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14. ABSTRACT This project is based on the observation in our laboratories that chemokines (small proteins produced by cells of the immune system released from injured cells during inflammatory processes) decrease the analgesic potency of opioid drugs. The hypothesis being tested was that blocking the activity of the chemokines by administering chemokine receptor antagonists (CRAs) would restore the analgesic potency of opioids. It was found that the combination of 2 CRAs, administered with a submaximal analgesic dose of morphine in the rat incisional pain assay, resulted in maximal analgesia and a 3.3-fold shift in the morphine dose-response curve to the left. The morphine-CRA combination resulted in down-regulation of a broad panel of immune mediators induced by the incision. CRAs also potentiated the analgesic potency of oxycodone and meperidine. In the rat cold-water tail flick test, a submaximal dose of morphine in combination with maraviroc alone, with AMD3100 plus maraviroc, or with four CRAs, produced significant synergistic increases in antinociception. In the mouse formalin pain test, AMD3100 plus morphine produced an additive effect on pain, and 4 CRAs plus morphine had a significant interaction. These results show that CRAs could lead to therapies that are "opioid-sparing", but still able to provide optimal analgesia.					
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1. INTRODUCTION:

Based on experiments carried out in our laboratories, we demonstrated that chemokines (small proteins produced by cells of the immune system released from injured cells during inflammatory processes) inhibit the analgesic ability of opioid drugs. We hypothesized that the effectiveness of these pain-relieving drugs would be enhanced by eliminating or reducing the activity of the chemokines by administering chemokine receptor antagonists (CRAs). We proposed that blockade of one or more chemokine receptor antagonists would increase the ability of a submaximal dose of opioid analgesics to relieve pain. Thus, taking CRAs in combination with opioids would improve pain control and potentially permit use of lower doses of opioids. Such an opioid-sparing strategy would have potential to reduce the undesirable and adverse effects of these drugs. With effective pain control and lower doses of opioids, treatment of conditions such as PTSD, for which drug cocktails that may include opioids are used, might also be improved. The project proposed and utilized five different rodent models of acute or chronic pain, having varying degrees of inflammation. The models used were hind paw skin incision in the rat (post-operative acute pain), cold water tail immersion in the rat (acute pain), formalin injection into the mouse paw (persistent pain), hind paw injection of carrageenan in the rat (inflammatory pain), and chemically induced neuropathic pain in the rat (chronic pain). In order to determine if specific treatment that includes CRAs alters levels of selected chemokines and cytokines in the different pain models, serum and lymphoid tissues were analyzed utilizing molecular and protein arrays, as well as multiplex quantitative analysis of protein levels of these molecules.

2. KEYWORDS

Pain; Pain Treatment; Analgesia; Nociception; Antinociception; Animal models of pain; Opioids; Morphine, Oxycontin; Meperidine; Chemokine Receptor Antagonists; Chemokines; Cytokines; Inflammation

3. ACCOMPLISHMENTS: OVERALL PROJECT SUMMARY

What were the major goals of the project?

1. Determine if chemokine receptor antagonists (CRAs) can diminish pain in 5 different pain models, and if they alter chemokine, cytokine and other mediator levels.
2. Determine if CRAs can enhance the actions of opioid analgesics.
3. Determine whether various combinations of CRAs are more efficacious than a single CRA when used in conjunction with an opioid, and if chemokine, cytokine and other mediator levels are altered.

What was accomplished under these goals?

1. Effect of Chemokine Receptor Antagonists (CRAs) alone and in combination with opioids using the rat Incisional Pain Model

This part of the study utilized two chemokine receptor antagonists (CRAs), alone, singly in combination with morphine, oxycodone or meperidine, or both in combination with morphine, oxycodone or meperidine. The results were published in two papers. **The first appeared in 2018 in the Journal of Pharmacology and Experimental Therapeutics, 367:433-441. DOI: 10.1124/jpet.118.252890** (and Appendix Item 1). What was discovered is that co-administration of chemokine receptor antagonists (CRAs) with morphine enhanced the analgesic potency of morphine on incisional pain in rats. Animals underwent incisional surgery on the left hind paw and pain responses were evaluated using von Frey filaments at various time points post-surgery between 15 and 360 minutes and daily between 24 and 72 hours. Dose-response curves for morphine, maraviroc (a CCR5 antagonist) and AMD3100 (a CXCR4 antagonist) alone were established. While morphine significantly reduced pain in a time- and dose-dependent manner, maraviroc and AMD3100 had no effect by themselves. Co-administration of either maraviroc or AMD3100 with morphine significantly increased morphine's analgesic effect on incisional pain, shifting the dose-response curve to the left 2.31-fold and 1.8-fold, respectively. Co-administration of both CRAs with morphine significantly shifted the morphine dose-response curve to the left 3.3-fold. The effect of treatments on mRNA levels in the draining popliteal lymph node for a panel of chemokines and

cytokines showed that message for many of these mediators was upregulated by the incision, and the combination of morphine with the CRAs markedly downregulated them. The data show that combining morphine with CRAs potentiates morphine's analgesic effect on incisional pain. Thus, the same analgesic effect of morphine alone can be achieved with lower doses of morphine when combined with CRAs. Using morphine in lower doses could reduce unwanted side effects and possibly inhibit development of tolerance and dependence. Two major figures from this paper are reproduced below. Figure 1 shows the shift in the dose-response curve of morphine in this incisional pain assay. Figure 2 shows the changes in levels of chemokines and cytokines as a result of the treatment with morphine alone, the chemokines alone, or the combination of morphine plus the chemokines.

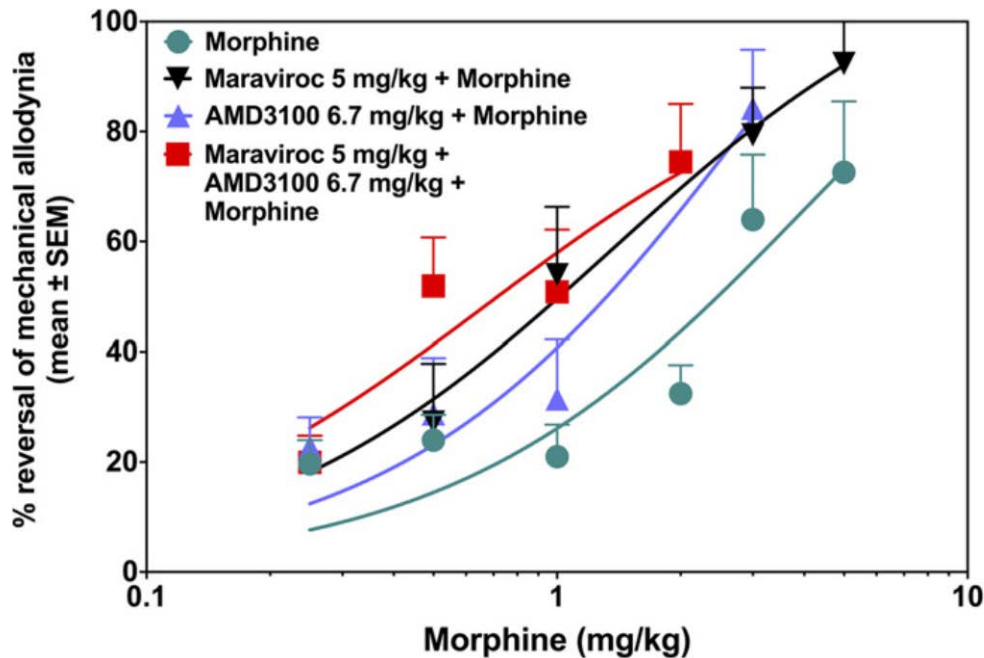


Fig. 1. Combination of morphine with 2 CRAs shifts the morphine dose-response curve to the left. Percent reversal of mechanical allodynia was calculated using data at 60 min. The combination of morphine with either AMD3100 or maraviroc individually, as well as the combination of morphine with both CRAs significantly shifted the morphine dose-response curve to the left. The paired Student's t-test showed, $*p < 0.05$, (n=6-10).

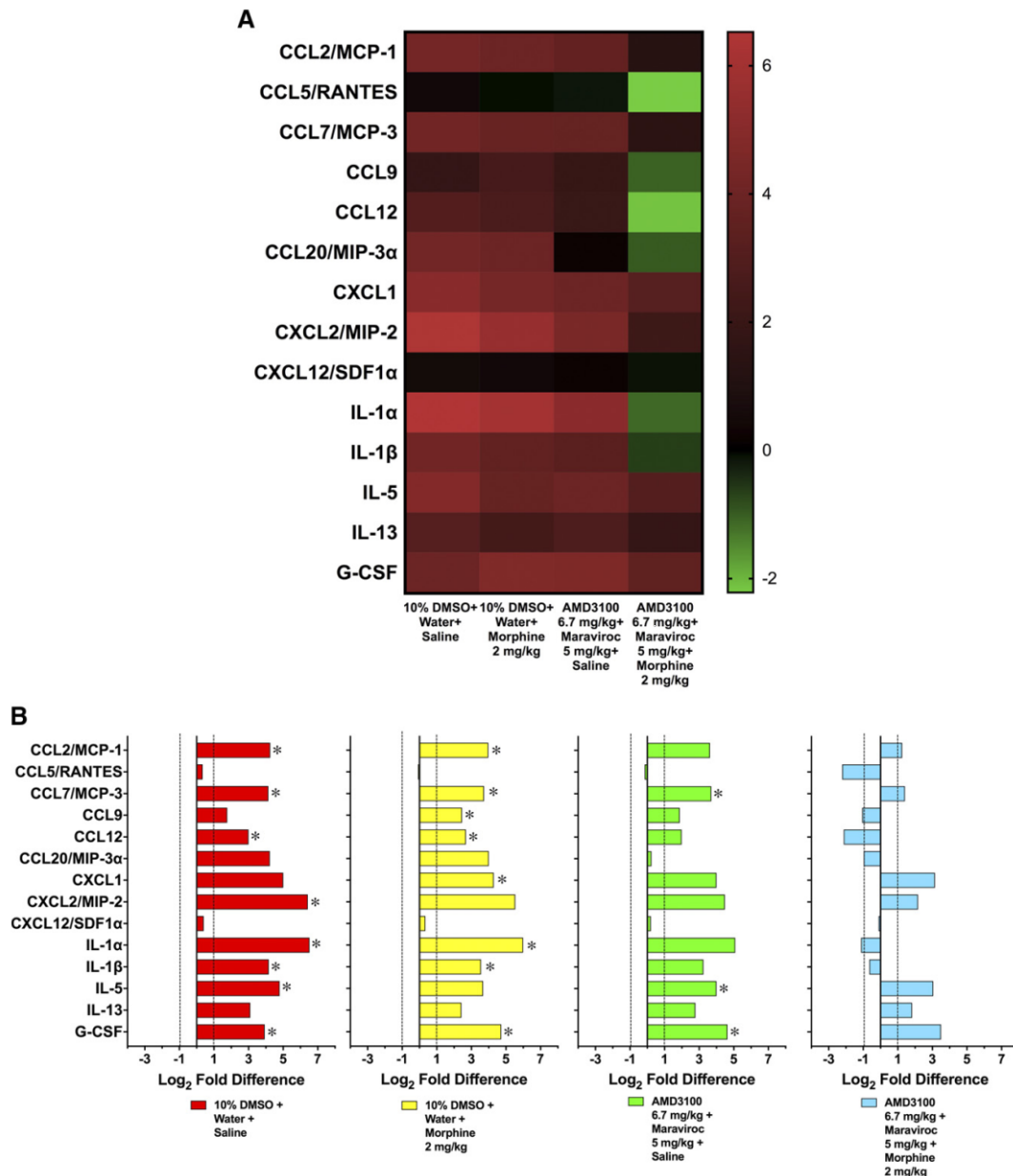


Fig. 2. Effect of incision alone and of treatment post-incision with morphine alone, AMD3100 + maraviroc, or morphine + AMD3100 + maraviroc on mRNA levels for a panel of chemokines and cytokines. The draining popliteal lymph node was harvested 1 hr post incision and was extracted and processed to obtain mRNA, which was analyzed using the RT² Profiler Array (see Methods). Panel A: mRNA expression heat map of the popliteal lymph nodes of rats receiving an incision with and without treatments. Treatments are indicated at the bottom of each column. Color scale is a representation of the log₂ fold change in mRNA levels of various immune mediators compared with untreated baseline lymph nodes, ranging from bright red (upregulated to over log₂ 6-fold) to bright green (downregulated to under log₂ -2 fold). Black represents 0-fold change in mRNA expression. Data are presented as the mean level of mRNA expression in lymph nodes from 3 individual animals. Panel B: Fold change of mRNA expression in popliteal lymph nodes of rats receiving an incision with and without treatments. The data represent the log₂ fold change in mRNA expression for a panel of immune mediators post incision, with or without treatments, compared with untreated, baseline popliteal lymph nodes. Dotted lines indicate limits of 2-fold up- or downregulation from baseline. Data are presented as the mean level of mRNA expression in lymph nodes from 3 individual animals. *p<0.05 vs. baseline.

It is evident from both panels A and B that the incision alone caused a statistically significant increase (starred bars) in mRNAs for several cytokines and chemokines, including CCL2/MCP-1, CCL7/MCP-3, CCL12, CXCL2/MIP-2, IL-1 α , IL- β , IL-5, and G-CSF. There was a trend towards increased mRNA levels for CCL20/MIP-3 α and CXCL1. Morphine alone at 2 mg/kg did not have a strong effect on the mRNA levels of mediators in this panel. The two CRAs, in spite of having little effect on pain, did reduce mRNA for 5 of the 8 mediators upregulated by the incision to a level that was not significantly above baseline. The combination of the suboptimal dose of morphine plus the two CRAs decreased the mRNA levels of all of the mediators that were significantly upregulated by the incision, so that none of them were significantly different from baseline values. mRNAs for several of the mediators were also more robustly downregulated by the morphine + two CRAs, than by the CRAs without morphine. (Baseline values are not available, but are intrinsic to the assay). Surprisingly, neither mRNA for CCL5/RANTES nor for CXCL12/SDF-1 α were upregulated by the incision alone, and there was no effect on mRNA for these chemokines in animals receiving the antagonists for their receptors (AMD3100 for CXCR4 and maraviroc for CCR5). Instead, the combination of morphine plus the two CRAs downregulated a broader panel of mRNAs for unrelated pro-inflammatory chemokines and cytokines.

As proposed in the original SOW, the applicability of these results to other opioid analgesics, oxycodone and meperidine, was also tested in the incisional pain assay. **The results of this study were published in the British Journal of Anaesthesia in 2019. DOI: 10.1016/j.bja.2019.01.021** (and Appendix Item 2).

As shown in Figure 3A, combining AMD3100 with oxycodone significantly shifted the oxycodone dose-response 2-fold to the left (paired Student's t-test, * $p=0.042$). The combination of two CRAs with oxycodone shifted the oxycodone dose-response 1.9-fold to the left, however, it was not statistically significant. For meperidine, the ED₅₀ values for meperidine alone were 15.39 mg kg⁻¹ (95% CL: 9.27 – 21.66); meperidine with maraviroc 19.20 mg kg⁻¹ (95% CL: 10.49 – 30.04); meperidine with AMD3100 18.46 mg kg⁻¹ (95% CL: 8.76 – 31.09); and meperidine with both CRAs, 6.57 mg kg⁻¹ (95% CL: 3.09 – 11.61). In contrast to oxycodone, combining meperidine with either CRA alone did not cause a significant shift in the meperidine dose-response curve. However, the combination of meperidine with both CRAs significantly shifted the meperidine dose-response 2.3-fold to the left (paired Student's t-test, * $p=0.014$).

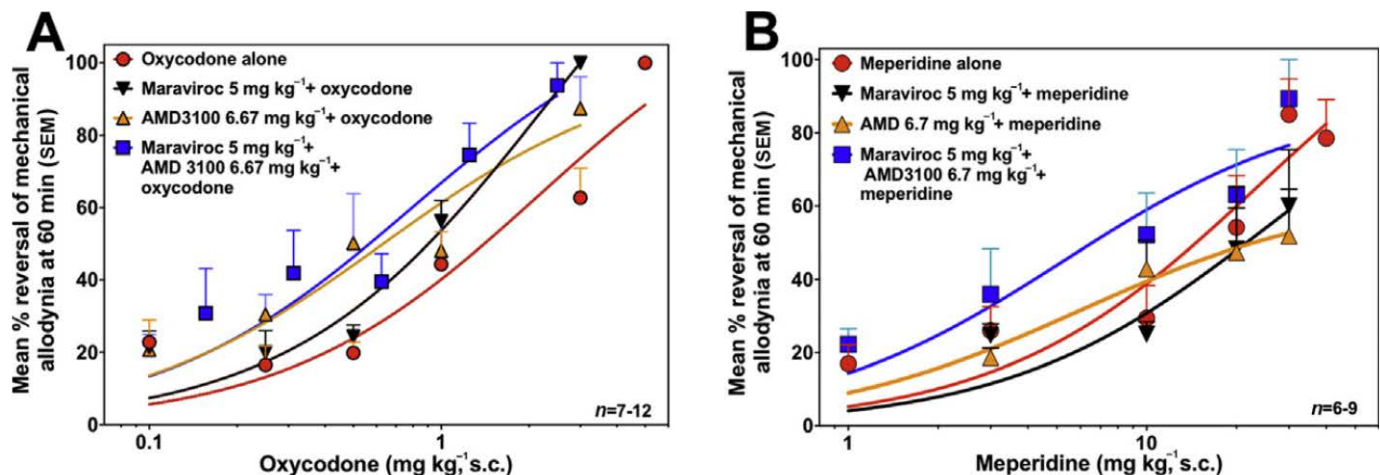


Fig 3. Dose-response curves for opioids alone and with chemokine receptor antagonists. (a) Combination of oxycodone with two chemokine receptor antagonists shifts the oxycodone dose-response curve to the left. Percent reversal of mechanical allodynia was calculated using data at 60 min after incision. (b) The combination of meperidine with two chemokine receptor antagonists shifts the meperidine dose-response curve to the left. Percent reversal of mechanical allodynia was calculated using data at 60 min after incision.

This study shows that simultaneous administration of one or two CRAs with oxycodone or meperidine enhances their analgesic potency on incisional pain in a manner similar to that previously reported for morphine. The differences between results obtained with morphine in contrast to oxycodone and meperidine might be due to affinity differences to mu opioid receptors among these opioids. Since oxycodone and meperidine have lower affinity for mu receptors than morphine, the effect of CRAs may be less robust. As all of these opioids may be used in the clinical setting, the extension of the work to show that the combination of submaximal analgesic doses of any of these opioids with CRAs results in enhanced analgesia, supports the viability of the approach.

