmacophores designed for the pathophysiology of neuropathic pain

Katherine E. Hanlon[a], **Dave S. Herman**[a], **Richard S. Agnes**[b], **Tally M. Largent-Milnes**[a], **Isuru R. Kumarasinghe**[b], **Sho W. Ma**[a], **Wenhong Guo**[a], **Yeon-Sun Lee**[a,b], **Michael H. Ossipov**[a], **Victor J. Hruby**[b], **Josephine Lai**[a], **Frank Porreca**[a], and **Todd W. Vanderah**[a]

[a] Department of Pharmacology, University of Arizona Health Sciences Center, P.O. Box 245050, 1501 N Campbell Ave., Tucson, Arizona 85724 USA

[b] Department of Chemistry, University of Arizona, P.O. Box 210041, 1306 E University Blvd, Tucson, Arizona 85721 USA

Abstract

The conventional design of high affinity drugs targeted to a single molecule has not resulted in clinically useful therapies for pain relief. Recent reviews have suggested that newly designed analgesic drugs should incorporate multiple targets. The distributions of cholecystokinin (CCK) and CCK receptors in the central nervous system (CNS) overlap significantly with endogenous opioid systems and can be dually targeted. CCK has been shown to act as an endogenous "anti-analgesic" peptide and neuropathic pain conditions promote endogenous CCK release in CNS regions of pain modulation. Administration of CCK into nuclei of the rostral ventromedial medulla induces pronociceptive behaviors in rats. RSA 504 and RSA 601 are novel bifunctional compounds developed to target neuropathic pain by simultaneously acting as agonists at two distinct opioid receptors and antagonizing CCK receptors in the CNS. RSA 504 and RSA 601 demonstrate agonist activity *in vitro* and antihypersensitivity to mechanical and thermal stimuli *in vivo* using the spinal nerve ligation model of neuropathic pain. Intrathecal administration of RSA 504 and RSA 601 did not demonstrate antinociceptive tolerance over 7 days of administration and did not display motor impairment or sedation using a rotarod. These are the first behavioral studies that demonstrate how multi-targeted molecule design can address the pathology of neuropathic pain. These compounds with δ and μ opioid agonist activity and CCK antagonist activity within one molecule offer a novel approach with efficacy for neuropathic pain while lacking the side effects typically caused by conventional opioid therapies.

Keywords

neuropathic pain; spinal nerve ligation; cholecystokinin; opioids

Corresponding Author: Todd W. Vanderah, Ph.D., University of Arizona, Department of Pharmacology, 1501 N. Campbell Ave., Tucson, AZ 85724, Phone: (520) 626-7801, Fax: (520) 626-4779, vanderah@email.arizona.edu.

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1. Introduction

Chronic pain affects 78 million people in the United States and at least 75 million Europeans (Breivik et al., 2006; Loeser and Bonica, 2001). For many patients chronic pain is untreated or undermanaged due to a lack of novel medications that are tolerable. Recently, the National Institutes for Health described the need for designing medications that may have multiple sites of action to better address the pathology of chronic and/or neuropathic pain (Woodcock et al., 2007), stimulating the development of mechanism-based, individualized pain therapies.

Neuropathic pain, demonstrated by hypersensitivity to noxious and innocuous stimuli, is defined in broad terms as pain caused by damage or dysfunction in the peripheral nervous system (Merskey, 2002) leading to dysfunction of pain fibers of the CNS (Ossipov et al., 2000). This type of pain is generally diverse with unpredictable etiology; additionally, some patients present with genetic conditions, making treatment outcome even more difficult to predict (Ossipov and Porreca, 2005). Opiate therapy is one of the most commonly prescribed treatments for chronic neuropathic pain, however opioids do not address the mechanisms of neuropathic pain and often have limited efficacy against this type of pain. Opioids are plagued by analgesic tolerance, addiction, medication overuse hypersensitivities and other physical side effects (Jensen *et al.*, 2001). Regardless of whether it is caused by a disease state or a nerve trauma, neuropathic pain generates unique challenges for the design of novel analgesic treatments due to the multiple mechanisms driving pain sequela.

Of particular importance are the descending facilitatory pathways of the brainstem, specifically CCK, which is co-localized in the CNS with endogenous opioids and opioid receptors (Ghilardi *et al.*, 1992; Zhang *et al.*, 2009). CCK release is increased in multiple models of injury in the descending pain facilitatory pathways (Kovelowski *et al.*, 2000; Friedrich and Gebhart, 2003). Together, these data suggest that CCK plays a significant role in pain processing and endogenous opioid activity. In addition to activating nociceptive descending pain facilitatory neurons (Heinricher and Neubert, 2004), CCK administered into the RVM of normal rats produces time dependent behavioral signs of mechanical and thermal hypersensitivity of the viscera and hindpaw (Friedrich and Gebhart, 2003; Xie *et al.*, 2005). Studies have demonstrated that exogenous opioid therapy also induces the release of CCK in the spinal cord, frontal cortex and brainstem (Becker *et al.*, 2000; Gustafsson *et al.*, 2001; Xie *et al.*, 2005). Conversely, administration of a CCK antagonist into the RVM produces a time dependent attenuation of thermal and mechanical hypersensitivity in the rat L_5/L_6 SNL model (Kovelowski *et al.*, 2000); the opioid-induced hyperalgesia model (Xie *et al.*, 2005); as well as a model of visceral hypersensitivity (Friedrich and Gebhart, 2003). Lastly, systemic CCK antagonists significantly enhance the analgesic effects of opioids for the treatment of neuropathic pain and attenuate the development of opioid-induced antinociceptive tolerance in animals and humans (Dourish *et al.*, 1990; McCleane, 1998; McCleane, 2002; McCleane, 2003; Nichols *et al.*, 1996; Wiesenfeld-Hallin *et al.*, 2002; Xie *et al.*, 2005).