Summary of Incisional Pain Studies: The work described above carried out under this grant establishes the validity of the hypothesis that combining submaximal analgesic doses of morphine or other opioid analgesics with one or two chemokine receptor antagonists (CRAs), results in maximal opioid analgesia. These studies provide an explanation for why patients with pain, which usually has an inflammatory component, can be refractory to opioid analgesia. The model shows that the chemokines released during the inflammation block signaling by the opioid receptors when they combine with morphine or another opioid analgesic that binds to the mu opioid receptor. This phenomenon is called “heterologous desensitization” between these two classes of G-protein-coupled receptors (GPCRs). What is not clear is how blocking just two of the chemokine receptors resulted in down-regulation of a wide panel of chemokines and cytokines in a manner that was not receptor specific. However, the major conclusion from this work is that combining an opioid at a reduced dose with one or more CRAs has the capacity to result in maximal analgesia. This strategy is thus “opioid sparing”, allowing use of less opioid, with potentially fewer side effects, such as respiratory depression, inhibition of GI transit, tolerance, and addictive potential.

2. Effect of Chemokine Receptor Antagonists (CRAs) alone and in combination with opioids using the rat cold-water tail flick (CWTF) test.

This second pain model uses a test that has been well established in the laboratory of Dr. Adler. A manuscript is in preparation showing the results of this test and of the formalin pain test in mice. As this work is not yet published, the methods and results are presented here in greater detail than what was given for the incisional pain test. The latency to flick the tail in cold water was used as the antinociceptive index, according to a standard protocol (Tiseo et al.1988. J. PET. 246:449) A 1:1 mix of ethylene glycol:water was maintained at -3°C in a circulating water bath. Rats were held over the bath with their tails submerged approximately halfway into the solution. All animals were tested at 60, 15 and 0 min before drug injection. For each animal, the first reading was discarded and the mean of the second and third readings was taken as the baseline value. A cutoff limit of 60 seconds was set to avoid damage to the tail. Rats were assigned to groups randomly. Experiments were carried out blind: the person scoring the test did not know whether the syringes contained the test compounds or vehicles. In these experiments, the CRAs, AMD3100 and maraviroc were used, as in the incisional pain test. In addition, two other CRAs with different receptors were also tested. AZD8797 binds to CX3CR1 and RS504393 binds to CCR2. These CRAs were injected by themselves or in combination with morphine. Animals were injected with either vehicle or different doses of morphine or CRA at different s.c. sites on the right and left dorsal flank areas. When morphine was combined with 2 CRAs, the CRA or vehicles were administered on either flank area and morphine or saline was given s.c. behind the neck. When combinations of 4 CRAs with morphine were used with sub-maximal doses of morphine, AMD3100 and maraviroc were given into separate flank areas, and RS504393 and AZD8797 were injected intraperitoneally (i.p.) into the right or left side of the cavity. Morphine was administered behind the neck. Control animals received only vehicle injections. Following injections, tail flick latencies were measured at 15, 30, 45, and 60 min by a person blinded to the treatments. The percent of maximal possible antinociception (% MPA) for each animal at each time was calculated using the following formula: $\% \text{MPA} = [(\text{test latency} - \text{baseline latency}) / (60 - \text{baseline latency})] \times 100$.

Statistical Method: The data are expressed as the mean +/- standard error. Statistical analysis of differences between groups was assessed using ANOVA, a linear (mixed) regression model for the bulk of the CWTF data, and linear regression analysis for the formalin data. Linear mixed models are an extension of ordinary regression models that allow one to properly account for the fact that multiple observations on the same experimental animal over time will be more correlated than observations across different animals

(Brown and Prescott, 1999; Duricki et al., 2016; Yoon et al., 2013). $P < 0.05$ was taken as the significant level of difference. Where appropriate, Sidak corrections for multiple comparisons are applied. Time is treated as a categorical variable throughout.

As in the incisional pain test, based on dose-response curves, none of the CRAs alone had analgesic activity (data not shown) in the CWTF test. In the CWTF test AMD3100 or maraviroc were each tested alone in combination with a suboptimal dose of morphine. As shown in Figure 3, combining AMD3100 with morphine (Fig. 3A), a significant effect on morphine-induced analgesia was not observed ($p = 0.21$). When a similar experiment was carried out combining morphine with maraviroc (Fig. 3B), and the results analyzed using a linear mixed model (dependent variable % MPA; independent variables morphine, maraviroc, the morphine-maraviroc interaction, and time (not shown)), a synergistic interaction of morphine and maraviroc ($F(1,68) = 4.56$, $p = 0.036$) with maximal analgesia peaking at 30 min ($F(3,213) = 34.9$, $p < 0.0001$) was identified.

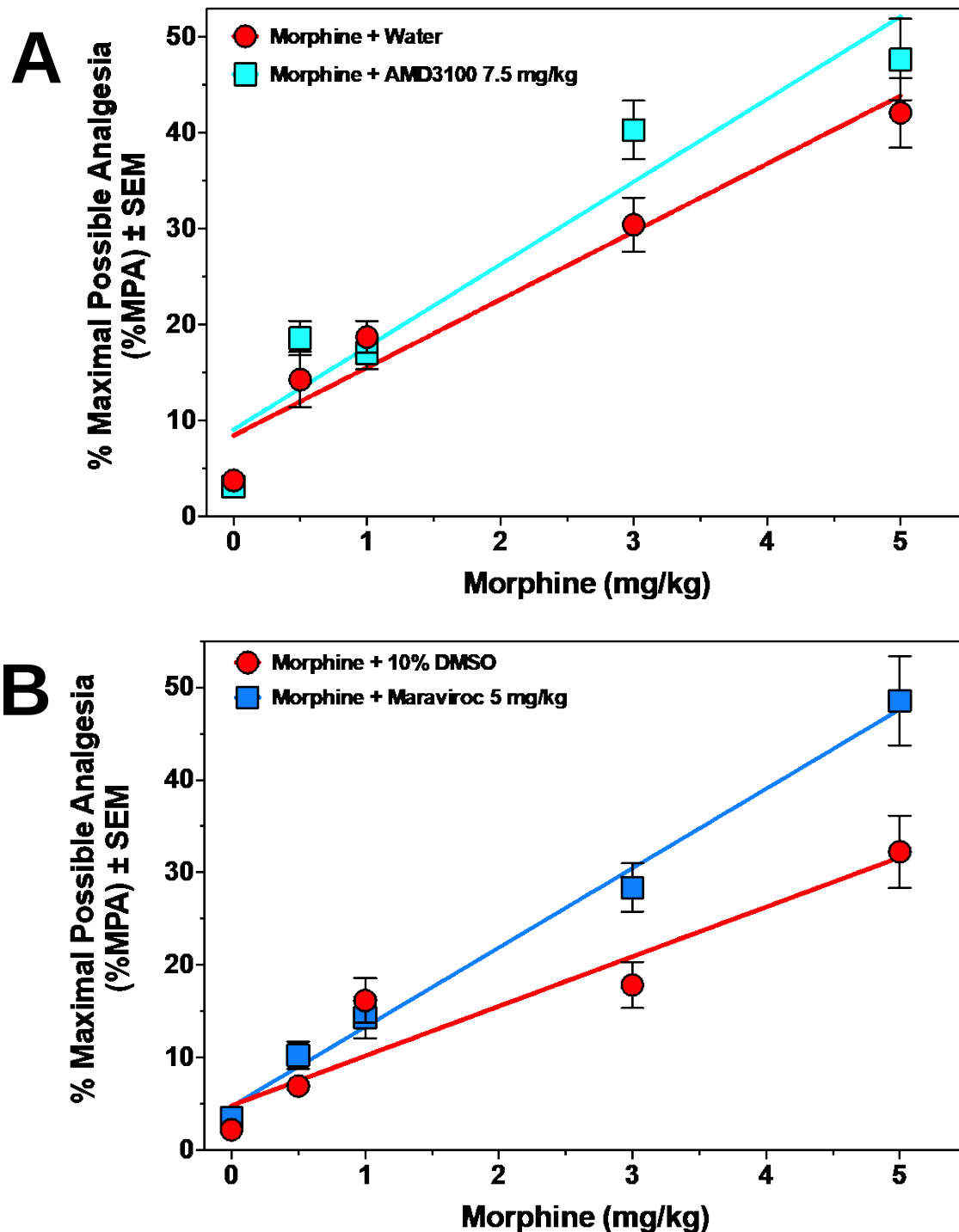


Figure 3. The effect of the combination of morphine plus maraviroc or AMD3100 in the CWFT test in rats. Panel A: Combining AMD3100 with morphine did not have an effect on morphine-induced analgesia ($p=0.21$). Panel B: Using linear mixed model (dependent variable % MPA; independent variables morphine, vehicle, maraviroc, and time), a synergistic interaction of morphine and maraviroc ($F(1,68)=4.56$, $p=.036$) was observed.

These findings were extended further by testing the combination of two CRAs, AMD3100 and maraviroc, with a submaximal analgesic dose of morphine in the CWTF test. Enhanced analgesia was observed compared to that for morphine alone. As shown in Figure 4, a synergistic interaction of morphine combined with the CRAs ($F(1,93)=5.61$, $p=0.02$), with maximal analgesia peaking at 30 min (time not shown, $F(1,288)=141.8$, $p<0.0001$), compared to morphine alone, was detected.

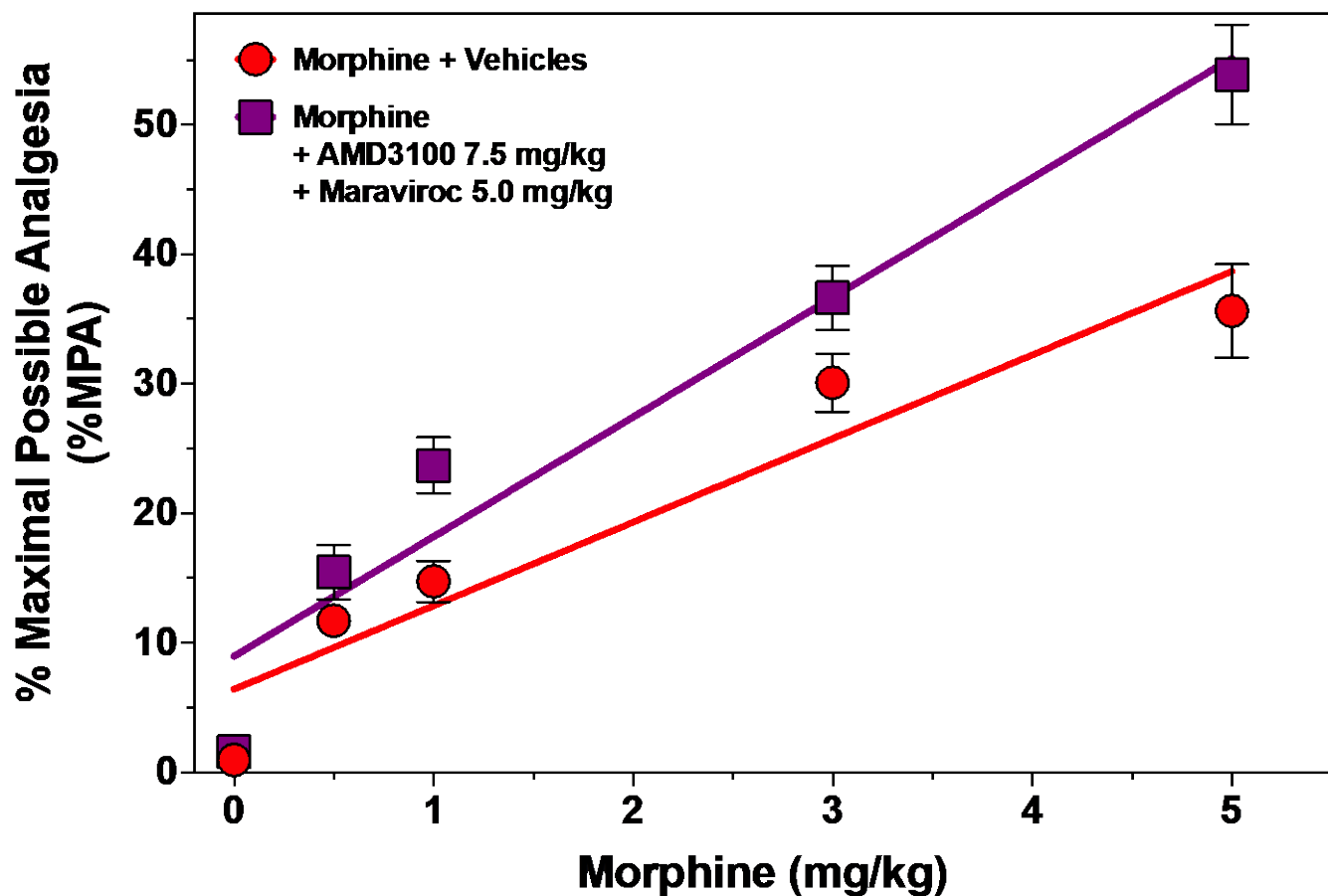


Figure 4. The effect of the combination of morphine plus both AMD3100 and maraviroc in the CWT test in rats. Using a linear mixed model (dependent variable % MPA; independent variables morphine, CRAs, the morphine-CRAs interaction, and time - not plotted), a synergistic interaction of morphine combined with the CRAs was found ($F(1,93)=5.61$, $p=0.02$).

It was decided to test the effect of using a combination of four different CRAs with morphine in the CWTF test. In this experiment, a single dose of morphine with intermediate efficacy was chosen, 3 mg/kg. Figure 5 presents the results showing the kinetic effect over 60 minutes. While the % MPA obtained using 3 mg/kg morphine alone was approximately 20%, combining the 3 mg/kg morphine dose with the 4 CRAs increased the % MPA to over 50%. Using a linear mixed model (dependent variable % MPA; independent variables morphine, CRAs, the morphine-CRAs interaction, and time, with a Toeplitz error structure), a synergistic interaction between morphine and the CRAs was observed ($F(1,35)=22.9$, $p<0.0001$), with maximal analgesia peaking between 30-45 min ($F(1,114)=27.5$, $p<0.0001$).

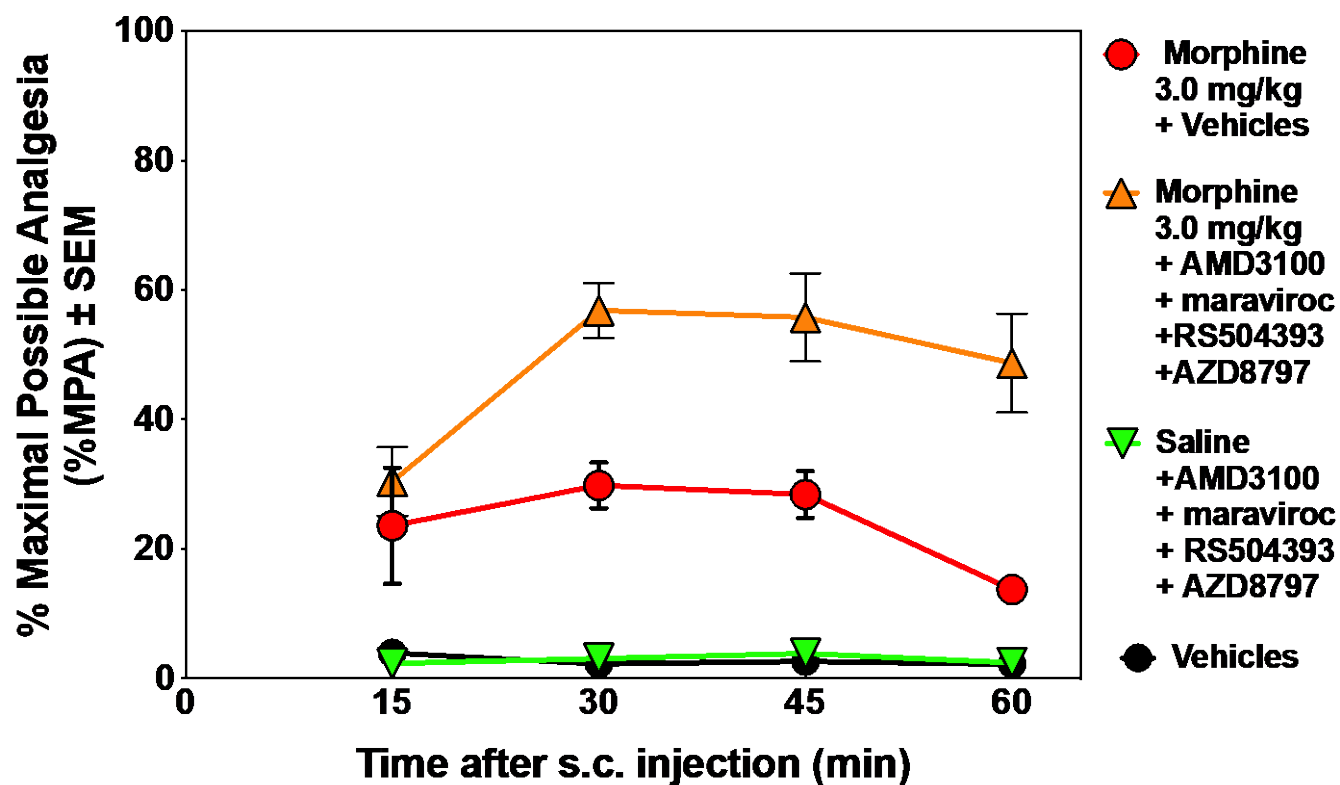


Figure 5. The effect of the combination of morphine (3.0 mg/kg) plus four CRAs: AMD3100 (7.5 mg/kg), maraviroc (5.0 mg/kg), RS504393 (1.0 mg/kg), and AZD8797 (1.0 mg/kg) in the CWT test in rats. Using a linear mixed model (dependent variable % MPA; independent variables morphine, 4CRAs, the morphine-4CRA interaction, and time, Toeplitz error structure), a synergistic interaction of morphine and CRAs ($F(1,35)=22.9$, $p<0.0001$) with maximal analgesia peaking between 30-45 min ($F(1,114)=27.5$, $p<0.0001$) was identified.

Summary of Cold-Water Tail Flick Test Study: The results above show that a single CRA (maraviroc), two CRAs or four CRAs, each targeting a different chemokine receptor, used in combination with submaximal doses of morphine, result in increased potency of morphine, as evidenced by greater analgesia in the CWTF test. The effects were morphine dose and time dependent.

3. Effect of Chemokine Receptor Antagonists (CRAs) alone and in combination with opioids using the mouse formalin test

The third pain test is a well-accepted model, carried out in mice, which involves the injection of formalin into the mouse foot. Mice were acclimated for 1 hr in the procedure room before taking any measurements. Animals were assigned to groups randomly. Experiments were carried out blind: the person scoring the test did not know whether the syringes contained the test compounds or vehicles. The standard formalin paw injection assay was used (Hunskar et al., 1985. J. Neuroscience Methods. 14:69; Hunskar and Hole, 1987. Pain. 30:103) in which a solution of 5% formalin prepared in 0.9 % saline (20 μ l) was injected into the dorsal surface of the left hind paw of Swiss-Webster mice (25-35 gram) to incite pain. To test the analgesic effect of morphine and CRAs, the drugs were administered just prior to injection of the formalin. Mice were then placed in a transparent box to observe the licking response. In this study, the second phase of licking in response to formalin, $t = +20$ min post injection was scored, which is considered a more chronic phase of the pain response. The duration time of formalin-injected paw licking was scored for the next 15 minutes (licking that occurred between 20 and 35 min post formalin injection) using a stopwatch by an observer blinded to the treatments. In these studies, responses of male and female mice were compared. The explanation of the statistical analysis used in the formalin test is given under the method for the CWTF test.

Figure 6 presents the effects of morphine, AMD3100, or maraviroc, each given alone, in male and female mice, on formalin-induced nociception. In this assay, the greater the licking time, the less the analgesia. Morphine induced antinociception in a dose-dependent manner in both male and female animals (Fig. 6A), however the opioid had greater potency in male mice (morphine-sex interaction $t(105) = 2.74$, $p = .007$). It was also observed that the overall licking response to the formalin injection was higher in female mice than in male mice $t(105) = 4.59$, $p < .0001$. Neither AMD3100 nor maraviroc had a significant effect on formalin-induced licking in either male or female mice (Fig. 6B and C).

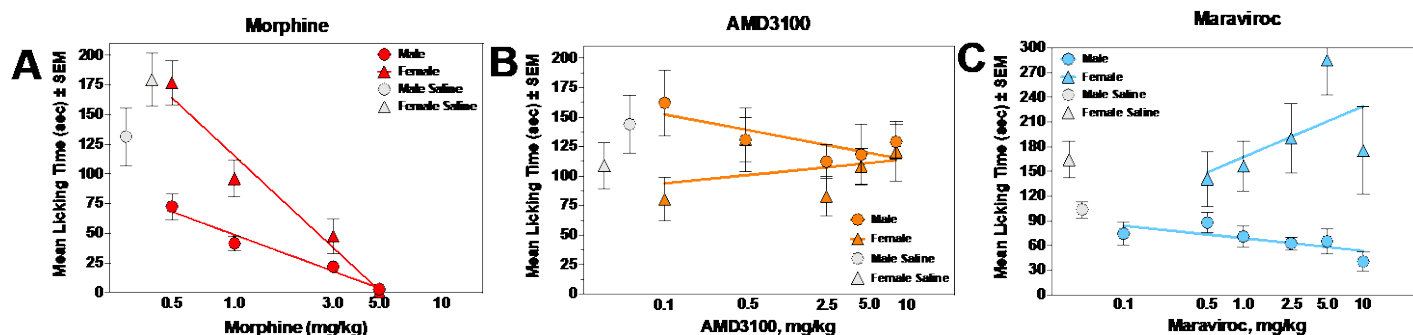
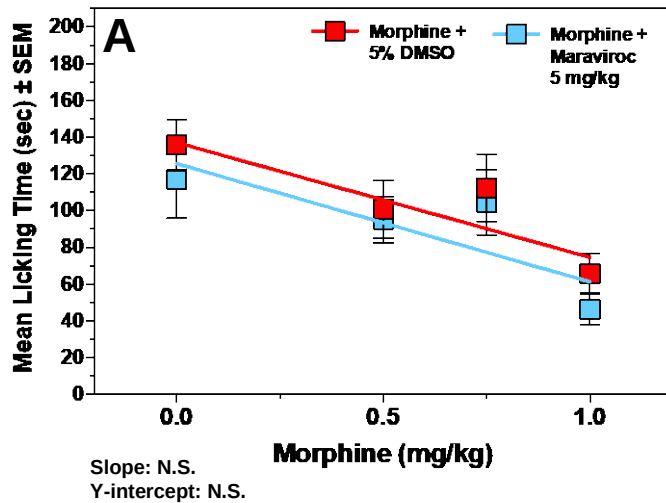


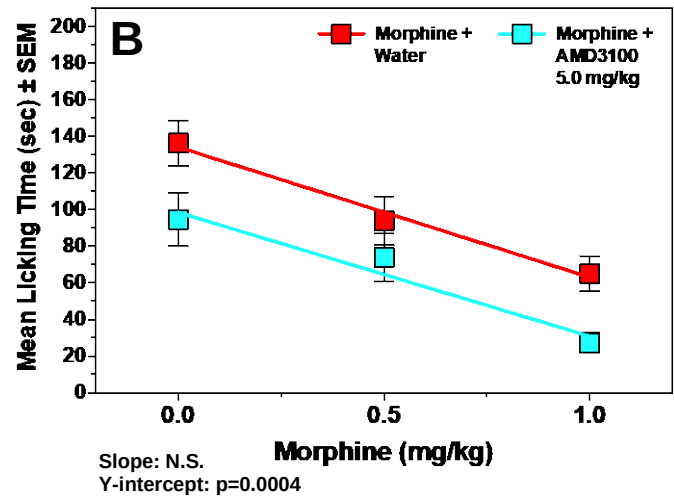
Fig. 6. Dose-response curves for morphine, AMD3100, and maraviroc in the formalin test in male and female mice. Panel A: Morphine induced antinociception in a dose-dependent manner in both male and female mice. Morphine showed greater potency in male mice (morphine-sex interaction $t(105) = 2.74$, $p = .007$). Higher baseline licking responses were observed in female mice than in male mice ($t(105) = 5.17$, $p < .0001$). Panels B and C: Neither AMD3100 nor maraviroc had an effect on formalin-induced licking in either male or female mice.

The combination of a sub-analgesic dose of morphine with a single CRA, either maraviroc or AMD3100 was also assessed in the formalin test. Figure 7A shows the pooled results for male and female mice of morphine at three different submaximal analgesic doses in combination with maraviroc. A significant effect of morphine was found ($t(248) = -3.41$, $p = .0008$) as expected, but there was no significant effect of maraviroc ($p = .22$), nor was there a significant interaction (change in slope of morphine response between morphine and morphine + maraviroc, $p = 0.95$). Similar results were observed when the data were broken out to show effects in male and female mice (Fig. 7C and D). When morphine at two different submaximal analgesic doses was tested in combination with AMD3100 (Figure 7B), significant effects of morphine ($t(215) = -4.73$, $p < .0001$) and AMD3100 ($t(215) = -4.32$, $p < .0001$) were detected, but the morphine plus AMD3100 interaction was not significant ($p = .59$), so the effect was additive rather than affecting the slope. When analyzed separately, both male and female mice replicated the overall pattern (Fig. 7C-F). In male mice, significant effects of morphine ($t(102) = -2.61$, $p = .01$) and AMD3100 ($t(102) = -4.00$, $p = .0001$) were observed, and the interaction was n.s. ($p = .59$); in female mice, significant effects of morphine ($t(108) = -4.81$, $p < .0001$) and AMD3100 ($t(108) = -2.87$, $p = .005$) were observed, but the interaction was also n.s. ($p = .84$).

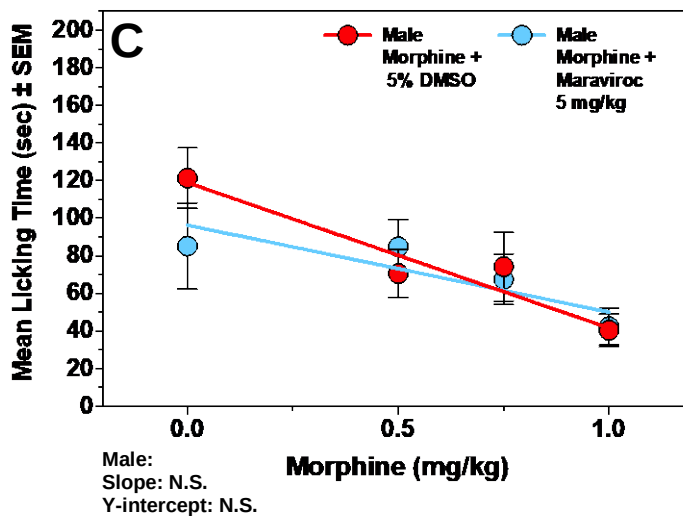
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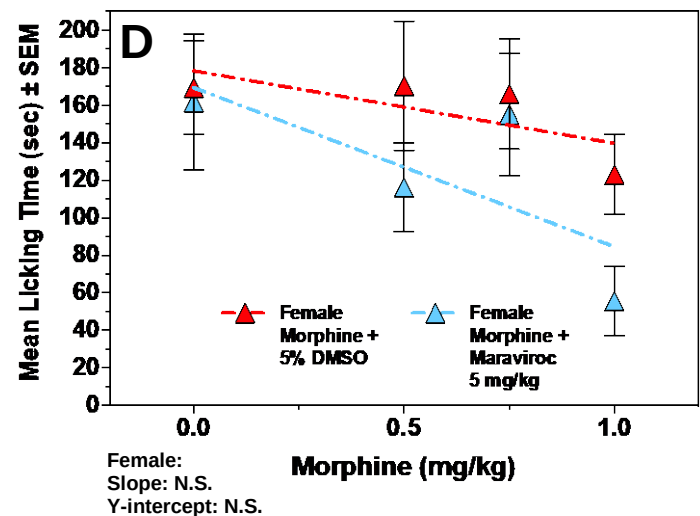
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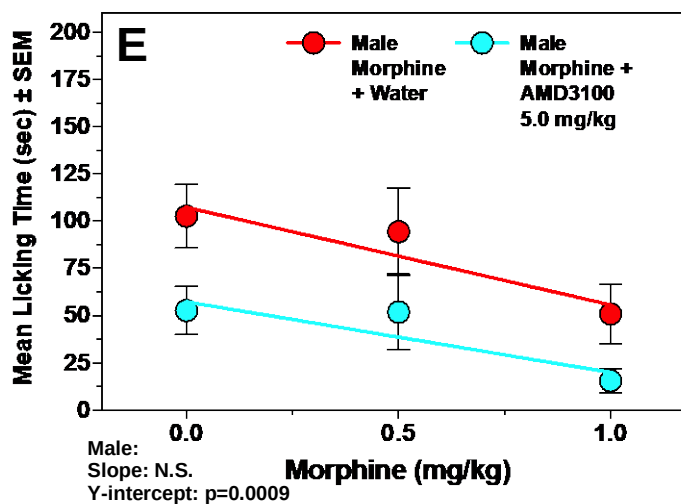
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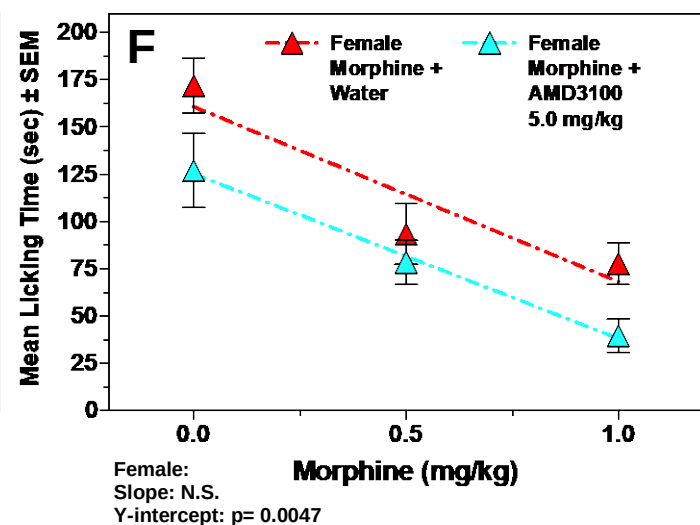


Figure 7. Combination of a submaximal analgesic dose of morphine with either maraviroc or AMD3100 overall and separately in male and female mice. Panel A: Submaximal analgesic dose of morphine

combined with Maraviroc produced a significant effect of morphine ($t(248) = -3.41, p=.0008$), but no effect of maraviroc ($p=.22$) nor interaction. Panel B: Submaximal analgesic dose of morphine combined with AMD3100 resulted in significant additive effects of morphine ($t(215) = -4.73, p<.0001$) and AMD3100 ($t(215) = -4.32, p<.0001$), with no interaction. Panel C and E: No effects of morphine or differential effects were found with respect to sex. Panels D and F: The overall pattern from Panel B replicated for both male and female mice. Males (Panel D, morphine $t(102) = -2.61, p<.01$, AMD3100 $t(102) = -4.00, p=.0001$) and Females (Panel F, $t(108) = -4.81, p<.0001$, AMD3100 $t(108) = -2.87, p=.005$).

In Figure 8, the combination of the 4 CRAs with morphine (1 mg/kg) significantly decreased licking time compared to morphine alone. A two-way ANOVA was estimated with total licking time as the dependent variable and morphine and 4CRAs, and the morphine/4CRA interaction as independent effects. Morphine significantly decreased licking as expected ($t(66) = -3.65, p=.0005$) and the interaction between morphine and the 4 CRAs was significant ($t(66) = -2.11, p=.039$). Based on our prior findings, we explored differential effects of sex on morphine and morphine/CRA response, and found no evidence for sex-related differences, so the responses of male and female animals were combined in the bar graph.

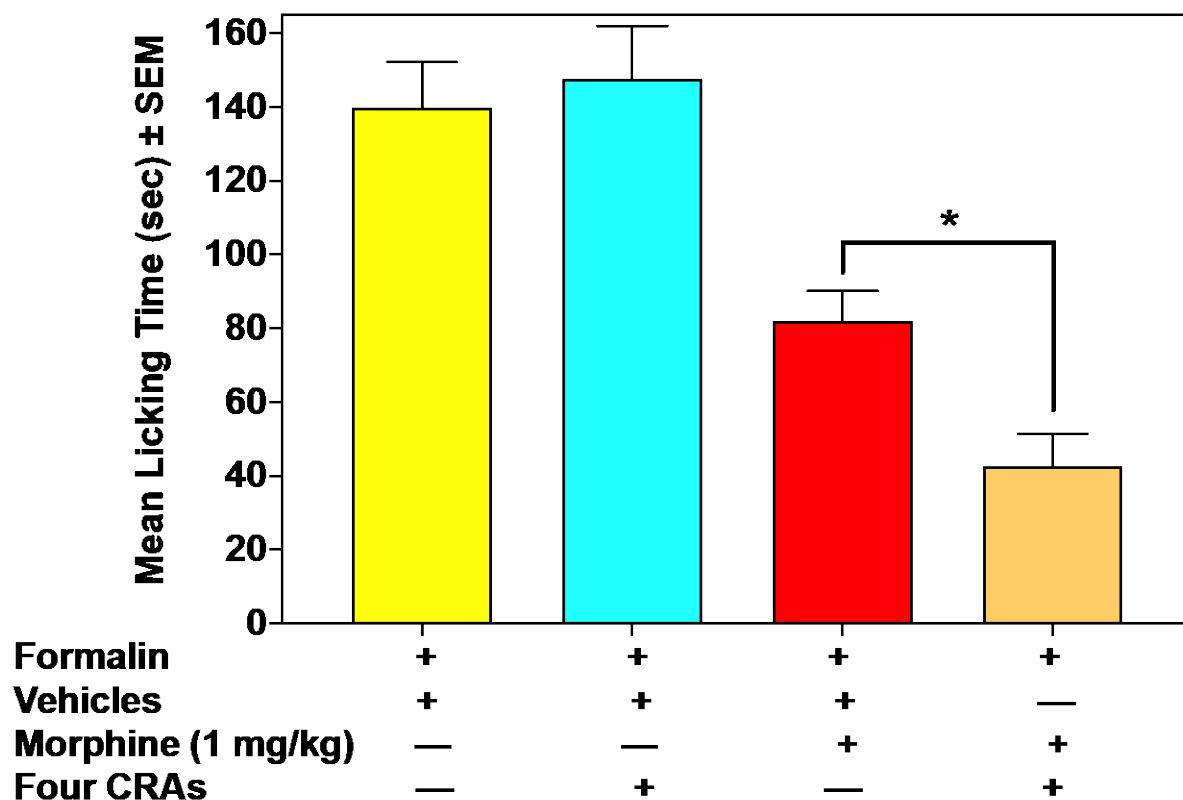


Fig. 8. Combination of 4CRAs with morphine (1 mg/kg). Two-way ANOVA was estimated with total licking as the dependent variable and morphine, 4CRAs, and the morphine/4CRAs interaction as independent effects. Morphine significantly decreased licking as expected ($F(1,66) = -3.65, p=.0005$) and the morphine/4CRAs interaction was significant ($t(66) = -2.11, p=.039$).

Summary of the formalin test data: The results for the formalin test show that submaximal analgesic doses of morphine in combination with AMD3100 produced an additive analgesic effect. Maraviroc did not produce a similar additive effect. The combination of four CRAs with morphine resulted in a significant increased potency of morphine. Comparing male and female mice, it was found that morphine had greater potency in male mice, which was superimposed on an overall greater licking response to the formalin injection in female mice. AMD3100 in combination with morphine produced an increase in the opioid potency in both male and female mice. No effect of the combination of morphine and maraviroc was observed in male or female mice. A manuscript describing the results for the CWTF test and the formalin test is in preparation.

4. Effect of Chemokine Receptor Antagonists (CRAs) alone and in combination with opioids using the rat carrageenan test

Two different methods were used to assess the efficacy of CRAs in carrageenan-induced pain. In both methods, carrageenan is injected into the plantar surface of the rat paw, and paw withdrawal latencies are measured using noxious heat from an infrared beam focused onto the plantar surface of paws in unrestrained rats using a Hargreaves' apparatus (Ugo Basile, Comerio, Italy). In the first method (Hargreaves et al., Pain. 1988. 32:77), after post-carrageenan baseline withdrawal latencies were taken, rats received a s.c. injection of the CRA(s), and then 20 min later received morphine. Thirty minutes later, paw withdrawal latencies were again measured. Results using this assay did not show an enhanced analgesic effect of morphine when combined with AMD3100, maraviroc, or the combination of the two CRAs (data not shown). It was decided to try a new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia (Kolosov et al., Pain Medicine. 2010. 11:106). In this second method, morphine, with or without addition of CRAs, was given two hr after the carrageenan injection into the paw of the rat. In more detail, the assay was carried out as follows. Rats were acclimated in a plexiglass box in the behavioral testing room for 1 hr. Baseline paw withdrawal latencies were measured for each paw three times in 5 min. Then, 100 µl of 2% carrageenan prepared in saline was injected into the plantar side of the left hind paw under 4% isoflurane anesthesia. As a control, saline (100 µl) was similarly injected into the right paw. Paw withdrawal latencies to heat were then measured at 2 and 3 hr post-carrageenan injection. Preliminary experiments established that the 2-hr time point was sufficient to demonstrate measurable decreased paw withdrawal latencies. Percent mean possible analgesia at the 2-hr time point was calculated using the formula:

% Maximal possible analgesia = [(Test latency at 10, 60, or 90 min – control latency)/(22 – control latency)]x100 (22 seconds = cut off time)

The results are shown in Figure 9 for the combination of AMD3100 and maraviroc with various doses of morphine.