An emerging trend encompasses the use of hetero-bivalent compounds in the development of novel therapeutics. Licofelone, a pyrrolizine analogue, simultaneously inhibits both cyclooxygenases and 5-lipoxygenase; currently in clinical trials for osteoarthritis, it is the first NSAID to inhibit leukotriene and prostaglandin formation simultaneously (Raynauld et al., 2008). The only centrally acting analgesic approved in the United States since the 1970's is itself a dual pharmacophore: tapentadol (Nucynta©), a μ opioid agonist and norepinephrine reuptake inhibitor in a single molecule (Frampton, 2010). Tapentadol sets itself apart from traditional opiate therapy with reduced side effects- most notably fewer patients experienced constipation, nausea, vomiting, pruritis and dizziness when compared

to oxycodone in a long term safety and tolerability trial (Wild et al., 2010). Dual target non analgesics have also shown efficacy in chronic pain states: duloxetine (Cymbalta©), an SNRI, has been approved in the treatment of fibromyalgia and diabetic neuropathy in addition to major depressive disorder and generalized anxiety disorder in the United States (Wright et al., 2011). SNRIs alone have not resulted in the treatment for all types of chronic, neuropathic pain suggesting alternative underlying disease states such as increased levels of CCK. The increasing occurrence of bifunctional compounds in pain therapies is not accidental: it has been suggested that multivalent therapies offer a greater disease specificity and greater affinity than existing monovalent therapies (Lezoualc'h et al., 2009).

The bi-functional compounds examined here, RSA 504 (H-Tyr[1]-DNle[2]-Gly[3]-Trp[4]-Nle[5]-Asp[6]-Phe[7]-NH$_2$) (Agnes *et al.*, 2008) and RSA 601 (H-Tyr[1]-DPhe[2]-Gly[3]-DTrp[4]-NMeNle[5]-Asp[6]-Phe[7]-NH$_2$) (Hruby *et al.*, 2003) were based on the structure of SNF9007, a novel peptide analogue of CCK. SNF9007 was found to act simultaneously at the δ and μ opioid receptors to produce antinociception (Williams *et al.*, 1994) but lacked efficacy due to some CCK agonist activity. The RSA compounds, designed to act as both an opioid agonists and CCK antagonists, have nanomolar binding affinity at both receptor classes with no CCK agonist activity and clear *in vitro* and *in vivo* antagonist activity at the CCK receptors. RSA 504 (913.02 g/mol) possesses Ki values of 23 nM and 27 nM at the δ and μ opioid receptors, respectively with Ki's of 11.2 nM and 16 nM at the CCK-1 and CCK-2 receptors, respectively (Agnes et al., 2008). RSA 601 (m.w. 962.04 g/mol) displays 0.55 nM and 5.7 nM affinity at the δ and μ opioid receptors, respectively, as well as 1100 and 1.6 nM affinity for the CCK-1 and CCK-2 receptors, respectively (Hruby et al., 2003).

Overall, many compounds for neuropathic pain have been designed to act at a single selective target in a particular animal model of pain; however, they have not resulted in advancing clinical therapies. The RSA 504 and 601 compounds were designed towards the pathology of neuropathic pain in which two pharmacophores with different mechanisms of action result in better, longer lasting efficacy over the current monotherapy. These single entity compounds with agonist activity at the δ/μ opioid receptors in addition to CCK antagonist activity demonstrate reduced adverse side effects versus multiple compounds with pharmacokinetic and/or drug interactions. We advance the idea that these dual acting compounds address the pathology seen in many chronic pain syndromes, offering a multi-targeted approach to significantly improve antinociceptive efficacy.

2. Results

2.1 Biological Activities *In Vitro*

The RSA compounds were designed towards the pathology of neuropathic pain containing both CCK antagonist and opioid agonist pharmacophores. To expand upon the *in vitro* activities published by the Hruby and colleagues (Agnes *et al.*, 2008; Hruby *et al.*, 2003) demonstrating 0.45 or 23 nM in the MVD for delta opioid receptor activity and 63 or 210 nM in GPI for mu opioid receptor activity for RSA 601 or 504 respectively. The biological activity of RSA 601 was assessed using GTPγS binding assay for opioid receptors. At the hDOR in the GTP-γ-S binding assay RSA 601 displayed an EC_{50} of 20.9 nM and E_{max} of 33.8 nM; at MOR the EC_{50} was 520 nM and demonstrated an E_{max} of 52.7 nM. RSA 504 shows similar activity in the GTP-γ-S binding assay with an EC_{50} of 5.5 nM and an E_{max} of 81 nM at the hDOR and an EC_{50} of 0.47 nM and E_{max} of 110 nM at the MOR (Agnes et al., 2008).

2.2 RSA 504 and 601 Attenuate Tactile Allodynia When Administered Intrathecally

The L_5/L_6 SNL model was used to evaluate efficacy against mechanical hypersensitivity. Prior to and after injury, all animals were evaluated for mechanical response to non-noxious probing of the left hind paw with calibrated von Frey filaments. The mean paw withdrawal threshold before SNL was 15.0 ± 0.0 g. Seven days after nerve ligation, the mean withdrawal threshold was 4.7 ± 1.02 g, indicating the development of tactile allodynia ($n = 74$). The animals were separated at random into i.t. treatment groups: 33% EtOH vehicle, morphine (10 μg), gabapentin (30 μg), RSA 504 (3, 10, or 30 μg), and RSA 601 (3, 10, or 30 μg). Each group was given an acute bolus i.t. Following treatment administration, behavioral measurements of tactile allodynia were obtained every 15 min for the first hour. Responses were compared to control animal paw withdrawal thresholds. Significant attenuation of mechanical hypersensitivity using RSA 504 (15, 30, and 45 min post administration) was observed at doses of 3 μg/5mL, 10 μg/5μL, and 30 μg/5μL on day 7 after nerve injury when compared to post-injury baselines. RSA 504 induced significant attenuation of mechanical hypersensitivity (30 min post administration) at doses of 10 μg/5μL and 30 μg/5μL when compared to vehicle. No significance was seen at the 3 μg/5μL dose of RSA 504 when compared to vehicle. (Figure 1A). A dose response curve was generated at peak effect (30 min) (Figure 1B). The A_{50} at 30 min was 4.67 (2.81 – 9.88 μg/5μL; 95% CI). For additional comparative purposes, AUCs were calculated and compared to vehicle (AUC=22.07 ± 3.02). RSA 504 at doses of 3 μg/5μL (AUC= 32.53 ± 4.39), 10 μg/5μL (AUC = 39.38 ± 3.17), and 30 μg/5μL (AUC= 44.80 ± 4.93) differed significantly from vehicle. Similarly, RSA 601 when administered spinally displayed a time- and dose-related attenuation of SNL-induced mechanical hypersensitivity on day 7 after injury with significant attenuation of mechanical hypersensitivity at doses of 10μg/5μL and 30μg/5μL at all time points when compared to vehicle or post-injury baseline. RSA 601 at 3 μg/5 μL induced significant attenuation of mechanical hypersensitivity at the 15, 30, and 45 min time points when compared to post injury baseline, but not when compared to vehicle (Figure 2A). A dose response curve was generated for the time of peak effect (30 min) (Figure 2B). The A_{50} value at this time was 3.91 (2.75 – 6.34 μg/5μL; 95% CI). AUCs were calculated and compared to vehicle. RSA 601 at doses of 10 μg/5μL (AUC = 46.28 ± 2.43) and 30 μg/5μL (AUC= 49.34 ± 5.41) differed significantly from vehicle. No significant difference was observed with comparison of morphine or gabapentin to vehicle or post injury baseline at any time points tested or with total AUC (morphine AUC = 34.96 ± 7.99; gabapentin AUC = 25.97 ± 6.68).