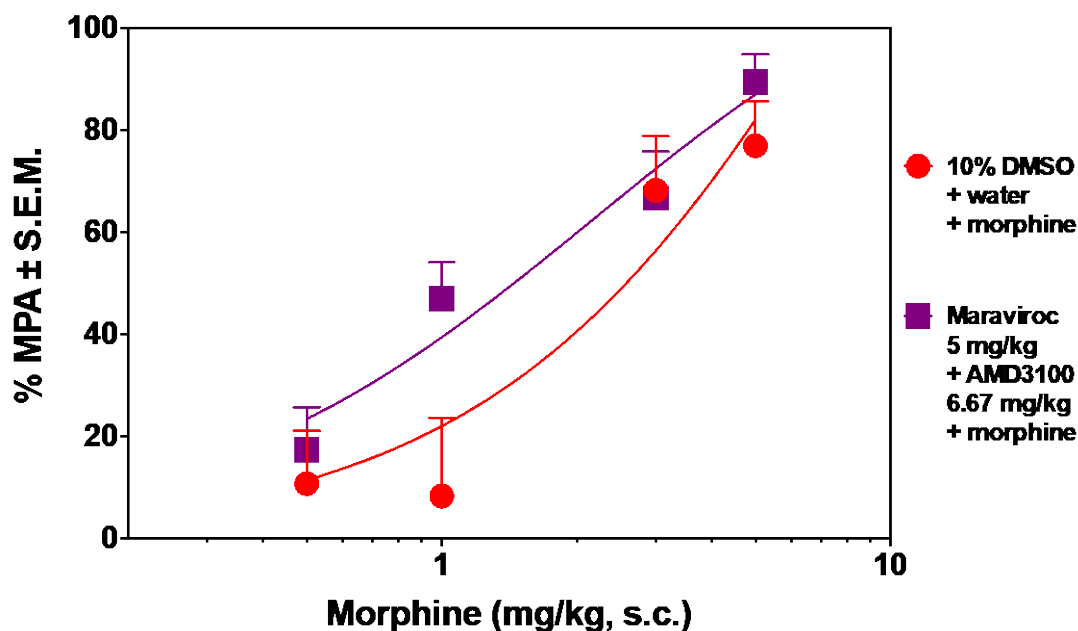


Fig. 9: Comparison of analgesia induced by various doses of morphine alone or in combination with AMD3100 and maraviroc in the carrageenan pain assay. The differences in the two curves were not statistically significant by the paired one-tailed Students' t-test.

Using this paradigm, the ability of the combination of maraviroc and AMD3100 to enhance the analgesic activity of a suboptimal dose of morphine was tested. The results (Fig.9) show that the ED₅₀ of morphine in this assay was 2.57 mg/kg, and it was reduced 1.7-fold with the addition of the CRAs. While there was a positive

trend towards enhanced analgesia with the addition of the CRAs, the difference compared to morphine alone was not significant.

Figure 10 shows the results of an RT-PCR array on tissues from the draining popliteal lymph node of the paw that was injected with carrageenan, and rats that received the various treatments shown in Figure 9.

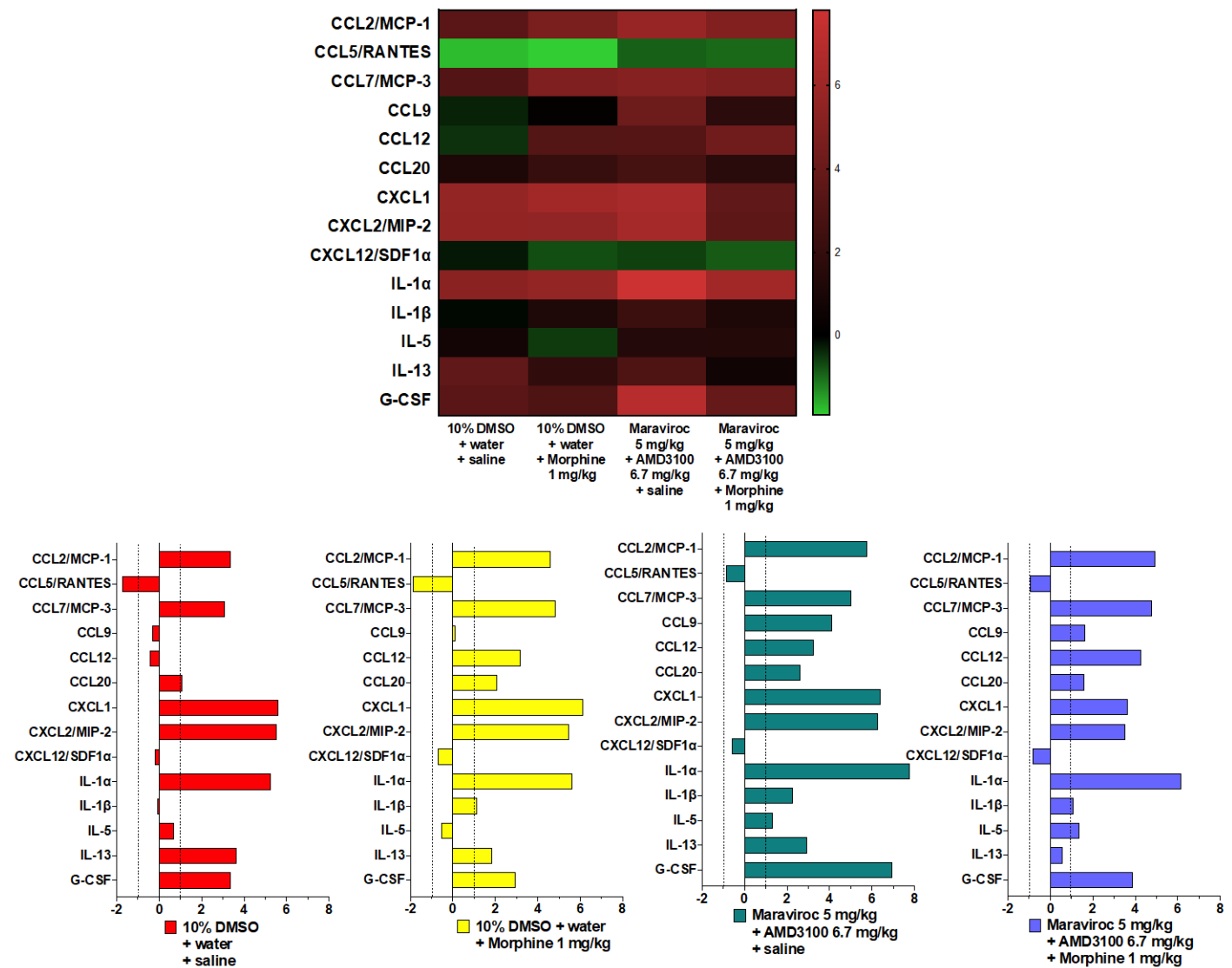


Fig. 10. Effect of carrageenan alone and of treatment with morphine alone, AMD3100 + maraviroc alone, or with morphine + AMD3100 + maraviroc on mRNA levels for a panel of chemokines and cytokines. The draining popliteal lymph node was harvested 1 hr post incision and was extracted and processed to obtain mRNA, which was analyzed using the RT² Profiler Array (see Methods). In both panels A and B changes in selected mediators are shown. Many mediators had minimal change and are omitted. Panel A: mRNA expression heat map of the popliteal lymph nodes of rats receiving carrageenan with vehicle controls or with treatments. Treatments are indicated at the bottom of each column. Color scale is a representation of the log₂ fold change in mRNA levels of various immune mediators compared with untreated baseline lymph nodes, ranging from bright red (upregulated) to bright green (downregulated). Black represents 0-fold change in mRNA expression. Panel B: Fold change of mRNA expression in popliteal lymph nodes of rats receiving carrageenan with and without treatments. The data represent the log₂ fold change in mRNA expression for a panel of immune mediators post incision, with or without treatments, compared with untreated, baseline popliteal lymph nodes. Dotted lines indicate limits of 2-fold up- or downregulation from baseline.

Interestingly, as in the incisional pain assay, the carrageenan insult did not upregulate CCR5 or CXCL12, the ligands for the receptors that are the targets of maraviroc and AMD3100. Several of the other immune mediators were increased at least 4-fold. The combination of morphine plus the two CRAs did not have a significant effect on production of the mediators which were upregulated by the carrageenan insult.

Summary of the carrageenan test data: Thus, in two different methods of testing analgesia using carrageen injection into the rat paw as the pain insult, no significant increase in analgesia was observed by adding CRAs to morphine. The immune assays did not show an effect of the CRAs in downregulating immune mediators that were upregulated by the carrageenan injection.

5. Effect of Chemokine Receptor Antagonists (CRAs) alone and in combination with opioids using the rat paclitaxel neuropathic pain assay

These experiments were begun by establishing the paclitaxel pain model in rats. We followed the method of Bennett (Xiao, W.H. et al. 2011. Neuroscience 199:461), which is widely referenced in the literature. Paclitaxel was obtained from the Temple University Hospital Pharmacy. It is the formulation used for chemotherapy in humans. The drug is suspended in a solution of cremophor and ethanol at a 1:1 ratio. The pain endpoint for this model is based on measurements of mechanical allodynia using Von Frey filaments. Animals were acclimated to the chambers where measurements were taken for 2 hours before the first paclitaxel injection. Animals were poked on the plantar surface of the left rear paw using filaments of increasing weight. The filament weight that caused paw withdrawal was scored. Following establishment of the baseline values, rats were given their first intraperitoneal injection of paclitaxel at 2 mg/kg on day 0. On days 2, 4, and 6 after the baseline day, they received additional injections. Rats were then tested for mechanical allodynia on days 9, 14, 21, and 28 after baseline measurements. As shown in Fig. 11, maximal allodynia was observed on day 21 after initiation of paclitaxel injections. Day 21 was, therefore, used as the time for further measurements on efficacy of pain therapies.

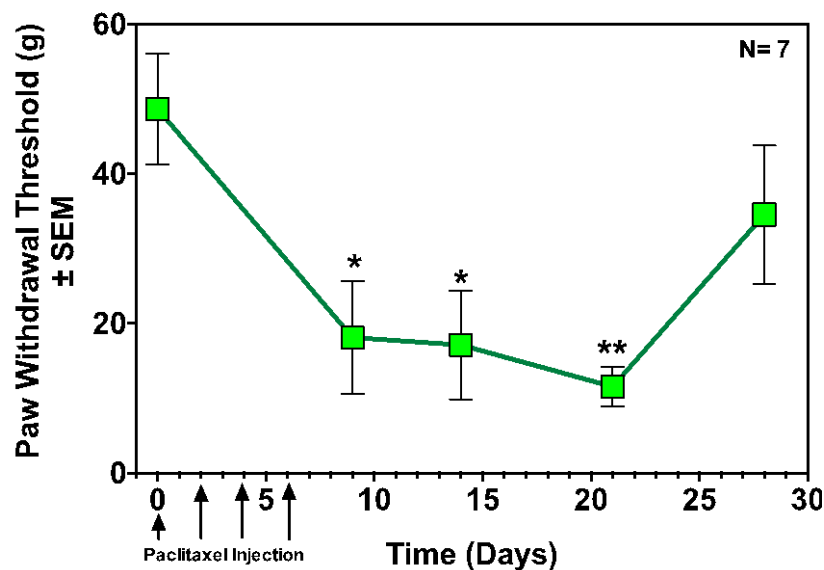


Figure 11. Time-course of development of mechanical allodynia following paclitaxel administration. Rats were given paclitaxel i.p. on days 0, 2, 4, and 6 after baseline measurements were taken on day 0. Mechanical allodynia was measured using Von Frey filaments of graded weights. One-way ANOVA followed by Dunnett's multiple comparison test revealed that significant pain was observed on days 9, 14, and 21 post baseline measurements and compared to baseline (day 0). $p < 0.05$ *, $p < 0.01$ **

A morphine dose-response curve was established in this assay as shown in Fig. 12. One hundred percent maximal possible analgesia was observed in rats given 10mg/kg. Doses of 5.0 mg/kg and 3.0 mg/kg produced 50 to 60% analgesia, and 1 mg/kg gave approximately 40% analgesia.

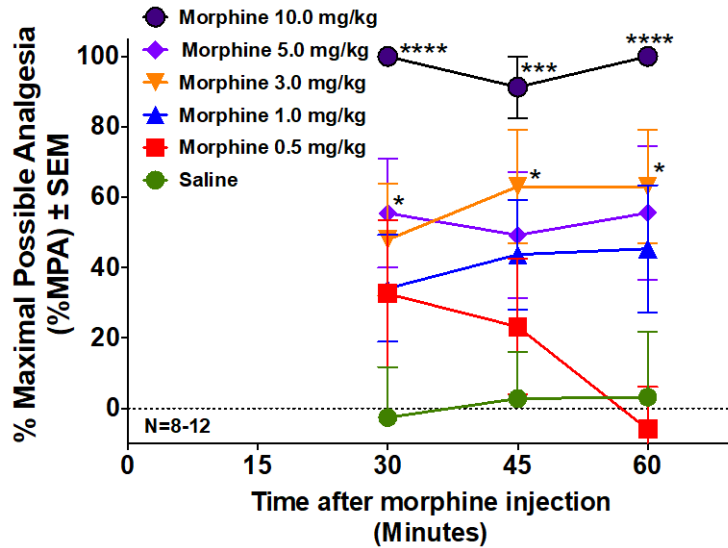


Fig. 12. Effect of different doses of morphine on paclitaxel-induced mechanical pain. Data were analyzed by two-way ANOVA followed by Dunnett's multiple comparison test. Compared to the saline control, * $p < .05$, *** $p < 0.001$, **** $p < 0.0001$.

AMD3100 (0.5 mg/kg to 10.0 mg/kg) and maraviroc (0.5 mg/kg to 5.0 mg/kg) were each tested over a range of doses and found to have no effect by themselves on paclitaxel-induced pain (data not shown). When each of these CRAs was combined with morphine at different doses, no significant enhancement of morphine analgesia was observed (Fig. 13A and B).

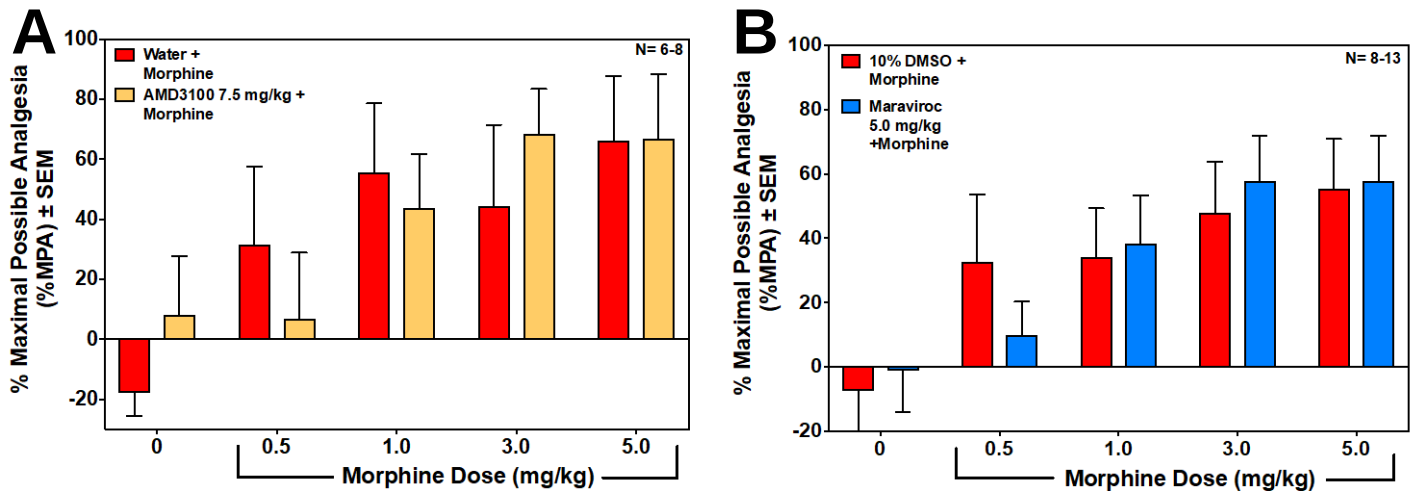


Fig. 13. Comparison of analgesic responses induced by morphine alone (0.5 to 5.0 mg/kg) with morphine plus AMD3100 or maraviroc in neuropathic pain induced by paclitaxel in rats. Pain was assessed using Von Frey filaments at 60 minutes post injection of morphine or morphine plus AMD3100 or plus maraviroc. Panel A: morphine plus AMD3100. Panel B: morphine plus maraviroc. No significant differences were seen between morphine alone and morphine plus AMD3100 or plus maraviroc by two-way ANOVA.

As the paclitaxel assay requires one month to obtain results, it was decided to move directly to testing a combination of 4 CRAs to potentially save time and animals. If this strategy were successful, it was then planned to work backwards and see the minimal number of CRAs that were effective. In the experiment shown in Figure 14, morphine was used alone at 3.0 mg/kg, and in combination with 4 CRAs: AMD3100 (5.0 mg/kg, s.c.) maraviroc (5.0 mg/kg, s.c.), AZD8797 (1.0 mg/kg, i.p.), and RS504393 (1.0 mg/kg, i.p). These CRAs targeted the following chemokine receptors, respectively, CXCR4, CCR5, CX3CR1, and CCR2, and are the same ones we used in the cold-water tail flick test assay.

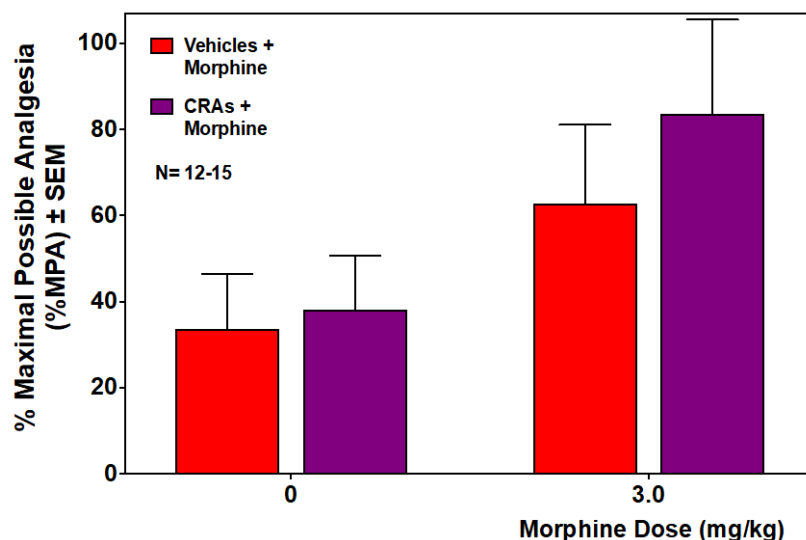


Fig. 14. Comparison of analgesic responses induced by morphine alone with morphine plus 4 CRAs in neuropathic pain induced by paclitaxel in rats. Pain was assessed using Von Frey filaments at 60 minutes post injection of morphine or morphine plus maraviroc. Differences between morphine alone and morphine plus 4 CRAs not significant by two-way ANOVA.

As shown in Figure 14, the combination of the 4 CRAs with morphine did not enhance the analgesic effect of morphine alone in this paclitaxel experiment.