2.3 RSA 504 and 601 Attenuate Thermal Hyperalgesia When Administered Intrathecally

The L_5/L_6 SNL model was also used to evaluate efficacy against thermal hypersensitivity. Prior to and after injury, all animals were evaluated for hyperalgesic response to noxious probing of the left hind paw with an infrared cylinder. The mean paw withdrawal latency before SNL was 22.65 ± 1.6 seconds (n=80). Seven days after nerve ligation, the mean withdrawal latency was 12.21 ± 0.26 seconds (n=80), indicating the development of thermal hyperalgesia. The animals were separated at random into i.t. treatment groups: 33% EtOH vehicle, morphine (10 mg), gabapentin (30 mg), RSA 504 (1, 10, or 30 μg), and RSA 601 (1, 6, or 30 μg). An acute bolus was given i.t. to rats that had undergone L5/L6 SNL 7 days earlier. Additionally, sham control groups were included in the studies in which the animals received the same surgery as SNL animals minus the ligation of the L5 and L6 spinal nerves. No change in paw withdrawal latency was observed in sham-operated animals from pre-surgical baseline (22.09 ± 0.76 seconds, n=18) to 7 day post-surgical baseline (20.61 ± 0.98 seconds, n=18). Sham animals were separated into three groups: 33% EtOH vehicle, RSA 504 (30 μg/5 μL) and RSA 601 (10 μg/5 μL). Following treatment administration, behavioral measurements of thermal hyperalgesia were obtained every 15 min for the first hour. Responses were compared to pre-injury and post- injury paw withdrawal thresholds, and those of vehicle-treated animals. Administration of RSA 504 at doses of 10 μg/5μL and

30 μg/5μL in SNL animals significantly attenuated thermal hyperalgesia at the 30 min time point as compared to vehicle and post-injury baseline, whereas the 1μg/5μL dose did not produce a significant effect compared to vehicle or post injury baseline (Figure 3A). Peak effect of RSA 504 in the SNL animals occurred 30 minutes after intrathecal administration. A dose response curve was generated at the 30 min time point with an A_{50} value of 6.09 (3.45 – 13.96 μg/5μL; 95% CI) (Figure 3B). AUCs were calculated and compared to vehicle (AUC=55.52 ± 2.74). RSA 504 at doses of 10 μg/5μL (AUC = 83.61 ± 6.40) and 30 μg/5μL (AUC= 111.10 ± 7.97) differed significantly from vehicle. RSA 504 in sham operated animals and vehicle in the SNL animals had no effect on thermal hypersensitivity. RSA 601 administered intrathecally also significantly attenuated thermal hyperalgesia. Doses of 6 μg/5μL and 10 μg/5μL induced significant antihyperalgesic response as compared with vehicle treated animals or post-injury baselines (Figure 4A), however the lowest dose tested did not produce a significant effect 1 μg/5μL when compared to vehicle treated animals. A dose response curve for thermal latency in the SNL animals was generated for peak effect (15 min) with an A_{50} value of 5.17 (3.66 – 7.83 μg/5μL; 95% CI) (Figure 4B). RSA 601 (10 μg/5μL) demonstrated significant antinociception in sham animals. AUCs were calculated and compared to vehicle. RSA 601 at the 6 μg/5μL (AUC = 80.45 ± 4.13) differed significantly from vehicle. As compared to vehicle or post injury baseline, both gabapentin (figure 3A) and morphine (data not shown) displayed significant efficacy at all time-points as well as total AUC (morphine AUC = 77.33 ± 6.71; gabapentin AUC = 104.72 ± 11.01). In both SNL and sham-operated animals, intrathecal injection with control vehicle had no significant effect.

2.4 Chronic Spinal Administration of RSA 504 Does Not Induce Opioid Antinociceptive Tolerance

The L_5/L_6 SNL model was used to evaluate efficacy against thermal hyperalgesia over a period of chronic RSA 504 administration. Animals were tested for thermal paw withdrawal latencies on treatment day one (i.e., 7 days after SNL injury). An antihyperalgesic dose response curve was generated on the first treatment day using RSA 504, 3 μg/5μL, 10 μg/5μL and 30 μg/5μL (Figure 5). In order to test whether RSA 504 induces tolerance to its antihyperalgesic effects, these same animals were administered RSA 504 30 μg/5μL for 6 days and re-evaluated for thermal paw withdrawal latencies on the seventh treatment day. Reconstruction of the dose response curve in these chronically treated animals yielded significant attenuation of thermal hyperalgesia at doses of 3 μg/5μL (p<0.01), 10 μg/5μL, and 30 μg/5μL as compared with baseline (Figure 5). The A_{50} of RSA 504 on day one was 5.89 (4.11 – 8.45 μg/5μL; 95% CI) and was not significantly different when compared to the reconstructed RSA 504 dose response curve on day 7 after chronic treatment with an A_{50} of 6.45 (3.16 – 13.7 μg/5μL; 95% CI) (Figure 5). Chronic treatment with vehicle had no effect (data not shown).

2.5 Intrathecal administration of RSA 504 and 601 do not induce motor impairment or sedation

Intrathecal administration of RSA 601 and 504 did not result in motor impairment as tested using the rotarod device (Figure 6) thus demonstrating that RSA 504 and 601 do not impair motor function or produce sedation.

2.6 *In Vivo* Demonstration of RSA 504 and RSA 601 CCK Antagonist Activity

Thermal hindpaw latencies were tested along with the RVM administration of RSA 504 and RSA 601 in with the presence of systemic naloxone (subcutaneous) to inhibit their opioid actions. Studies were performed in order to evaluate whether *in vivo* compound activity was being produced as a result of their CCK antagonist activity (Table 1). Thermal hypersensitivity was determined by paw withdrawal latencies to infrared radiant heat applied

to the plantar aspect of the hind paw. Morphine produced significant (* p<0.01) analgesia as compared with baseline. Naloxone fully reversed this analgesia. CCK-8 administration alone produced thermal hypersensitivity as compared with baseline. RSA 504 and RSA 601 reversed the CCK-8 hypersensitivity and this effect was not significantly attenuated by naloxone as compared to CCK alone. These data suggest that RSA 504 and RSA 601, in the presence of naloxone in order to block the opioid effects, demonstrated an *in vivo* inhibition of the CCK-induced thermal hypersensitivity.

3. Discussion

Widespread systemic changes and often prolonged treatment periods make many of the classic analgesics less viable for neuropathic pain. This may be due to issues of tolerance, adverse side effects, medication overuse hypersensitivities or simply a difference in the underlying pain mechanisms. While there are several mechanisms that are likely to play a role in this decreased efficacy, one such mechanism may be the increased activity of endogenous CCK at both spinal and supraspinal sites.

The PAG and RVM have been shown to mediate descending modulatory pathways that play a role in neuropathic pain (Behbehani, 1995; Mason, 2001; Ossipov et al., 2000; Urban and Gebhart, 1999). Several studies have shown that descending facilitation from the RVM is driven endogenously by CCK (Kovelowski et al., 2000; (Friedrich and Gebhart, 2003) and that inhibition is regulated by GABAergic interneurons (Cho and Basbaum, 1991; Gilbert and Franklin, 2001; Heinricher *et al.*, 1991; Kovelowski *et al.*, 2000; Moreau and Fields, 1986). CCK and opioid receptors as well as the associated endogenous peptides are not only co-localized in many of the pain modulating areas of the CNS (Ghilardi *et al.*, 1992; Zhang *et al.*, 2009), but also share some structural and pharmacological similarities (Hruby *et al.*, 1994; Slaninova *et al.*, 1991). Here we demonstrate, in rats with SNL, that morphine given spinally lacks efficacy for mechanical hypersensitivity after nerve ligation. However, when administered spinally, both RSA 504 and RSA 601 effectively inhibited tactile and thermal hypersensitivity caused by L_5/L_6 SNL at doses that do not produce sedation. By having dual pharmacophores, opioid agonist/CCK antagonist activity, the RSA compounds are more likely to reverse nerve injury-induced hypersensitivities.

Previous studies have demonstrated that a single injection of a CCK-2 antagonist into the RVM reversed L_5/L_6 SNL-induced mechanical and thermal hypersensitivity in a time dependent manner with peak effect occurring ten minutes post injection (Kovelowski *et al.*, 2000). Similarly, mechanical hypersensitivity from nerve injury was reversed by spinal co-administration of a CCK-2 antagonist and morphine (Dourish *et al.*, 1990) or spinal CCK-2 antagonist and inhibitors of endogenous enkephalin (Nichols *et al.*, 1996). Studies by Friedrich and Gebhart (Friedrich and Gebhart, 2003) demonstrated that a CCK-2 antagonist administered into the RVM dose- and time-dependently reversed a visceral hypersensitive response induced by chronic inflammation of the GI tract. These data suggest that peripheral nerve injury to the lumbar region or chronic inflammation of visceral afferents results in the increase in endogenous CCK in the spinal cord and RVM promoting behavioral signs of pain and, therefore, a compound that acts to inhibit CCK receptors and activate opioid receptors is more likely to inhibit neuropathic and inflammatory pain.

Although in rodent studies the CCK-2 receptor does seem to be the primary arbiter of CCK descending modulation of the endogenous opioid system, this may not be the case in humans. Human trials conducted by McCleane evaluated the effects of the CCK-2 specific antagonist L365,260 in conjunction with morphine and did not show any augmentation to the analgesic effects of morphine (McCleane, 2003). Conversely, proglumide, a non specific CCK inhibitor, did augment the analgesic efficacy of morphine in human trials (McCleane,

1998), suggesting that in humans both CCK-1 and CCK-2 may play a role in pain modulation. Therefore, a non-selective CCK antagonist may be the most appropriate for clinical development. The compounds tested here have binding affinity in the nanomolar range at both CCK receptors, and simultaneously agonize δ and μ opioid receptors; a characteristic that may further increase utility of these compounds. Along with increases in affinity and specificity, patient care is also improved by simultaneously counteracting multiple facets of one disease state, lowering required doses, and reducing the risk of side effects and drug-drug interactions.

Our studies demonstrate that RSA 601, and to a lesser extent RSA 504, resulted in a significant increase in thermal thresholds above baseline in sham animals. By measuring their ability to attenuate CCK-8$_{sulf}$ induced thermal hyperalgesia using naïve animals in the presence of naloxone, the RSA compounds were shown to act as CCK antagonists *in vivo*. Since these compounds have opioid agonist activity, we blocked their ability to act at opioid receptors using naloxone and found that the CCK antagonist pharmacophore was active against CCK-8-induced thermal hyperalgesia confirming their dual acting biological activity. Preclinical studies have shown that long-term opioids can elicit abnormal pain states manifested as paradoxical algesia and hyperesthesias (Mao *et al.*, 1995; Trujillo and Akil, 1991; Woolf, 1981; Yaksh and Harty, 1988). The development of thermal hyperalgesia in response to either repeated injections or constant infusion of morphine has also been reported (Mao et al., 1994), 1995). Thus, opioids given over time may maintain their level of efficacy, but the concurrent development of hyperalgesia could serve to counteract the antinociceptive effect of opioids, producing an impression of tolerance (Colpaert, 1996; Laulin et al., 1998; Laulin et al., 1999). Systems that may be activated upon sustained morphine administration include the descending pain facilitatory pathways from the RVM up-regulating the endogenous pronociceptive transmitter CCK.

Using *in vivo* microdialysis, Afrah and colleagues (Afrah *et al.*, 2001) demonstrated that peripheral nerve injury results in a 3 fold increase in spinal endogenous CCK levels 7 days after injury. Likewise, using *in vivo* microdialysis we have demonstrated a 2 to 3 fold increase in endogenous CCK in the RVM, 7 days after L_5/L_6 SNL (abstract SfN, Vanderah Lab, 2008). The exogenous administration of CCK into the RVM results in a time dependent mechanical and thermal hypersensitivity as well as visceral hyperalgesia in naïve animals (Friedrich and Gebhart, 2003; Xie *et al.*, 2005). CCK is thought to promote pain by activating a pain facilitatory pathway in the RVM. This pathway has been described as neurons that fire when pain is present, termed "ON cells" and are directly activated by the administration of CCK (Heinricher and Neubert, 2004). Collectively, these data strongly suggest that nerve injury or chronic inflammation results in the increase of endogenous CCK in the spinal cord and RVM resulting in pain facilitation, strongly supporting the concept of having a compound such as RSA 504 and 601 that not only activates opioid receptors but also blocks CCK receptors to inhibit neuropathic pain and antinociceptive tolerance.