In studies measuring the levels of chemokines, cytokines and other mediators in the incisional pain assay, the carrageenan assay and the formalin assay, the draining popliteal lymph node was used as the source of the tissue to carry out mRNA arrays. In the paclitaxel assay, there is no wound from which to collect a draining node. It was decided to use spleen and serum to look for changes in a spectrum of mediators. Figure 15 shows the results of the mRNA array using samples collected on day 21 at the conclusion of the pain experiment shown in Figure 14 only for paclitaxel plus vehicle compared to paclitaxel plus morphine. The only mediators that were increased greater than 2-fold by paclitaxel were CXCL2, CCL20, CCL22 and IL-21. CCL2, CCL17, CCL19 and CCL21 were downregulated by morphine treatment of the paclitaxel animals. However, none of these results were robust. Serum is not as amenable to analysis for mRNA levels as is tissue. Therefore, the Proteome Profiler Array (R&D Systems, Minneapolis, MN) was used to measure changes in protein levels of 10 chemokines, 10 cytokines, and 9 other mediators in serum (Fig. 16).

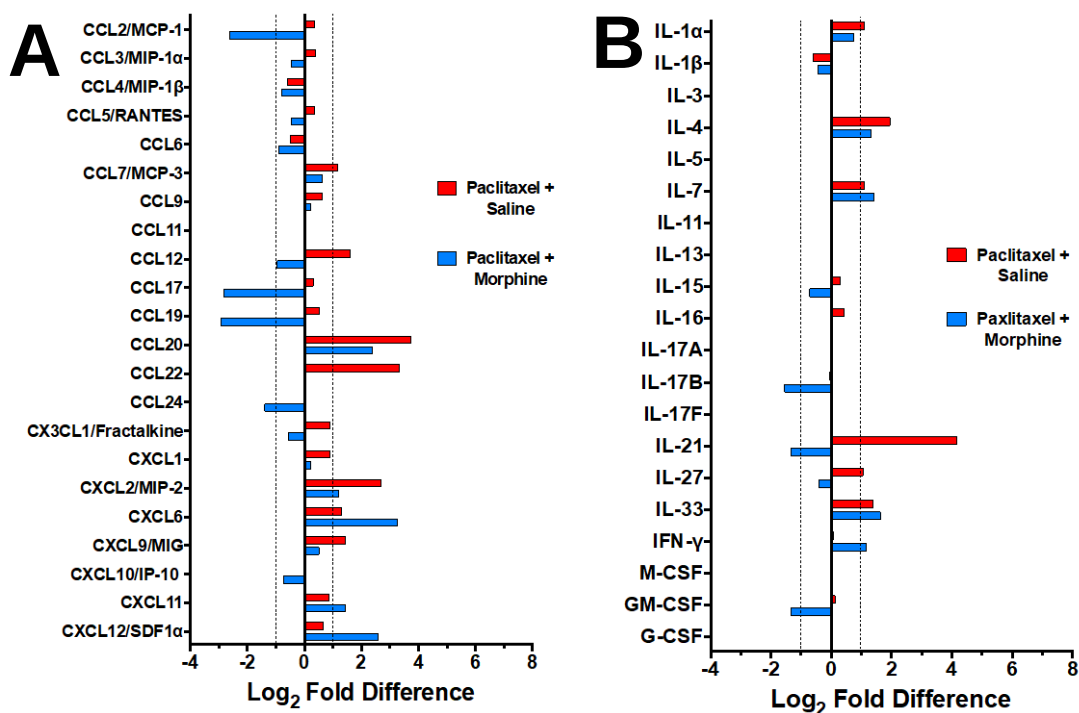
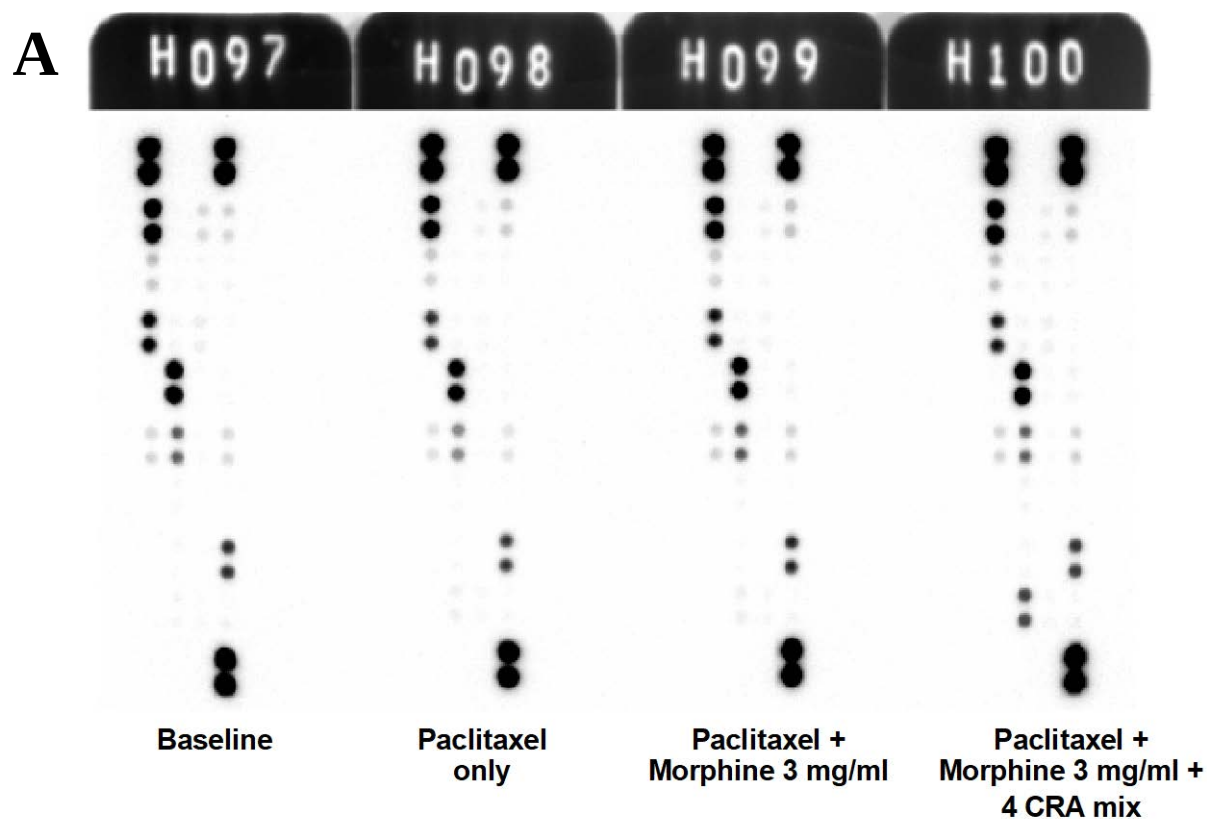
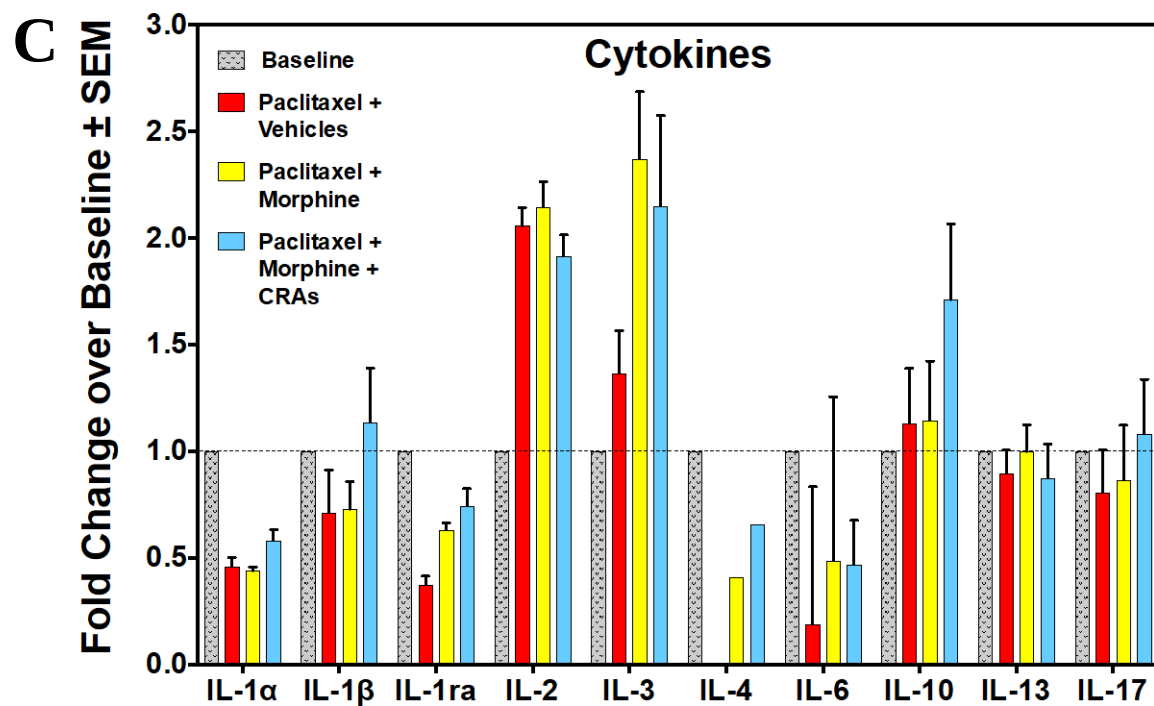
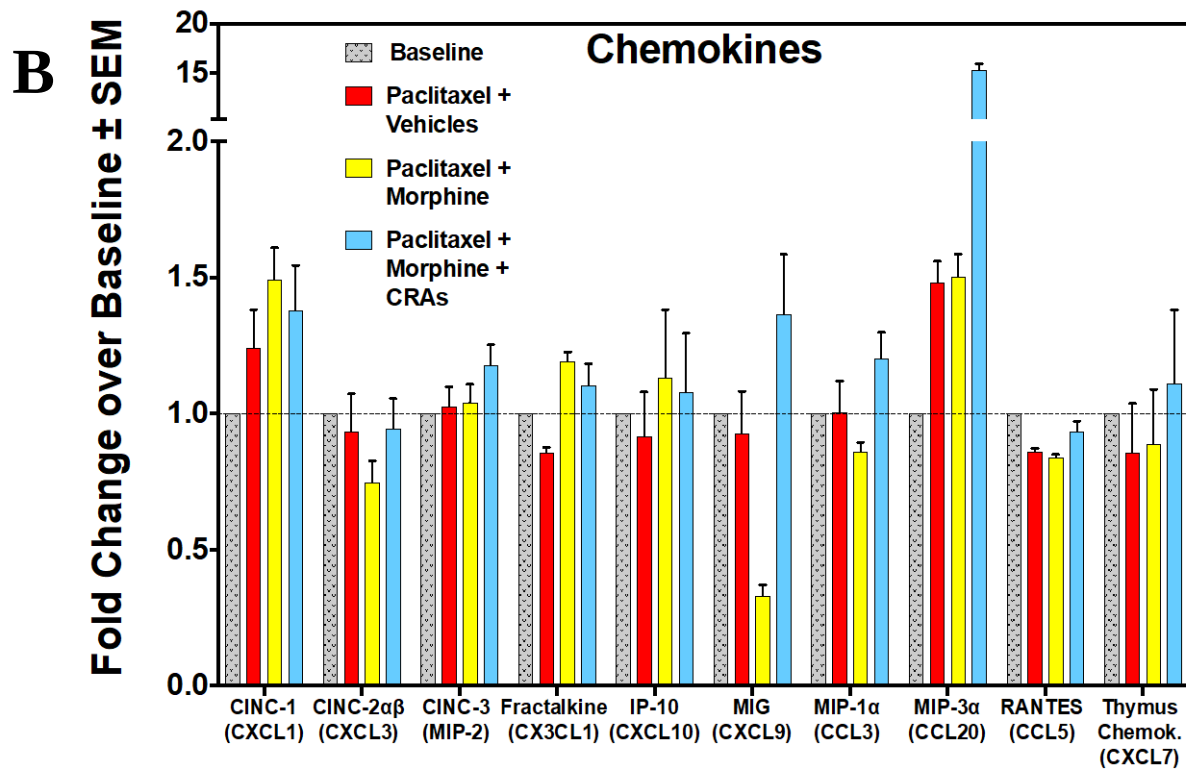


Figure 15. Fold change in levels of mRNA for a panel of chemokines (panel A) and cytokines (panel B) in spleens of rats harvested 21 days after administration of paclitaxel to induce neuropathic pain. Data are log₂ of fold change in gene expression compared with untreated baseline spleen levels. Dotted lines indicate limits of 2-fold up- or downregulation from baseline.





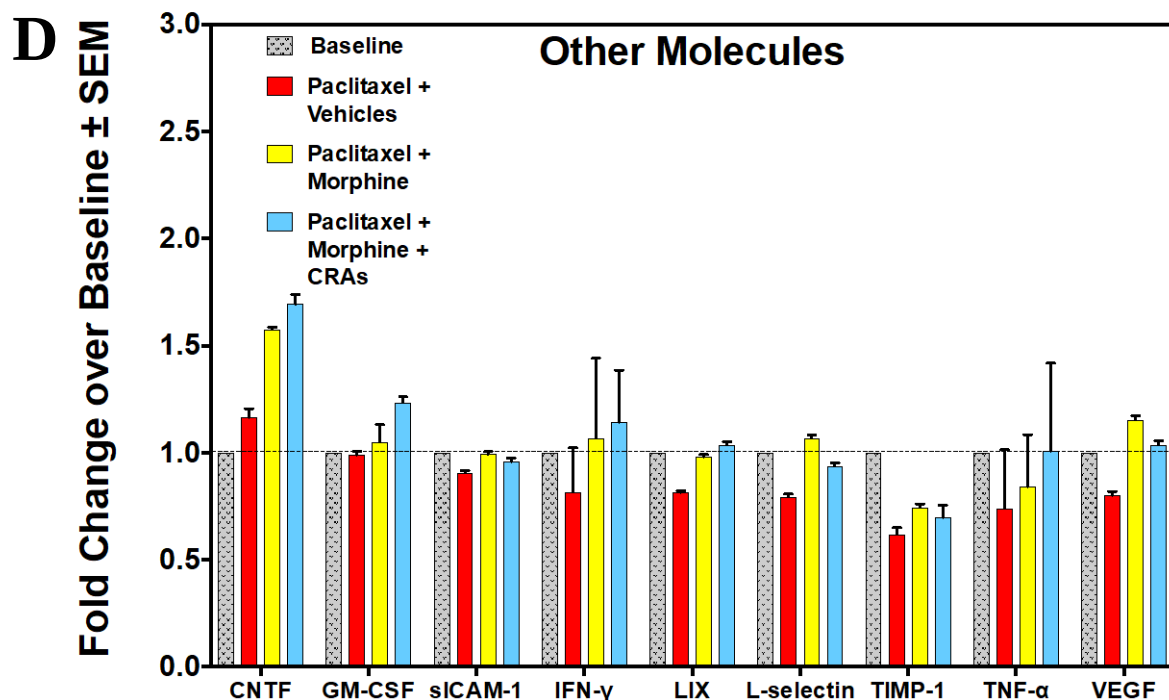


Figure 16. Levels of chemokines and other mediators in serum of rats given paclitaxel for 21 days and treated with morphine alone or morphine plus 4 CRAs. Each mediator was tested in duplicate using pooled serum from 4 to 7 rats. Results were averaged to obtain values shown in panels B-D. Panel A: Digital camera image of array membrane chemiluminescence. Panels B-D: Levels of mediators at baseline (animals with no paclitaxel treatment) were given a value of 1.0. Fold change over baseline was calculated for other groups. CRAs were AMD3100, maraviroc, AZD8797, and RS504393. Data are mean fold change over baseline $\pm$ SEM.

At 21 days post paclitaxel the only mediator with greater than a 2-fold change following paclitaxel treatment (red bars) was IL-2, a T-cell growth factor. Morphine in paclitaxel-treated rats had little effect on any of the mediators except IL-3, where the level was 2-fold higher than with paclitaxel alone, a weak response. The combination of morphine with the 4 CRAs in the paclitaxel-treated animals markedly increased CCL20, 15-fold above the levels observed with paclitaxel alone or paclitaxel plus morphine. The significance of this finding is not readily obvious, as the 4 CRAs used in the pain test did not target CCR6, the receptor for CCL20.

Summary of the paclitaxel test data: No significant increase in analgesia was observed by adding either AMD3100 or maraviroc each alone to morphine in the paclitaxel test. No significant increase in analgesia was observed by adding four CRAs to morphine in this test. Studies of immune mediators showed that, in aggregate, paclitaxel treatment, at the time point that showed maximal neuropathic pain, did not result in a robust inflammatory response.

What opportunities for training and professional development has the project provided?

In addition to training laboratory personnel for the specific tasks required to conduct the research for this project, laboratory personnel were part of the group that met every 2 weeks to discuss results, progress, and plans for the next phases of the project, including specific experiments planned. Those in charge of each experiment presented the results to the group. Everything was discussed openly, including new ideas and interpretations of experimental results. Preparation of presentations for meetings were also reviewed by all of the members of the research team. Papers published by other investigators that were pertinent to this project were frequently reviewed at the biweekly meetings, thereby educating everyone about cutting-edge developments in the pain field, and especially anything pertaining to immune responses and pain. This inclusionary strategy was essentially a tutorial for younger members of the team in terms of analysis of results,

new procedures, implications of the experiments, considerations of procedures that can confound interpretation of results, and proper application of statistics. In addition, all members of the team contributed ideas and information that was included in the quarterly and annual reports.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report (Final Report)

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The results obtained in this project have theoretical and practical implications in the field of pain management. The results show that chemokines produced during inflammatory pain can block the analgesic activity of opioids, and that chemokine receptor antagonists (CRAs) can prevent or unblock the anti-analgesic activity of these immune mediators. The robust finding that the combination of a suboptimal dose of morphine with two chemokine receptor antagonists (CRAs), maraviroc and AMD3100, is able to provide near-maximal analgesia in the incisional pain assay in rats, and to shift the morphine dose-response curve significantly to the left (3.3-fold) is a novel observation in the pain field. The fact that similar results were obtained using oxycodone and meperidine shows that this strategy is applicable to opioid analgesics as a class of drugs. The fact that significant potentiation of morphine analgesia was also obtained in two other pain tests, the cold-water tail flick test in rats and the formalin pain test in mice, shows that the results are generalizable to several different types of pain. In all of these studies the CRAs were given peripherally. The translational implication of these results is that use of a CRA in combination with morphine for treatment of certain types of pain may significantly reduce the dose of morphine required. Such combinations could lead to combination therapies that are “opioid sparing” but still able to provide optimal analgesia. This “opioid sparing” should lead to prescribing (and treating) severe pain with lower doses of opioids and thus contribute to aiding the current opioid crisis.

What was the impact on other disciplines?