Here we demonstrate that chronic administration of RSA 504 does not induce the onset of opioid antinociceptive tolerance: a promising finding consistent with previous findings that a CCK antagonist can reverse morphine-induced antinociceptive tolerance (Dourish *et al.*, 1990). Likewise, several studies in humans with chronic pain have demonstrated significant opioid sparing effects by co-administering a CCK antagonist with an opioid and/or pain relief (McCleane, 1998; McCleane, 2002; McCleane, 2003). Unfortunately, such human studies cannot proceed since CCK antagonists, approved for human use, are no longer being manufactured most likely due to expired patents.

Over the past twenty years companies have continued to design highly selective compounds for a single target in order to attenuate neuropathic pain, however, such strategies have not

resulted in a plethora of highly efficacious compounds that lack unwanted side effects. By designing compounds that target μ and δ opioid receptors, as well as the CCK receptors, we have produced novel compounds that demonstrate better, longer lasting antinociceptive efficacy in a neuropathic pain model. Novel compounds are being designed to target multiple sites that may offer better long-term efficacy and/or lack side effects due to over stimulation of one molecular target. Here we report *in vivo* behavioral studies with a compound designed to have agonist and antagonist activity at two distinctly different receptors for neuropathic pain. CCK is upregulated under conditions of neuropathic pain and therefore a compound is needed to block its pro-algesic effects. Unlike morphine, RSA 504 with CCK antagonist activity and opioid agonist activity did not result in antihyperalgesic tolerance over a 7 day period in nerve injured animals suggesting that the sustained administration of such dual acting pharmacophores may be effective over a long period of time in chronic pain patients. The results of this study show that the design of single compounds with dual pharmacophores leads to promising therapeutic agents for the treatment of neuropathic pain lacking unwanted side effects such as tolerance, sedation and opioid-induced hyperalgesia.

4. Experimental Procedure

4.1 Peptides

The peptides reported here were synthesized by standard solid phase peptide synthesis methods (Agnes et al., 2008; Hruby et al., 2003) by the Hruby lab in the University of Arizona department of Chemistry.

4.2 *In Vitro* Assays

4.2.1 GTPγS Binding—GTPγS binding was done using membranes from cells that express the human δ or rat μ opioid receptors. The procedure for this analysis was based on that of Lorenzen et al. (Lorenzen et al., 1993). Reactions were initiated by the addition of an aliquot of membrane preparation (15 μg) to a final volume of 300 μL of incubation mix [50 mM HEPES, pH.7.4, 1 mM EDTA, 5 mM $MgCl_2$, 30 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 100 μM phenylmethylsulfonyl fluoride, 0.1% bovine serum albumin, 0.1 nM [^{35}S]GTPγS (1250 Ci/mmol)] and indicated concentration range of agonist and incubated for 60 min at 30°C. Basal level of [^{35}S]GTPγS binding was defined as the amount bound in the absence of agonist. Nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS. Reactions were performed in triplicate and terminated by rapid filtration through Whatman GF/B filters presoaked in water followed by four washes with ice-cold wash buffer (50 mM Tris, 5 mM $MgCl_2$, and 100 mM NaCl, pH 7.4). The radioactivity was determined by liquid scintillation counting. Data were fitted by nonlinear least-squares analysis using GraphPad Prism. 4.3 *In Vivo* Assays 4.3.1 Animals The experiments contained herein were carried out using male Sprague Dawley rats (250–350g; Harlan; Indianapolis, IN). All animals were maintained on a 12/12 hr light/dark cycle (lights on at 07:00 am) and provided food and water ad libitum except as noted during the experimental procedures. All experiments were performed under an approved protocol by the Institutional Animal Care and Use Committee of the University of Arizona, and in accordance with policies and guidelines for the care and use of laboratory animals as adopted by International Association for the Study of Pain and the National Institutes of Health.

4.3.2 L₅/L₆ SNL surgery—SNL injury was induced in male Sprague Dawley rats as described by Chung and colleagues (Kim and Chung, 1992). Anesthesia was induced with 2% isoflurane in O_2 at 5 L/min and maintained with 2.5% isoflurane in O_2. The dorsal vertebral column from L_4 to S_2 was exposed; the L_5 and L_6 spinal nerves were tightly ligated distal to the dorsal root ganglion using 4-0 silk suture. The incision was closed and

the animals were allowed to recover for 5–7 days. Rats that exhibited motor deficiency (such as paw dragging) or failure to exhibit subsequent tactile allodynia were excluded from further testing (< 5%). Sham control rats underwent the same operation and handling as the experimental animals, but without ligation of the L5/L6 spinal nerves.

4.3.3 Intrathecal Catheter Surgery—Male Sprague Dawley rats were prepared for intrathecal drug administration as described by Yaksh and Rudy (Yaksh and Rudy, 1976) by placing anesthetized (ketamine/xylazine 100 mg/kg, intraperitoneal) animals in a stereotaxic head holder. The cisterna magna was exposed, an incision was made and animals were implanted with a catheter (PE: 10, 8 mm) that terminated in the lumbar region of the spinal cord. The animals were allowed to recover 5–7 days post-surgery before any pharmacological manipulations were made. Any rats showing impaired motor skills (e.g. paralysis) or greater than 10% weight loss were excluded from experiments (< 5%).

4.3.4 RVM Cannulation—Male Sprague Dawley rats were cannulated as described by the Porreca group (Roberts et al., 2009). Rats were anesthetized (ketamine/xylazine 100 mg/kg, intraperitoneal) and placed into a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). A 2 cm incision was made in the scalp, and the underlying connective tissue was retracted with hemostats to expose the skull. Paired guide cannulae 1.2 mm apart (26GA, #C235G-1.2 mm; Plastics One Inc., Roanoke, VA, USA) were directed towards the RVM (10.8 mm caudal to bregma, 0.6 mm to each side of the sagittal suture, and 7.0 mm ventral to the dura mater surface). Bone wax was used to seal the hole around the cannula. The paired guide cannula was secured to the skull using stainless steel screws and dental acrylic. A dummy cannula (#C235DC; Plastics One Inc.) was inserted to prevent contaminants from entering the RVM guide cannula. Rats were given an injection of antibiotic (amikacin C, 5 mg/kg, intraperitoneal) and allowed to recover for 7 days.