The results showing interactions between chemokines and opioids in pain perception adds to a small literature implicating these mediators in pain pathways. Basic research on the mechanisms by which these molecules interact to result in analgesia could open new frontiers in the understanding of pain pathways and analgesic mechanisms, as well as in the development of new molecules to alleviate pain. The finding that use of chemokine receptor antagonists in pain assays downregulates mRNA for a broad panel of immune mediators is a novel finding and one for which a theoretical explanation needs to be sought.

What was the impact on technology transfer?

The findings were published and more are being prepared for publication. As some of the CRAs are FDA-approved (AMD3100 and maraviroc), interested parties, including the government, the armed forces or industry, could move forward with translation of these findings into clinical trials.

What was the impact on society beyond science and technology?

If these studies lead to a way to reduce the doses of opioids needed for optimal analgesia, there is the potential to make an impact on the opioid crisis through decreasing the doses of opioid analgesics needed to control pain.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

There were no changes

Actual or anticipated problems or delays and actions or plans to resolve them

There were no problems or delays

Changes that had a significant impact on expenditures

No changes

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None to report

Significant changes in use or care of human subjects

Not applicable. No human subjects

Significant changes in use or care of vertebrate animals

During the project all amendments to IACUC 4486 were approved by the Temple University Animal Use Committee and by ACURO.

Significant changes in use of biohazards and/or select agents

None to report

6. PRODUCTS**Journal publications.**

Inan, S., T. K. Eisenstein, M. N. Watson, M. Doura, J. J. Meissler, C. S. Tallarida, X. Chen, E. B. Geller, S. M. Rawls, A. Cowan, M. W. Adler. Co-Administration of Chemokine Receptor Antagonists with Morphine Potentiates Morphine's Analgesic Effect on Incisional Pain in Rats. *J Pharmacol Exp Ther*. 2018. 367:433-441. DOI: 10.1124/jpet.118.25289. Acknowledgement of federal support: yes.

Inan, S., M. N. Watson, M. Doura, C. S. Tallarida, J. J. Meissler, X. Chen, E. B. Geller, A. Cowan, S. M. Rawls, T. K. Eisenstein, M. W. Adler. 2019. Chemokine receptor antagonists enhance the antinociceptive activity of oxycodone and meperidine on incisional pain in rats. *Br. J. Anaesth*. 122(6):e213-e215. DOI: 10.1016/j.bja.2019.01.021. Acknowledgement of federal support: yes.

Eisenstein, T.K., X. Chen, S. Inan, J.J. Meissler, C.S. Tallarida, E.B. Geller, S.M. Rawls, A. Cowan, M.W. Adler. Chemokine receptor antagonists in combination with morphine as a novel strategy for opioid dose reduction in pain management. *Military Medicine*. Invited submission, in press. Acknowledgement of federal support: yes.

Chen, X., S. Inan, E. M. Eisenstein, J. J. Meissler, E. B. Geller, C. Tallarida, M. Watson, M. Doura, A. Cowan, S. Rawls, M. W. Adler, T. K. Eisenstein. Chemokine Receptor Antagonists as a Novel Strategy for Opioid Dose Reduction for Pain Management. Manuscript in preparation. Acknowledgement of federal support: yes.

Books or other non-periodical, one-time publications.

None

Other publications, conference papers, and presentations.**1. Poster at the 24th conference of the Society on Neuroimmune Pharmacology, Chicago, IL, April 2018.**

Meissler, J., S. Inan, M. Watson, M. Doura, S. Rawls, A. Cowan, M. Adler, T. Eisenstein. Co-administration of chemokine receptor antagonists with morphine attenuated upregulation of chemokine and cytokine expression induced by incisional pain in rats.*

2. Poster at the College on Problems of Drug Dependence 80st annual meeting in San Diego, CA, June 2018.

X. Chen, T. K. Eisenstein, S. Inan, C. Tallarida, A. Cowan, E. B. Geller, J. J. Meissler, S. Rawls, M. N. Watson, M. Doura & M. W. Adler. CXCR4 & CCR5 chemokine receptor antagonists significantly enhanced morphine-induced antinociception in female, but not male, mice in the formalin test.*

3. Poster at the International Narcotics Research Conference meeting in San Diego, CA, June, 2018.

Saadet Inan, Mia N Watson, Menahem Doura, Christopher S Tallarida, Xiaohong Chen, Joseph J Meissler, Ellen B Geller, Scott M Rawls, Alan Cowan, Toby K Eisenstein, Martin W Adler. Co-administration of chemokine receptor antagonists with opioids: Potentiation of analgesic effects on incisional pain in rats.*

4. Oral presentation at the Military Health System Research Symposium, Kissimmee, FL, August 2018.

Saadet Inan, Mia N Watson, Menahem Doura, Christopher S Tallarida, Xiaohong Chen, Joseph J Meissler, Ellen B Geller, Scott M Rawls, Alan Cowan, Toby K Eisenstein, Martin W Adler. Chemokine receptor antagonists as a novel strategy to enhance pain management.*

5. Poster at the NIH Conference on “The Opioid Crisis and the Future of Addiction and Pain Therapeutics”. Bethesda, MD. February 7-8, 2019.

Inan, S., X. Chen, J.J. Meissler, M. Duora, M.N. Watson, C. Tallarida, E.B. Geller, S.M. Rawls, A. Cowan, T.K. Eisenstein, M.W. Adler. Co-administration of chemokine receptor antagonists with opioids in rodents: A novel strategy to spare opioids for treatment of pain.*

6. Poster presented at the NIH Conference on “The Opioid Crisis and the Future of Addiction and Pain Therapeutics”. Bethesda, MD. February 7-8, 2019.

Ruff, M.R., J. Zhang, S. Inan, T.K. Eisenstein, M.W. Adler. Multi-chemokine receptor antagonist peptide R103 blocks inflammation and pain: Potentiates opioid potency.

7. Invited oral symposium talk at the College on Problems of Drug Dependence 81st annual meeting, San Antonio, TX, June 2019. S. Inan. Enhancement of opioid analgesia using chemokine receptor antagonists.*

8. Poster at the Military Health System Research Symposium, Kissimmee, FL, August 2019. Inan, S., X. Chen, J.J. Meissler, M. Duora, M.N. Watson, C. Tallarida, E.B. Geller, S.M. Rawls, A. Cowan, T.K. Eisenstein, M.W. Adler. Chemokine receptor antagonists increase opioid potency in incisional pain in rats.*

- **Website(s) or other Internet site(s)**

None

- **Technologies or techniques**

None

- **Inventions, patent applications, and/or licenses**

None

- **Other Products**

The results obtained in this project have major implications for improved clinical interventions in the treatment of pain. The new strategy of combining reduced doses of opioids with chemokine receptor antagonists holds out the promise of being able to lower the dose of an opioid by combining it with one or more chemokine receptor antagonists.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Martin W. Adler, Ph.D.

Project Role: PI

Nearest person months worked: 4

Contribution to Project: Overall direction of this project, including involvement in planning and design of all phases of the research, as well as choice of drugs and doses. Manuscript preparation

Name:	Toby K. Eisenstein, Ph.D
Project Role:	Co-Investigator
Nearest person months worked:	3
Contribution to Project:	Responsible for all experiments related to measurements of chemokines after induction of pain in the various models, including following the use of chemokine receptor antagonists and opioids. Participated in experimental design and interpretation of results. Preparation of reports and manuscripts.
Name:	Alan Cowan, Ph.D.
Project Role:	Co-Investigator
Nearest person months worked:	2
Contribution to Project:	Prime responsibility for oversight of all experiments involving testing of the drugs in pain models and in evaluation of the results.
Name:	Scott M. Rawls, Ph.D.
Project Role:	Co-Investigator
Nearest person months worked:	1
Contribution to Project:	Responsible for oversight of experimental design and interpretation of dose-response, time-response, and multiple drug studies particularly in regard to choice and use of CRAs.
Name:	Xiaohong Chen, M.D.
Project Role:	Associate Scientist
Nearest person months worked:	9
Contribution to Project:	In charge of conducting experiments with animals and preparing test solutions; Data graphing and presentation.
Name:	Saadet Inan, Ph.D.
Project Role:	Associate Scientist
Nearest person months worked:	5
Contribution to project:	In charge of conducting experiments with animals and preparing test solutions; Data graphing and presentation.
Name:	Ellen B. Geller, B.A., M.A.
Project Role:	Scientific Coordinator
Nearest person months worked:	3
Contribution to project:	Assists in coordinating the work from all of the laboratories involved in this study and keeping the inventory for and dispensing any controlled substances used in this project. Prepared IACUC renewals. Assisted in manuscript preparation.
Name:	Joseph Meissler, B.Sc.
Project Role:	Laboratory Manager
Nearest person months worked:	4
Contribution to project:	Responsible for measurement of chemokines and cytokines after the various treatments. Coordinated data processing and presentation.

Name: Menahem Doura, Ph.D
 Project Role: Technician
 Nearest person months worked: 1
 Contribution to project: Assisted Drs. Chen and Inan in conducting experiments and collecting data.

Name: Emily Dziedowiec, B.S.
 Project Role: Technician
 Nearest person months worked: 1
 Contribution to project: Assisted Drs. Chen and Inan in conducting experiments and collecting data.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Scott Rawls had a change in Other Support as detailed below.

RAWLS, SCOTT
ACTIVE SUPPORT

R01 DA045499-02 (Rawls & Unterwald - MPI)
 07/01/18 – 4/30/23 1.8 calendar
 NIH/NIDA \$245,636

“Chemokine CXCL12/CXCR4 system and synthetic cathinones”

The goal is to investigate how the chemokine CXCL12 (SDF-1 α), and its receptor, CXCR4, are influenced by cathinones and cocaine, and, in turn, how modulation of the CXCL12/CXCR4 system affects behavioral and neurochemical effects related to psychostimulant abuse. Our central hypotheses are that CXCL12 and CXCR4 are affected by psychostimulant exposure and withdrawal, and that pharmacological modulation of CXCR4 will reduce the reinforcing, motivational, and drug seeking effects of cocaine through modulation of mesolimbic dopamine and corticolimbic glutamate.

Overlap: None

R41 DA047169-01 (Rawls - PI)
 07/01/18 – 6/30/20 (NCE) 1.2 calendar
 NIH/NIDA \$113,565/year

“Trigriluzole for the Treatment of Cocaine Addiction”

This proposal describes the first comprehensive in vivo comparison of the efficacy of the glutamate-based compounds riluzole (RLZ) and our more pharmaceutically beneficial tripeptide prodrug trigriluzole (TRLZ). We will test TRLZ and RLZ in preclinical models of COC addiction based on the facilitation of COC relapse in rats by enhanced glutamate transmission in the nucleus accumbens and the highly attractive pharmacodynamic profile of RLZ, a pharmacologically-diverse, FDA-approved drug that reduces glutamate transmission by multiple mechanisms including enhancement of astrocytic glutamate reuptake by upregulation of the transporter GLT-1, reduction of neuronal glutamate release, and reduction of neuronal excitability by ion channel modulation.

Overlap: None

R01DA046833 NIH/NIDA (Ramirez & Potula – MPI; Rawls – Co-I)
 09/30/18 – 06/30/23 0.6 calendar
 NIH/NIDA \$336,987/year

“Brain Endothelial EVs Role in the Neuropathology of Drugs of Abuse and HIV”

The goal of this project is to investigate whether brain endothelial derived extracellular vesicles serve as entry passes for immune cells to gain access into the central nervous system. The degree of EV production (MVs and exosomes) will be characterized as a function of psychostimulant type using a novel microfluidic model of the neurovascular unit with primary human cells. Furthermore, EC-EV release will be correlated to

the phases of addiction in an in-vivo self-administration model as well as in a humanized mouse model of HIV infection with or without drug administration. Outcomes will offer crucial insight to brain endothelial EV production, mechanisms of immune cell infiltration into the CNS, and also reveal targets for pharmacological interventions that promote BBB protection in HIV and substance use disorder.

in these reward-associated areas, we will test the use novel anti-inflammatory agents, including non-psychoactive cannabinoid derivatives to counter the alterations observed after injury.

Overlap: None

What other organizations were involved as partners?

Not applicable. Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

None

QUAD CHARTS:

Final report so not applicable

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Three appendix items – 2 publications and 1 manuscript in press

Coadministration of Chemokine Receptor Antagonists with Morphine Potentiates Morphine's Analgesic Effect on Incisional Pain in Rats

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ABSTRACT

Crossdesensitization between opioid and chemokine receptors and involvement of chemokines in pain modulation are well established. We investigated if coadministration of chemokine receptor antagonists (CRAs) with morphine would enhance the analgesic potency of morphine on incisional pain in rats. Animals underwent incisional surgery on the left hind paw and pain responses were evaluated using von Frey filaments at various time points postsurgery between 15 and 360 minutes and daily between 24 and 72 hours. Dose-response curves for morphine, maraviroc (a CCR5 antagonist), and AMD3100 (a CXCR4 antagonist) alone were established. While morphine significantly reduced pain in a time- and dose-dependent manner, maraviroc and AMD3100 had no effect by themselves. Coadministration of either maraviroc or AMD3100 with morphine significantly increased morphine's analgesic effect on

incisional pain, shifting the dose-response curve to the left 2.3- and 1.8-fold, respectively. Coadministration of both CRAs with morphine significantly shifted further the morphine dose-response curve to the left 3.3-fold. The effect of treatments on mRNA levels in the draining popliteal lymph node for a panel of chemokines and cytokines showed that message for many of these mediators was upregulated by the incision, and the combination of morphine with the CRAs markedly downregulated them. The data show that combining morphine with CRAs potentiates morphine's analgesic effect on incisional pain. Thus, the same analgesic effect of morphine alone can be achieved with lower doses of morphine when combined with CRAs. Using morphine in lower doses could reduce unwanted side effects and possibly block development of tolerance and dependence.

Introduction

Crosstalk between opioid and chemokine receptors via heterologous desensitization is well established (Grimm et al., 1998; Ali et al., 1999; Zhang et al., 2003, 2004; Chen et al., 2004; Heinisch et al., 2011). Opioid receptors are expressed on macrophages, monocytes, and T and B lymphocytes, and their activation with an agonist induces changes in some chemokine levels and chemokine receptor expression (Chuang et al., 1995; Wick et al., 1996; Grimm et al., 1998; Bidlack, 2000; Wetzel et al., 2000; Guo et al., 2002; Mahajan et al., 2002; Happel et al., 2008). It has been shown that met-enkephalin and morphine can inhibit chemotaxis of human neutrophils and peripheral blood mononuclear cells to several chemokines including CXCL1/IL-8, CCL5/RANTES, and CCL2/MCP-1 via phosphorylation of the

intracellular tails of the chemokine receptors (Grimm et al., 1998). In the central nervous system, chemokine receptors are expressed on microglia, astrocytes, oligodendrocytes, and neurons. They play a role in brain development, angiogenesis, and development of inflammatory as well as degenerative conditions in the central nervous system (Kim and Broxmeyer, 1999; Ragozzino, 2002; Tran and Miller, 2003). Studies using different rodent models show that chemokines play a role in pain (Tanaka et al., 2004; Dansereau et al., 2008; Peters and Eisenach, 2010; Lee et al., 2013). Central or peripheral administration of chemokines enhances pain perception (Szabo et al., 2002; Zhang et al., 2005; Benamar et al., 2008). Furthermore, their central administration decreases the analgesic effect of opioids, providing evidence that heterologous desensitization between opioid and chemokine receptors can occur in the central nervous system as well (Szabo et al., 2002; Chen et al., 2007a,b). Szabo et al. (2002) showed that administering either CXCL12/SDF-1 α or CCL5/RANTES into the periaqueductal gray (PAG) area blocked the analgesic effect of the mu-selective opioid, DAMGO, given 30 minutes later into the PAG area, as measured by cold water tail flick (CWTF) assay. Pretreatment of animals with an antibody against CCL5/RANTES inhibited the hyperalgesia

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<https://doi.org/10.1124/jpet.118.252890>.

ABBREVIATIONS: ANOVA, analysis of variance; CL, confidence limit; CRA, chemokine receptor antagonist; CWTF, cold water tail flick; HIV, human immunodeficiency virus; PAG, periaqueductal gray.

induced by the chemokine in the CWTF assay (Benamar et al., 2008). It has been shown that chemical and inflammatory-induced pain stimuli are significantly reduced in CCR5 knockout mice compared with wild-type mice (Lee et al., 2013). Furthermore, these researchers also reported that mu-opioid receptor immunoreactivity in the PAG area is higher in CCR5 knockout mice than wild-type mice. Interestingly, gp120, a human immunodeficiency virus (HIV)-1 coat protein that binds to both CCR5 and CXCR4, had a similar desensitizing effect as a chemokine on morphine analgesia in rats using the CWTF assay (Chen et al., 2011). When AMD3100, a CXCR4 receptor antagonist, was given before gp120, it restored the analgesic effect of morphine. Also, electrophysiological studies of PAG neurons showed that gp120 (Chen et al., 2011) and CXCL12 as well as CX3CL1 (Heinisch et al., 2011) inhibit morphine-induced hyperpolarization and reduction of input resistance, which provides evidence for crosstalk between chemokine receptors and mu-opioid receptors on a cellular level. Thus, ligands that bind to chemokine receptors can desensitize mu-opioid receptors and decrease the analgesic effect induced by mu agonists.

In painful syndromes involving inflammation, patients can be refractory to the analgesic effects of morphine. We hypothesized that chemokine receptor antagonists (CRAs), used in combination with morphine, will produce effective analgesia with smaller doses of morphine. Blocking chemokine receptors with a CRA will reduce inflammation, and therefore pain, and will prevent the crossdesensitization induced by chemokine ligands, thereby increasing opioid receptor activity. Such combinations will likely have the added advantage of reducing unwanted side effects of opioids and potential development of tolerance and dependence. Maraviroc (a CCR5 receptor antagonist) and AMD3100 were chosen as the CRAs because both compounds are Food and Drug Administration–approved for human use (maraviroc: an HIV-1 entry inhibitor used as an antiretroviral for HIV-1 treatment; AMD3100: used in lymphoma and multiple myeloma treatment). CRAs were coadministered with morphine in a rat assay of incisional pain (model for clinical postsurgical pain), in which mechanical allodynia is measured.

Materials and Methods

Animals. Animal care and experimental procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee of Temple University. Male Sprague-Dawley rats (200–240 g; Taconic Biosciences, Germantown, NY) were used in these experiments. They were housed (two in a cage) in a temperature-controlled environment with a 12-hour light-dark cycle and were supplied with food and water ad libitum. Rats were acclimated for a week in the central animal facility before surgery and behavioral testing. Two days before the surgery and behavioral testing, rats were acclimated in individual transparent cubicles with a wire mesh floor for 2 days (1 hour each day).

Surgery. On the day of surgery, rats were acclimated for 30 minutes and then their individual baseline values for paw withdrawal threshold were measured using a series of von Frey filaments (North Coast Medical, Inc., Gilroy, CA), with gradually increasing equal logarithmic bending forces (equivalent to 2, 4, 6, 8, 10, 15, 26, and 60 g force). The filaments were applied to the plantar side of each hind paw in an ascending manner. Each filament was tested five consecutive times a few seconds apart. A positive response was defined as quick withdrawal or paw flinching after the application of a filament.