4.3.5 Drug Administration—RSA 504 and RSA 601 were dissolved in 33% EtOH and brought to volume with MilliPore H_2O. CCK-8_{sulf} was dissolved in Millipore H_2O. Morphine sulfate was dissolved in 0.9% saline. Deltorphin (Bachem) was dissolved in 30% DMSO and brought to volume with 0.9% saline. Naloxone (RBI) was dissolved in 0.9% saline. For intrathecal compound administration, all compounds were injected in a 5 μL volume followed by a 9 μL saline flush. Blinded testing took place 15, 30, 45 and 60 min after drug injection, and dose–response curves were generated from data gathered at the time of peak effect. RVM drug administrations were performed by slowly expelling 0.5 μL bilaterally through an injection cannula protruding 1 mm beyond the tip of the guide to prevent backflow of drug into the guide cannula. Naloxone (10 mg/kg), when used, was administered subcutaneously 20 minutes before behavioral testing.

4.3.6 Behavioral Assessment

4.3.6.1 Antinociceptive Testing: Antinociception was assessed using the 52°C warm water tail-flick test in male Sprague Dawley rats (Vanderah et al., 2008). The latency to the first sign of a rapid tail flick was taken as the behavioral endpoint. Each rat was first tested for baseline latency by immersing the distal third of the tail in the water and recording the time to response. Rats not responding within 10 s were excluded from further testing (average latency = 4.26 ± 0.2s). One hour after baseline latencies were measured, rats were administered RSA 504, RSA 601, or vehicle and tested for tail or hindpaw withdrawal latency at 15, 30, 45, and 60 min post injection in a blinded fashion.

%Antinociception Calculation

$$\% \text{ Activity} = 100 \times (\text{withdrawal latency after drug treatment} - \text{baseline latency})/(\text{cutoff latency} - \text{baseline latency})$$

4.3.6.2 Thermal Hypersensitivity: Thermal hypersensitivity was assessed using the rat plantar test following a modified method of Hargreaves and colleagues (Hargreaves et al., 1988). Male Sprague Dawley rats were allowed to acclimate within Plexiglas enclosures on a clear glass plate. A mobile radiant heat source (Ugo Basile, Italy) was located under the glass plate and focused onto the hind paw. Paw withdrawal latencies were recorded in seconds at 15 minute intervals for 60 minutes. An automatic cut off point of 33.0 s was set to prevent tissue damage. The apparatus was calibrated to give a paw withdrawal latency of approximately 20 seconds on the uninjured paw. The radiant heat source was activated with a timer and focused onto the plantar surface of the hindpaw. A motion detector which halted both heat source and timer when the paw was withdrawn determined paw-withdrawal latencies. The ipsilateral paws of SNL and sham animals were tested using the radiant heat source. The contralateral paws were not tested so that the injured paw would not be forced to bear weight unnecessarily. No thermal hypersensitivity was seen in sham animals. Crossover studies were performed when possible to reduce the total number of animals used: SNL or sham animals receiving vehicle during acute phase testing received a dose of either RSA 504 or 601 a minimum of 5 hours later for additional acute testing after repeating post-injury baseline testing.

<u>Injury % Activity Calculation (% Antihyperalgesia)</u>

$$\% \text{ Activity} = 100 \times (\text{withdrawal latency post drug} - \text{post surgery baseline latency})/(\text{pre surgery baseline latency} - \text{post surgery baseline latency})$$

<u>Sham operated percent activities (% analgesia)</u>

$$\% \text{ Activity} = 100 \times (\text{withdrawal latency post drug} - \text{post sham baseline latency})/(\text{pre sham baseline latency} - \text{post sham baseline latency})$$

4.3.6.3 Mechanical Hypersensitivity: The assessment of mechanical hypersensitivity consisted of measuring the withdrawal threshold of the paw ipsilateral to the site of nerve injury in response to probing with a series of calibrated von Frey filaments. Prior to the SNL surgery, male Sprague Dawley rats were tested for pre-injury baseline mechanical sensitivity. Each filament was applied perpendicularly to the plantar surface of the left hind paw of rats kept in suspended wire-mesh cages. 7 days post SNL surgery on the left hind limb, measurements were taken both before (post-injury baseline) and after administration of RSA 504, RSA 601, or vehicle at 15 minute intervals for 60 minutes. The withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength (`up–down' method) analyzed using a Dixon non-parametric test (Chaplan *et al.*, 1994) and expressed as the mean withdrawal threshold. SNL animals were tested with the von Frey filaments while sham animals were not since maximum threshold was set to 15 grams. Higher calibrated filaments (>15g) result in the actual lifting of the paw that may be misinterpreted as a mechanical withdrawal (the higher calibrated filaments support the weight of the paw). Crossover studies were performed when possible to reduce the total number of animals used: SNL or sham animals receiving vehicle during acute phase testing

received a dose of either RSA 504 or 601 a minimum of 5 hours later for additional acute testing after repeating post-injury baseline testing.

<u>Injury % Activity Calculation (% Antiallodynia)</u>

$\%\ \text{Activity} = 100 \times (\text{withdrawal threshold post drug}$
$-\text{post surgery baseline threshold})/(\text{pre surgery baseline threshold} - \text{post surgery baseline threshold})$

<u>4.3.6.4 Motor Function:</u> Male Sprague Dawley rats were trained to ambulate on a rotarod device (Columbus Instruments International, Columbus, OH) as previously described (Vanderah et al., 2008) until all could remain on the device for a duration of 180 s at a speed of 10 revolutions per minute. The rats were tested again after administration of RSA 504, RSA 601 or vehicle, and the time the rats were able to remain on the device without falling was recorded at 15 minute intervals for 60 minutes. The κ opioid agonist U50,488 was used as a positive control (not shown). A maximum cutoff time of 180 s was used.

4.4 Statistical Analysis

[35S]GTPγS binding data were analyzed by non-linear regression analysis using GraphPad Inplot. For binding affinity, the K_i value(s) for each ligand was calculated from the IC_{50} value(s) based on the Cheng and Prusoff equation from at least three independent experiments. For GTPγS binding, potency was expressed as $\log EC_{50} \pm$ S.E.M. Maximal effect was expressed as $E_{max} \pm$ S.E.M.

Thermal and tactile hypersensitivity data were analyzed as previously published using one-way analysis of variance followed by students Neuman-Kuels testing for multiple comparisons in FlashCalc (Vanderah *et al.*, 2000; Xie *et al.*, 2005; Zhang *et al.*, 2009). Differences were considered to be significant if $p \leq 0.05$. When possible, potencies (or A_{50}) were determined by regression analysis of dose–response curve (log dose [x] vs response [y]) using a 95% confidence interval according to the method of analysis of the Graded Dose–Response (Tallarida and Murray, 1987). For the calculations of A_{50}'s, the minimal possible response was set to 0%.