Surgery was performed as previously described by Brennan et al. (1996) under isoflurane anesthesia (4% isoflurane for induction and

2.5% isoflurane for maintenance of anesthesia) in aseptic conditions. A 1-cm longitudinal incision (starting 0.5 cm from the proximal edge of the heel and extending toward the toes) was made with a scalpel through skin and the fascia of the plantar side of the left hind paw. The plantaris muscle was exposed, elevated, and incised longitudinally. Following bleeding control with gentle pressure, the skin was closed with two single interrupted sutures using 5-0 nylon. Animals were brought back to the individual chambers for mechanical allodynia testing. The time when surgeries were completed was designated as time 0. The animals awoke on the average approximately 5–8 minutes after the end of the surgery, and fully regained consciousness by the first testing time, which was 15 minutes postsurgery. Paw withdrawal thresholds were recorded at time points postsurgery (15, 30, 45, 60, 120, 240, and 360 minutes; 24, 48, and 72 hours) by a person blinded to the treatments. Morphine, chemokine receptor antagonists, or their vehicles were injected subcutaneously at $t = 25$ minutes postsurgery. Right and left flank areas, as well as behind the neck area (for the third injection), were used to administer compounds or vehicle.

Compounds. Morphine sulfate pentahydrate (NIDA, Bethesda, MD) was dissolved in sterile saline. Maraviroc and AMD3100 were purchased from Sigma-Aldrich (St. Louis, MO). Maraviroc was dissolved in 10% dimethylsulfoxide (Sigma-Aldrich), and AMD3100 was dissolved in sterile, pyrogen-free water (Aqualite System, Lake Forest, IL).

Experimental Design. Morphine, chemokine receptor antagonists, or their vehicles alone or in combination studies were injected subcutaneously at $t = 25$ minutes postsurgery. Dose-response curves for morphine (1–10 mg/kg), maraviroc (0.5–5 mg/kg), and AMD3100 (1–10 mg/kg) were established. For combination experiments, fixed doses of maraviroc (2.5 and 5 mg/kg), AMD3100 (2.5 and 6.7 mg/kg), or both (maraviroc at 5 mg/kg and AMD3100 at 6.7 mg/kg) were coadministered with different doses of morphine to establish dose-response curves. In the experiments for coadministration of one CRA with morphine, rats were injected with vehicle plus morphine, a fixed dose of CRA plus morphine, CRA plus saline, or the two vehicles. Morphine doses used for these experiments were 0.25–5 mg/kg. For the coadministration of two CRAs with morphine, animals received one of the following: two vehicles plus morphine, CRAs plus morphine, CRAs plus saline, or three vehicles. Morphine doses used for these experiments were 0.25–2 mg/kg. Percentage of reversal of mechanical allodynia was calculated using the following formula:

$$\begin{aligned} \% \text{ reversal of mechanical allodynia} \\ = [(\text{test threshold}) - (\text{predose threshold}) / (\text{baseline threshold}) \\ - (\text{predose threshold})] \times 100 \end{aligned}$$

where the test threshold represents the paw pressure response to the filament at the testing time point; the predose threshold represents the response at 15 minutes after surgery and before treatments; and the baseline threshold represents the baseline value before surgery. The percentage of reversal of mechanical allodynia was calculated individually for each rat, and then the mean, S.D., and S.E.M. values were calculated for individual groups. In drug combination studies, the percentage of reversal of mechanical allodynia at 60 minutes was used because morphine's effect was maximal at 60 minutes postsurgery.

Quantitation of mRNA for Immune Mediators. The level of mRNA for a panel of immune mediators was determined using a commercial array, the RT₂ Profiler PCR Array for Rat Inflammatory Cytokines and Receptors (Qiagen, Inc., Germantown, MD). The draining popliteal lymph nodes were collected from animals subjected to the same surgery and treatments as shown in Fig. 7, but they were sacrificed at 1 hour postincision. Tissues were processed by the Qiagen RNeasy Microarray Tissue Mini Kit following the manufacturer's protocols. Individual mRNA preparations were quantitated by a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) to determine mRNA concentrations in nanograms per microliter. mRNAs from individual lymph nodes were used to generate cDNAs

using the RT² First Strand Kit (Qiagen, Inc.), which were then assayed using the RT² Profiler PCR Array with the RT² SYBR Green ROX qPCR Mastermix (Qiagen, Inc.). This array has probes for mRNA for 84 immune mediators, which include 22 chemokines, 20 cytokines, 21 receptors, and 21 other molecules that are involved in the inflammatory response. The polymerase chain reaction array was run on an ABI StepOne Plus RT-PCR thermocycler (Applied Biosystems, Foster City, CA) using the cycling conditions given in the protocol supplied with the RT² Array. Data were processed by the online GeneGlobe Data Analysis Center (Qiagen, Inc.). The results obtained are expressed as the log₂ fold difference in mRNA expression for each mediator in the array of the treated groups compared with the level of mRNA expression in the baseline untreated control.

Statistical Analysis. Data for morphine, maraviroc, and AMD3100 time-course and dose-response curves as well as data on the combination of morphine with each or both CRAs were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's or Sidak's multiple comparisons tests.

To establish the ED₅₀ values and 95% confidence limits (CLs) for morphine and for the combination of morphine with a single CRA or with both CRAs, data from dose-response curves measuring percentage of reversal of mechanical allodynia at 60 minutes were used and analyzed by nonlinear regression analysis. To compare dose-response curves for morphine alone and in combination with CRAs, the paired Student's *t* test was used (Tallarida et al., 2014). Data are expressed as mean ± S.E.M., and *P* < 0.05 was accepted as statistically significant. GraphPad Prism version 7, (GraphPad, La Jolla, CA) was used for data analysis. Statistics on the results of the RT² Profiler Arrays were done by Student's *t* test, using the online GeneGlobe Data Analysis Center (Qiagen, Inc.).

Results

Morphine, but Not Maraviroc or AMD3100, Induces Analgesia in a Time- and Dose-Dependent Manner. Figures 1–3 show the time- and dose-dependent percentages of reversal of mechanical allodynia for rats receiving morphine alone, maraviroc alone, or AMD3100 alone, respectively. Baseline (preincision) paw withdrawal thresholds were similar for all rats in the different groups (33.91 ± 2.28 g), and they dropped abruptly 15 minutes after the incision in all groups (3.22 ± 0.20 g). Time 0 is the time surgery is completed; 15 minutes postsurgery is the time that baseline measurements were taken; and 25 minutes postsurgery is the time when drugs were administered. Morphine demonstrated an analgesic effect in a time- and dose-dependent manner on incisional pain. Morphine at 5 and 10 mg/kg induced significant analgesia compared with saline (*P* < 0.001). The analgesic effect started at 45 minutes, reached a maximum at 60 minutes, lasted until 120 minutes, and disappeared by 240 minutes postsurgery. Morphine at 1 and 3 mg/kg did not produce analgesia, as shown in Fig. 1, where two-way ANOVA revealed significant effects of treatment [*F*(4,240) = 22.91, *P* < 0.0001] and time [*F*(5,240) = 9.08, *P* < 0.0001], and a significant interaction [*F*(20,240) = 1.786, *P* = 0.022]. Paw withdrawal thresholds were also measured at 24, 48, and 72 hours, but no analgesia was observed at these time points (data not shown). The ED₅₀ value for morphine was calculated as 2.34 mg/kg (95% CL: 1.69–3.17) (Table 1). Neither maraviroc nor AMD3100 had an analgesic effect on incisional pain (Figs. 2 and 3, respectively).

Effect of Coadministration of Maraviroc or AMD3100 with Morphine on Morphine's Analgesic Activity. The following experiments were carried out to test the effect of combining a CRA with morphine. First, fixed doses of maraviroc (2.5 or 5 mg/kg) were given with different doses

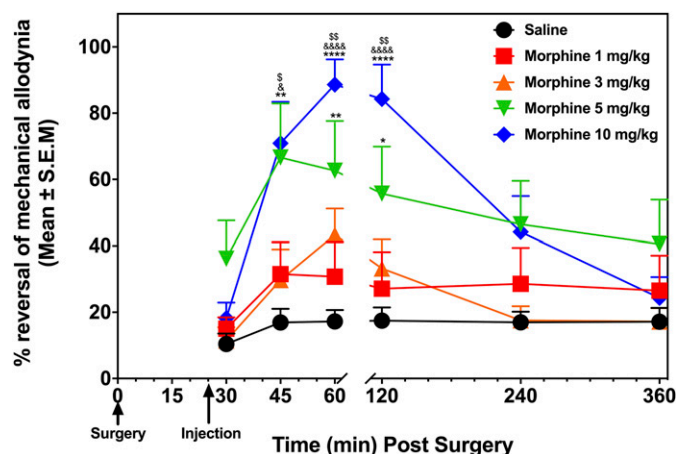


Fig. 1. Morphine time- and dose-response results on incisional pain. After baseline measurements, rats underwent surgery (*t* = 0). At 15 minutes, postsurgery baseline was recorded. Animals were injected with either saline or morphine (subcutaneously) at *t* = 25 minutes and paw withdrawal thresholds were measured at 30, 60, 120, 240, and 360 minutes. Percentage of reversal of mechanical allodynia was calculated. Morphine induced analgesia in a time- and dose-dependent manner. The analgesic effect started at 45 minutes, reached a maximum at 60 minutes, and lasted for 120 minutes postsurgery. Paw withdrawal thresholds also were measured at 24, 48, and 72 hours. No analgesia was observed at these time points (data not shown). Two-way ANOVA followed by Tukey's multiple comparisons test was applied. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 compared with saline; &*P* < 0.05; &&&&*P* < 0.0001 compared with 1 mg/kg; &*P* < 0.05; &&*P* < 0.01 compared with 3 mg/kg (*n* = 8–10).

of morphine. Maraviroc at 2.5 mg/kg was coadministered with morphine at doses ranging from 1 to 5 mg/kg. Since the maximal effect with morphine was established at 60 minutes postsurgery, the percentage of reversal of mechanical allodynia was calculated for all groups at this time point. As seen in Fig. 4A, coadministration of maraviroc at 2.5 mg/kg with morphine at 5 mg/kg significantly increased the analgesic effect compared with 10% dimethylsulfoxide plus morphine (5 mg/kg) at *P* < 0.05, where two-way ANOVA revealed significant effects of treatment [*F*(1,53) = 6.746, *P* = 0.012] and dose [*F*(3,53) = 16.36, *P* < 0.0001], and a significant interaction [*F*(3,53) = 3.304, *P* = 0.02]. While morphine alone at 5 mg/kg reversed mechanical allodynia by 49.8% ± 13.1%, addition of maraviroc at 2.5 mg/kg increased this effect to 100% ± 0% (*P* < 0.01). The ED₅₀ value for the combination of morphine and maraviroc at 2.5 mg/kg was calculated as 1.95 mg/kg (95% CL: 1.08–2.96). In a subsequent experiment, the dose of maraviroc was increased to 5 mg/kg and used in combination with morphine at doses ranging from 0.5 to 5 mg/kg, where two-way ANOVA revealed significant effects of treatment [*F*(1,69) = 5.993, *P* = 0.016] and dose [*F*(4,69) = 12.62, *P* < 0.0001] but not a significant effect of interaction [*F*(4,69) = 1.088, *P* = 0.36]. The ED₅₀ value for this combination was calculated as 1.01 mg/kg (95% CL: 0.60–1.56) (Table 1). Coadministration of maraviroc 5 mg/kg with morphine significantly shifted the morphine-dose response to the left 2.3-fold (*P* < 0.05, paired Student's *t* test) (Fig. 7).

Similar experiments were carried out with AMD3100 (2.5 and 6.7 mg/kg) with morphine (doses ranging from 1 to 5 and 0.5 to 3 mg/kg, respectively). The results for coadministration of AMD3100 2.5 mg/kg with morphine at 5 mg/kg, where two-way ANOVA revealed a significant effect of dose [*F*(4,61) = 9.776,

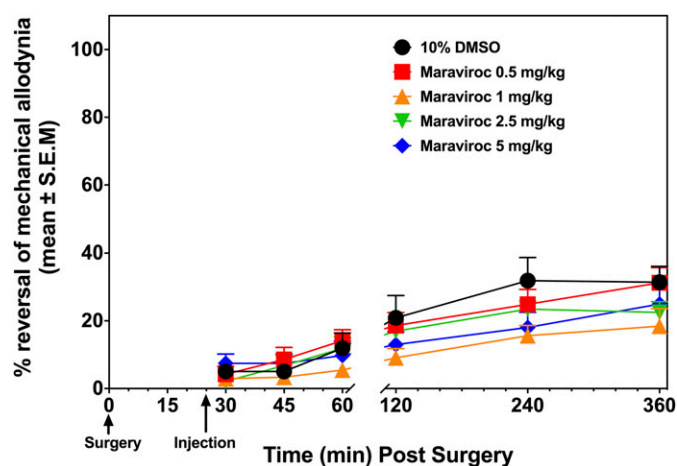


Fig. 2. Maraviroc time- and dose-response results on incisional pain. Determination of baseline values and surgery are the same as in Fig. 1. Animals were then injected with either 10% dimethylsulfoxide (DMSO) or maraviroc (subcutaneously) at $t = 25$ minutes and paw withdrawal thresholds were measured at 30, 60, 120, 240, and 360 minutes postsurgery. Percentage of reversal of mechanical allodynia was calculated. Two-way ANOVA followed by Tukey's multiple comparisons test revealed no significant differences between doses at any of the time points tested ($n = 8-10$).

$P < 0.0001$) but not of treatment [$F(1,61)=1.27$, $P = 0.26$] and interaction [$F(4,61)=1.88$, $P = 0.12$], and AMD3100 at 6.7 mg/kg with morphine at 3 mg/kg, where two-way ANOVA revealed a significant effect of dose [$F(4,69)=20.27$, $P < 0.0001$] but not of treatment [$F(1,69)=3.51$, $P = 0.06$] and interaction [$F(4,69)=1.66$, $P = 0.16$], are shown in Figs. 5A and B, respectively. The ED_{50} values for these combinations were 2.61 mg/kg (95% CL: 1.78–3.41) and 1.32 mg/kg (95% CL: 0.85–1.89), respectively (Table 1). Coadministration of AMD3100 6.7 mg/kg with morphine significantly shifted the morphine-dose response to the left 1.8-fold ($P < 0.05$, paired Student's t test) (Fig. 7).

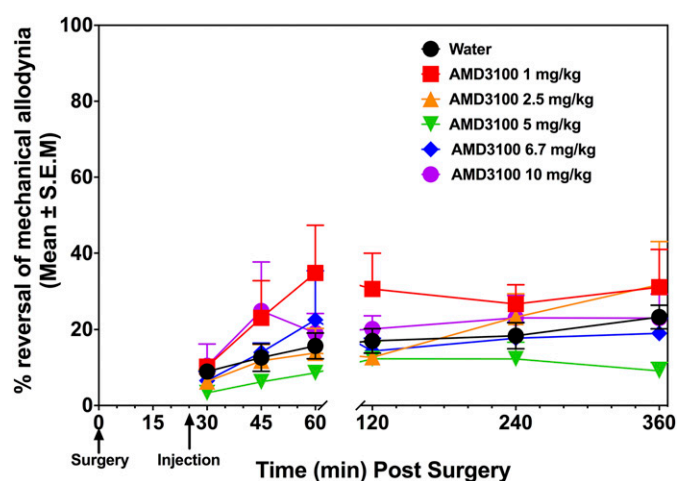


Fig. 3. AMD3100 time- and dose-response results on incisional pain. Determination of baseline values and surgery are the same as in Fig. 1. Animals were injected with either water or AMD 3100 (subcutaneously) at $t = 25$ minutes and paw withdrawal thresholds were measured at 30, 60, 120, 240, and 360 minutes postsurgery. Percentage of reversal of mechanical allodynia was calculated. No analgesia was observed with AMD3100. Two-way ANOVA followed by Tukey's multiple comparisons test revealed no significant differences ($n = 8-10$).

TABLE 1

Calculated ED_{50} values plus 95% confidence limits for morphine in combination with fixed doses of maraviroc or AMD3100, or a combination with morphine plus maraviroc and AMD3100

Treatment	Calculated ED_{50} of Morphine	95% Confidence Interval
	mg/kg	
Morphine alone	2.34	1.69–3.17
Morphine + Maraviroc	1.01	0.60–1.56
Morphine + AMD3100	1.32	0.85–1.89
Morphine + Maraviroc + AMD3100	0.71	0.42–1.14

Coadministration of Both Maraviroc and AMD3100 with Morphine Significantly Enhances the Potency of Morphine.

Since maraviroc and AMD3100 were each able to increase the analgesic activity of morphine, the effect of a combination of both CRAs with morphine was tested. Empirically, it was decided to test the highest doses of each of the CRAs with doses of morphine that alone gave below 40% analgesia. As shown in Fig. 6, coadministration of both CRAs with morphine significantly potentiated morphine's analgesic effect, where two-way ANOVA revealed significant effects of treatment [$F(1,76) = 17.01$, $P < 0.001$] and dose [$F(4,76) = 8.11$, $P < 0.001$] and a significant interaction [$F(4,76) = 2.67$, $P = 0.038$]. A significant increase in reversing mechanical allodynia was observed starting with doses of morphine as low as 0.5 mg/kg ($P < 0.05$). A dose of only 2 mg/kg of morphine, which is one-fifth of its maximal effective dose, reversed mechanical allodynia by $74.5\% \pm 11.1\%$ (Fig. 6, $P < 0.001$). The ED_{50} value for this combination of two CRAs plus morphine was calculated as 0.71 mg/kg (95% CL: 0.42–1.14), which shifted the morphine dose-response 3.3-fold to the left ($P < 0.05$). Dose-response curves for morphine alone, maraviroc (5 mg/kg) plus morphine, AMD3100 (6.7 mg/kg) plus morphine, and maraviroc (5 mg/kg) plus AMD3100 (6.7 mg/kg) plus morphine are shown in Fig. 7.