Abbreviations

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AUC	area under curve
CCK	cholecystokinin
CGRP	calcitonin gene related peptide
CNS	central nervous system
hDOR	human δ opioid receptor
GABA	γ-aminobutyric acid

| HEK | human embryonic kidney cells |
IMDM	Iscove's modified Dulbecco's medium
MOR	μ opioid receptor
MS	morphine sulfate
NOS	nitric oxide synthase
NRI	norepinephrine reuptake inhibitor
PAG	periaqueductal gray
PKC	protein kinase C
RVM	rostral ventromedial medulla
SNL	spinal nerve ligation
SNRI	serotonin-norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor

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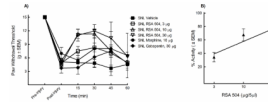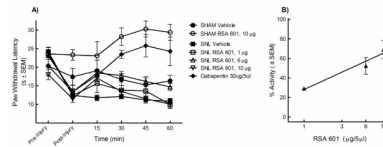
Figure 1.
Mechanical hypersensitivity in L_5/L_6 spinal nerve ligated male Sprague Dawley rats (post-injury, day 7) was significantly reversed by RSA 504: (**A**) Dose- and time-related curves of RSA 504 (intrathecal) in nerve injury-induced mechanical hypersensitivity using calibrated von Frey filaments applied to the plantar aspect of the hind paw ± SEM. RSA 504 significantly reversed paw withdrawal thresholds at all three doses 3µg/5µl ($p<0.05$; n=9), 10µg/5µl ($p<0.05$; n=10), and 30µg/5µl ($p<0.05$; n=10)(p values calculated at the 30 min time point). No significant effect was seen in vehicle (n=10) (intrathecal) treated, morphine treated (n=6), or gabapentin treated animals (n=6). (**B**) Antiallodynic dose-response curve ± SEM for RSA 504 (intrathecal) was plotted at the time of peak effect (30 min) with an A_{50} of 4.67 (2.81 – 9.88 µg; 95% CI).
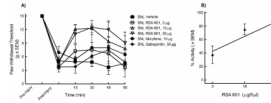
Figure 2.
Mechanical hypersensitivity in L_5/L_6 spinal nerve ligated male Sprague Dawley rats (post-injury, day 7) was significantly reversed by RSA 601: (**A**) Dose- and time-related curves of RSA 601 (intrathecal) in nerve injury-induced mechanical hypersensitivity using calibrated von Frey filaments applied to the plantar aspect of the hind paw ± SEM. RSA 601 significantly reversed paw withdrawal thresholds at all three doses 3μg/5μl ($p < 0.05$; n=9), 10μg/5μl ($p < 0.05$; n=12), and 30μg/5μl ($p < 0.05$; n=9)(p values calculated at the 30 min time point). RSA 601 did not significantly change paw withdrawal thresholds in vehicle control (n=9) animals, morphine treated animals (n=6), or gabapentin treated animals (n=6) from baseline thresholds. (**B**) Antiallodynic dose-response curve ± SEM for RSA 601 (intrathecal) was plotted at the time of peak effect (30 min) with an A_{50} of 3.91 (2.75 – 6.34 μg; 95% CI).
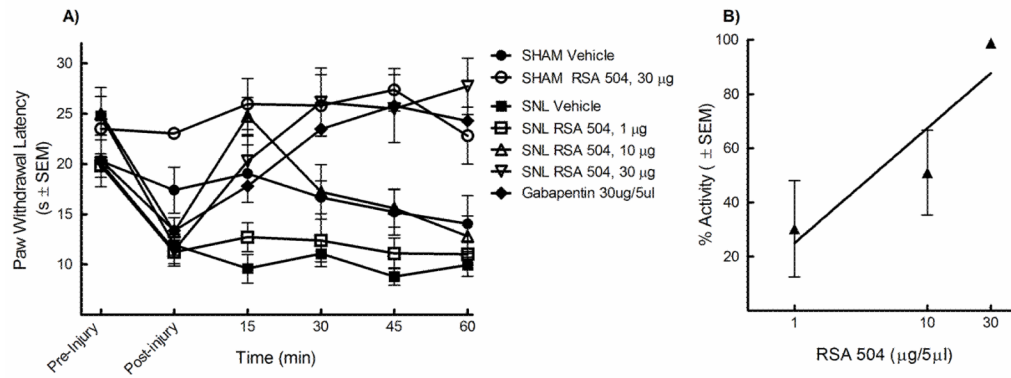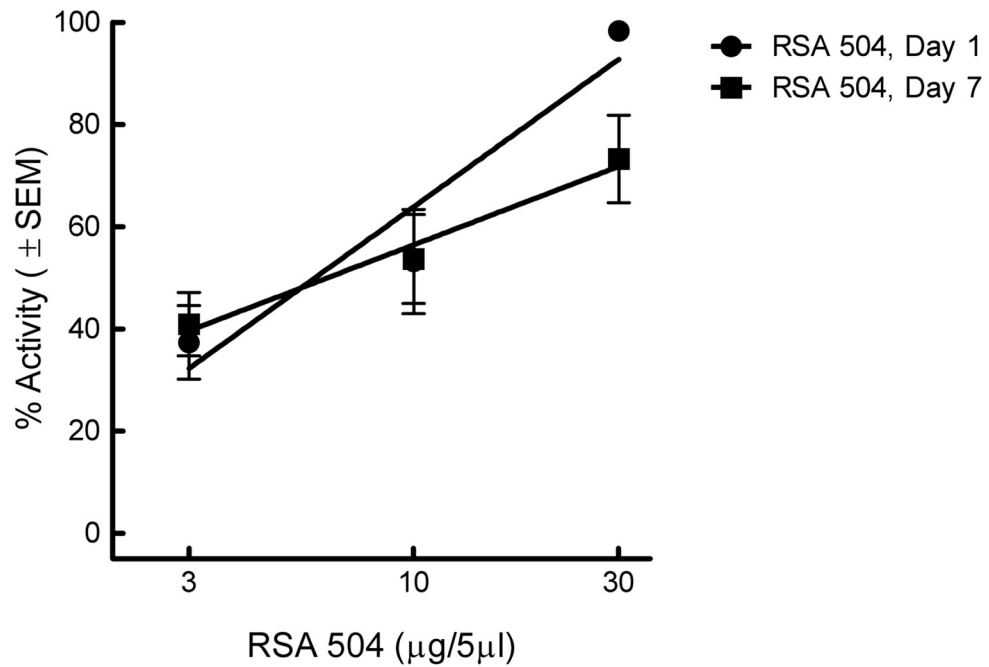
Figure 3.
Thermal hypersensitivity in L_5/L_6 spinal nerve ligated male Sprague Dawley rats (post-injury, day 7) was significantly reversed by RSA 504: (**A**) Dose- and time-related curves of RSA 504 (intrathecal) in nerve injury-induced thermal hypersensitivity using infrared radiant heat applied to the plantar aspect of the hind paw ± SEM. RSA 504 significantly reversed paw withdrawal latencies at doses of 10μg/5μl ($p<0.05$; n=9) and 30μg/5μl ($p<0.05$; n=10) but not at 1μg/5μl (n=8) (p values calculated at the 30 min time point). RSA 504 significantly increased paw withdrawal latencies in sham animals ($p<0.05$; n=10) at the 45 min time point. Gabapentin (n=6) and morphine (n=6) significantly increased paw withdrawal latencies at all timepoints ($p<0.05$). No significant effect was seen in vehicle (intrathecal) treated SNL (n=8) or Sham animals (n=6). (**B**) Antihyperalgesic dose-response curve ± SEM for RSA 504 (intrathecal) was plotted at the time of peak effect (30 min) with an A_{50} value of 6.09 (3.45 – 13.96 μg; 95% CI).
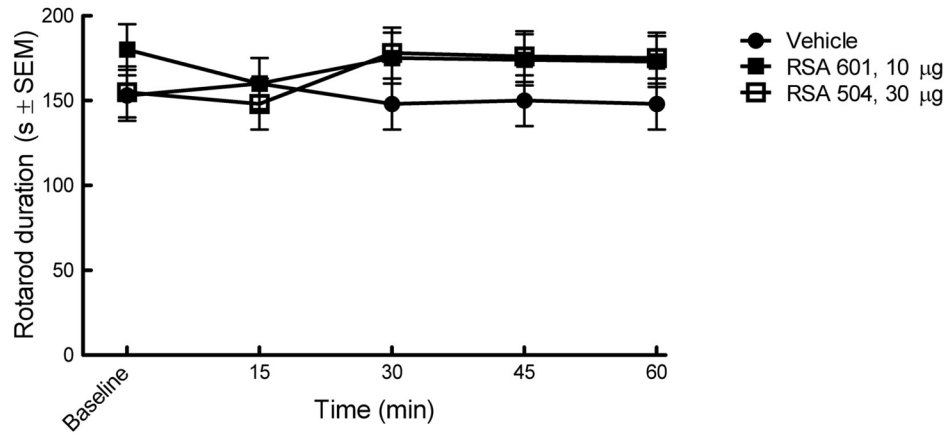
Figure 4.
Thermal hypersensitivity in L_5/L_6 spinal nerve ligated male Sprague Dawley rats (post-injury, day 7) was significantly reversed by RSA 601: (**A**) Dose- and time-related curves of RSA 601 (intrathecal) in nerve injury-induced thermal hypersensitivity using infrared radiant heat applied to the plantar aspect of the hind paw ± SEM. RSA 601 significantly reversed paw withdrawal latencies at the doses of 6μg/5μl (p<0.05; n=9) and 10μg/5μl (p<0.05; n=9) (p values calculated at the 15 min time point). RSA 601 significantly increased paw withdrawal latencies in sham animals (p<0.01; n=6) at the 45 min time point. Gabapentin (n=6) and morphine (n=6) significantly increased paw withdrawal latencies at all timepoints (p<0.05). No significant effect was seen in vehicle (intrathecal) treated SNL animals (n=10). (**B**) Antihyperalgesic dose-response curve ± SEM for RSA 601 (intrathecal) was plotted at the time of peak effect (15 min) with an A_{50} value of 5.17 (3.66 – 7.83 μg; 95% CI).

Figure 5.
Antihyperalgesic dose-response curve ± SEM for intrathecal injection of RSA 504 in SNL animals does not result in tolerance: DRCs were generated at the time of peak effect (30 min) on day 1 (i.e., 7 days after SNL) and day 7 (i.e., 14 days after SNL) in the same animals. After testing on day one animals received sustained injections of RSA 504 (30 μg/5μl/day for 6 days; n=10). The DRC was re-constructed on day 7. The A_{50} value on day 1 was 5.89 (4.11 – 8.45 μg; 95% CI); the A_{50} value on day 7 was 6.45 (3.16 – 13.74 μg; 95% CI).

Figure 6.
RSA 504, 601 or vehicle administered intrathecally did not result in motor impairment or sedation using the rotarod test: Animals were measured for their ability to stay on a rotating rod for 180 seconds ± SEM. No significant differences were seen between RSA 504 treated animals, RSA 601 animals or vehicle treated animals (n=6 per tested group).

Table 1

Thermal behavioral response in animals treated with RSA 504, RSA 601 and naloxone Thermal hypersensitivity was determined by paw withdrawal latencies to infrared radiant heat applied to the plantar aspect of the hind paw of male Sprague Dawley rats. The analgesic properties of RSA 504 (RVM; 60 ng/0.5μl) and RSA 601 (RVM; 60 ng/0.5μl) were compared with that of morphine sulfate (RVM; 60 ng/0.5μl) both with and without pretreatment (−20min) of naloxone (subcutaneous; 10mg/kg) and with pre-administration (−10min) of CCK-8$_{sulf}$ (RVM; 30 ng/0.5 μL). (*, **, ***, ****, or *****) denote significance when compared to baseline. († or ††) denote significance when compared to administration of RSA 504 alone. (††† or ††††) denote significance when compared to administration of RSA 601 alone.

Compound	Paw Withdrawal Latency (Sec ± SEM)	SEM	n
Baseline	20.74	0.29	24
Morphine Sulfate (MS)	30.54*	2.83	7
Naloxone (NX)	19.16	1.10	7
MS + NX	18.70**	3.72	6
Cholecystokinin (CCK)	12.32***	1.49	6
RSA 504	26.52****	1.79	6
RSA 504 + CCK	22.35†	0.30	6
RSA 504 + CCK + NX	20.15††	0.55	6
RSA 601	24.21*****	2.52	8
RSA 601 + CCK	21.76†††	1.27	6
RSA 601 + CCK + NX	20.81††††	1.30	6