Effect of Treatment with Morphine and CRAs on mRNA Levels for Chemokines and Cytokines. Lymph node samples were processed to obtain mRNA as described in *Materials and Methods*. The objective was to screen a large number of immune mediators to assess how the surgery and treatments with morphine, CRAs, or the combination of morphine plus CRAs altered levels of chemokines and cytokines. Although mRNA levels for 84 analytes were measured by the RT² Profiler Array, Fig. 8 only presents the results for the ones of interest or with significant changes. It is evident from Fig. 8, A and B, that the incision alone caused a statistically significant increase (starred bars) in mRNAs for several cytokines and chemokines, including CCL2/MCP-1, CCL7/MCP-3, CCL12, CXCL2/MIP-2, IL-1 α , IL-1 β , IL-5, and G-CSF. There was a trend toward increased mRNA levels for CCL20/MIP-3 α and CXCL1. Morphine alone at 2 mg/kg did not have a strong effect on the mRNA levels of mediators in this panel. The two CRAs, in spite of having little effect on pain, did reduce mRNA for five of the eight mediators upregulated by the incision to a level that was not significantly above baseline. The combination of the suboptimal dose of morphine plus the two CRAs decreased the mRNA levels of all of the mediators that were significantly upregulated by the incision, such that none of them were significantly different from baseline values. mRNAs for several of the mediators were also more robustly down-regulated by the morphine + two CRAs than by the CRAs

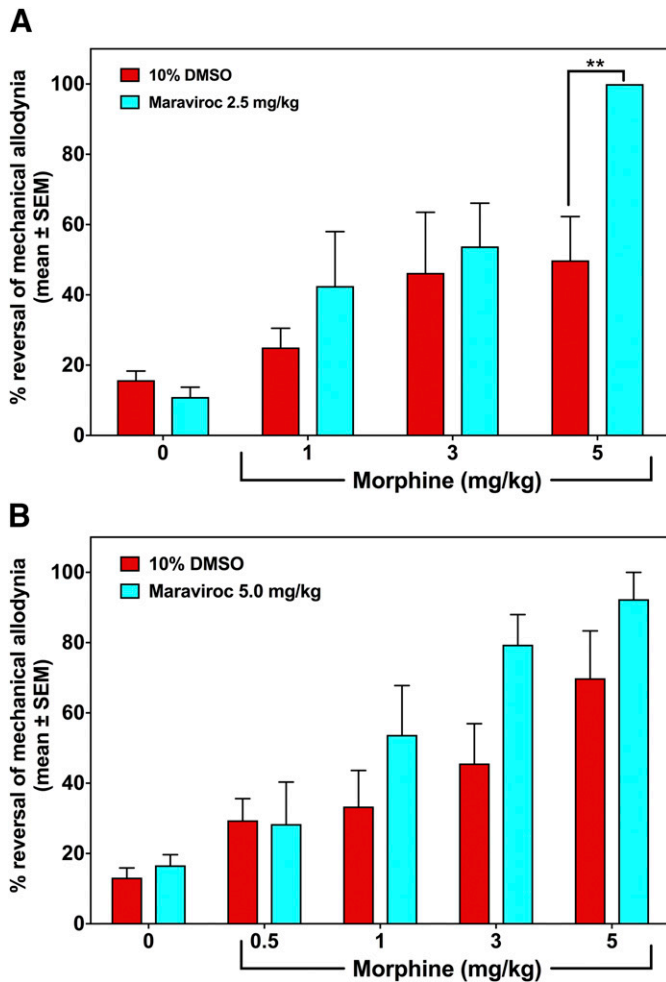


Fig. 4. Effect of the coadministration of maraviroc with morphine on incisional pain. Determination of baseline values and surgery are the same as in Fig. 1. (A) Animals were injected at $t = 25$ minutes with 10% dimethylsulfoxide (DMSO) + morphine (subcutaneously) or maraviroc (2.5 mg/kg) + morphine (subcutaneously). (B) Animals were injected at $t = 25$ minutes with 10% DMSO + morphine (subcutaneously) or maraviroc (5 mg/kg) + morphine (subcutaneously). Paw withdrawal thresholds were measured from 30 to 360 minutes. Percentage of reversal of mechanical allodynia was calculated using data at 60 minutes. Coadministration of maraviroc (2.5 mg/kg) with morphine (5 mg/kg) significantly increased morphine's analgesic effect (A). Two-way ANOVA followed by Sidak's multiple comparisons test showed $**P < 0.01$ ($n = 8-10$).

without morphine. (Baseline values are not available, but are intrinsic to the assay.) Surprisingly, neither mRNA for CCL5/RANTES nor for CXCL12/SDF-1 α were upregulated by the incision alone, and there was no effect on mRNA for these chemokines in animals receiving the antagonists for their receptors (AMD3100 for CXCR4 and maraviroc for CCR5). Instead, the combination of morphine plus the two CRAs downregulated a broader panel of mRNAs for unrelated proinflammatory chemokines and cytokines.

Discussion

The present study tested the hypothesis that simultaneous administration of one or two CRAs with a subanalgesic dose of morphine would increase morphine's analgesic potency. It is shown here for the first time that a CRA, having no analgesic effect by itself, when given together with a subanalgesic dose

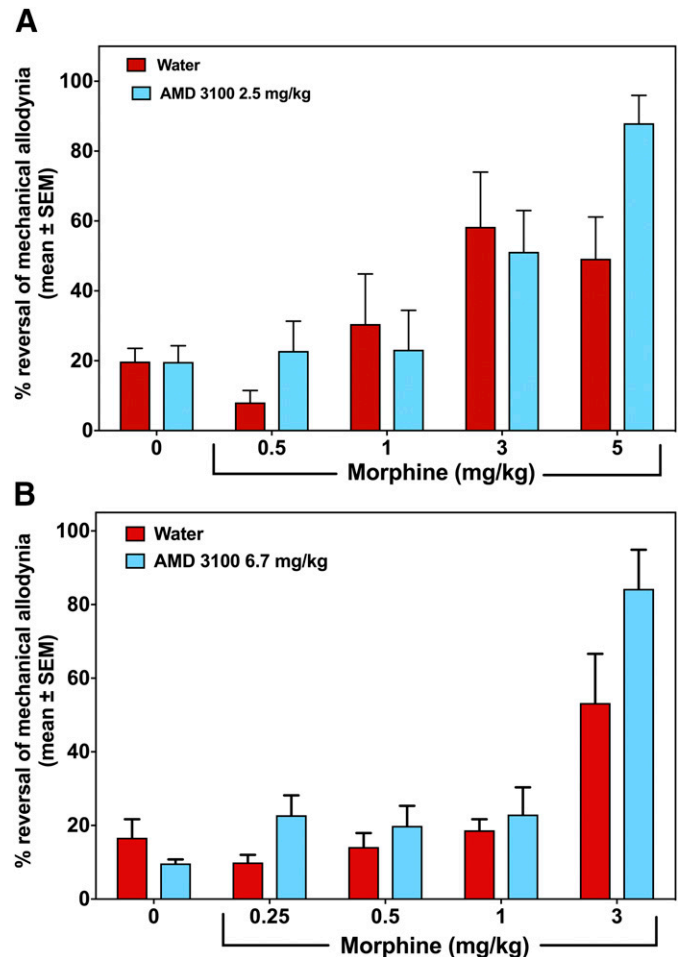


Fig. 5. Effect of coadministration of AMD3100 with morphine on incisional pain. Determination of baseline values and surgery are the same as in Fig. 1. (A) Animals were injected at $t = 25$ minutes with water + morphine (subcutaneously) or AMD3100 (2.5 mg/kg) + morphine (subcutaneously). (B) Animals were injected at $t = 25$ minutes with water + morphine (subcutaneously) or AMD3100 (6.7 mg/kg) + morphine (subcutaneously). Paw withdrawal thresholds were measured at time points between 30 and 360 minutes. Percentage of reversal of mechanical allodynia was calculated using data at 60 minutes. Coadministration of AMD3100 (2.5 mg/kg) with morphine (5 mg/kg) (A) and coadministration of AMD3100 (6.7 mg/kg) with morphine (3 mg/kg) (B) show a trend to increase analgesia (two-way ANOVA revealed significance only for dose) ($n = 8-10$).

of morphine, modestly enhances analgesia. However, when two CRAs were administered together with morphine, there was a much more robust and significant enhancement in the analgesic effect of morphine on incisional pain. To our knowledge, this is the first report showing that a combination of two CRAs with morphine enhances analgesia. When administered together with morphine, either maraviroc or AMD3100 each separately reduced the ED₅₀ value of morphine from 2.34 to 1.01 and 1.32 mg/kg, respectively. When both CRAs were combined with morphine, they further potentiated and reduced the ED₅₀ value of morphine to 0.71 mg/kg. Compared with morphine alone, this is a 3.3-fold reduction of the morphine dose. In these experiments, it should be noted that rats were not pretreated. The drugs were administered therapeutically, which simulates the human postsurgery condition, and is a more stringent paradigm than a prophylactic experimental design. Furthermore, this

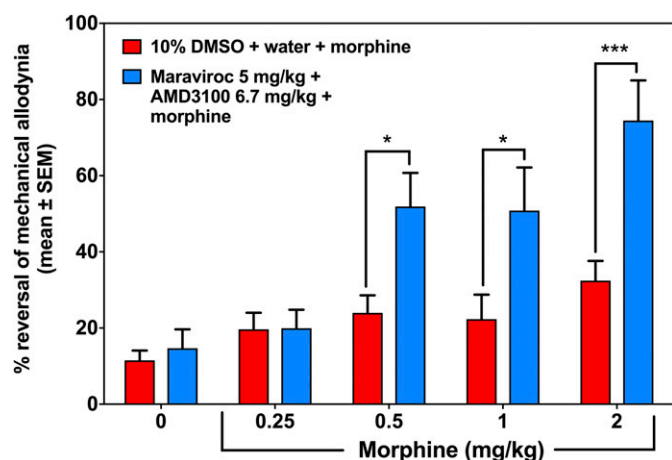


Fig. 6. Effect of coadministration of maraviroc (5 mg/kg) and AMD3100 (6.7 mg/kg) with morphine on incisional pain. Determination of baseline values and surgery are the same as in Fig. 1. At $t = 25$ minutes, animals were injected with either 10% dimethylsulfoxide (DMSO) + water + saline, 10% DMSO + water + morphine, maraviroc (5 mg/kg) + AMD3100 (6.7 mg/kg) + saline, or maraviroc (5 mg/kg) + AMD3100 (6.7 mg/kg) + morphine. Percentage of reversal of mechanical allodynia was calculated using data at 60 minutes. Combining two CRAs with subanalgesic doses of morphine (0.5, 1, and 2 mg/kg) significantly increased morphine's analgesic effects. Two-way ANOVA followed by Tukey's multiple comparisons test showed $*P < 0.05$; $**P < 0.01$ ($n = 6-10$).

study is the first to test the effects of maraviroc and AMD3100 by themselves, as well as in combination with morphine, on incisional pain. Maraviroc has been studied for neuropathic pain (Kwiatkowski et al., 2016; Piotrowska et al., 2016) and for remifentanyl-induced hyperalgesia (Li et al., 2016) in rodents. Kwiatkowski et al. (2016) administered maraviroc intrathecally before chronic constrictive nerve injury, and then continued by intrathecal injection for 7 days. Maraviroc significantly reduced mechanical allodynia by itself, and significantly increased analgesic effects of both morphine and buprenorphine on neuropathic pain. Also, chronic oral administration of a dual CCR2 and CCR5 antagonist, RAP-103, induced analgesia to neuropathic pain in rats induced by chronic, constrictive nerve injury (Padi et al., 2012). Li et al. (2016) also administered maraviroc intrathecally before remifentanyl and found that pretreatment with maraviroc inhibited remifentanyl-induced thermal and mechanical allodynia. Akgün et al. (2015) showed that intrathecal administration of a synthesized bivalent compound, which has both mu-opioid agonist and CCR5 antagonist activity, was highly potent in preventing inflammatory and neuropathic pain compared with morphine in mice pretreated with lipopolysaccharide. The findings of that study support the present study in that the result was to increase the potency of morphine's analgesic effect when combined with a CCR5 antagonist.

Previously, our laboratories have shown that individual administration of several different chemokines into the PAG area, before morphine administration, blocked the analgesic effect of morphine in the CWTF test (Szabo et al., 2002). There is evidence that opioid receptors and chemokine receptors can form heterodimers (Chen et al., 2004), which lead to heterologous desensitization of the opioid receptors. Furthermore, single neurons in the PAG area express both opioid and chemokine receptors, which would allow for heterodimer formation (Heinisch et al., 2011). The hypothesis to explain the current results, showing that the combination of a

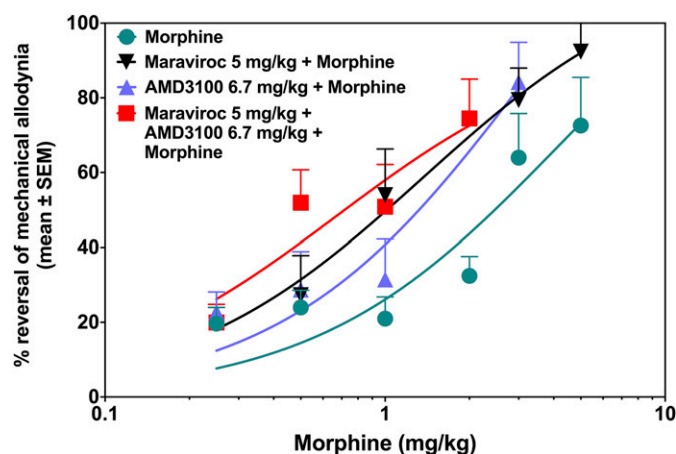


Fig. 7. Combination of morphine with two CRAs shifts the morphine dose-response curve to the left. Percentage of reversal of mechanical allodynia was calculated using data at 60 minutes. The combination of morphine with either AMD3100 or maraviroc individually, as well as the combination of morphine with both CRAs, significantly shifted the morphine dose-response curve to the left. The paired Student's t test showed $*P < 0.05$ ($n = 6-10$).

chemokine receptor antagonist with morphine can potentiate the activity of morphine, is that the CRA is preventing crossdesensitization of the opioid receptor by chemokines released in the inflammatory response resulting from the incision. It would be consistent with the hypothesis that two CRAs would be superior to a single CRA by preventing crossdesensitization at multiple chemokine receptors. The predicted result would be to increase the availability of additional mu receptors.

The present study is unique in that the maraviroc was administered systemically. Furthermore, the effect on incisional pain was evaluated using the drugs therapeutically rather than prophylactically. Finally, maraviroc increased morphine's analgesic effect on postsurgery pain. The reason why maraviroc by itself was ineffective in the present study may be that maraviroc was not administered chronically; it was given therapeutically after the insult, and the pain induced by the incision may have different mechanisms than neuropathic pain. Additionally, in a clinical study performed in patients with rheumatoid arthritis treated with methotrexate, maraviroc was found ineffective (Fleishaker et al., 2012).

AMD3100 has also been studied for possible analgesic effects in neuropathic pain in rodents. Intrathecal administration of AMD3100 significantly reduced mechanical allodynia induced by sciatic nerve ligation (Luo et al., 2016) and HIV gp120 (Huang et al., 2014). Additionally, systemic administration of AMD3100 improved mechanical allodynia in diabetic neuropathy (Menichella et al., 2014) and following spinal nerve ligation (Xie et al., 2016). In contrast to these reports, in the present study there was no analgesic effect when AMD3100 was given alone. Possible reasons for this discrepancy are that in the current studies the CRA was administered as a single dose, one time, and in a different pain model.

In the present study, changes in chemokine and cytokine mRNA expression induced by incision and the various treatments were studied in popliteal lymph nodes. Several cytokine and chemokine mRNAs, including those for CCL2/MCP-1, CCL7/MCP-3, CCL12, CXCL2/MIP-2, IL-1 α , IL-1 β , IL-5, and

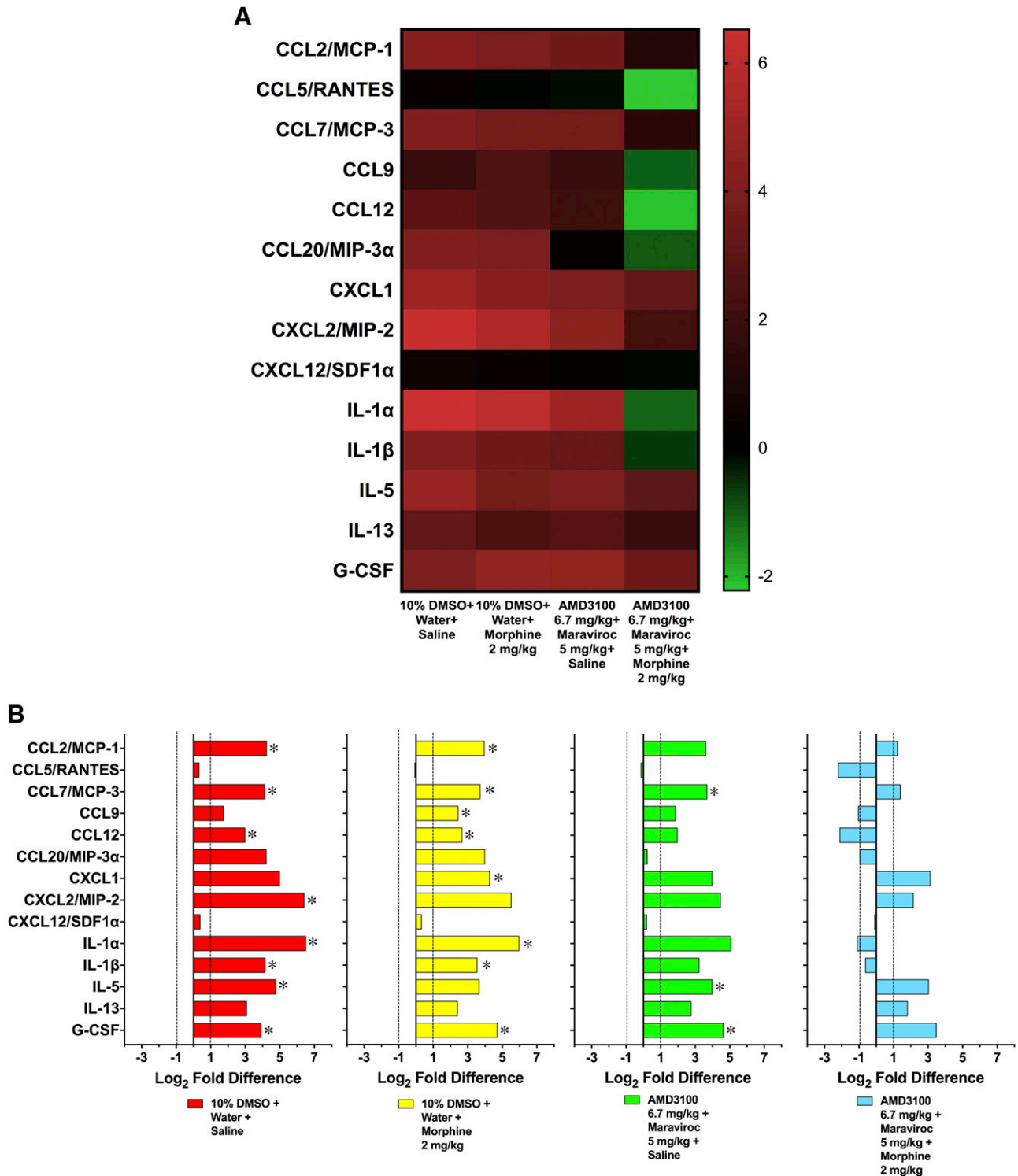


Fig. 8. Effect of incision alone and treatment postincision with morphine alone, AMD3100 + maraviroc, or morphine + AMD3100 + maraviroc on mRNA levels for a panel of chemokines and cytokines. The draining popliteal lymph node was harvested 1 hour postincision and was extracted and processed to obtain mRNA, which was analyzed using the RT² Profiler Array (see *Materials and Methods*). (A) mRNA expression heat map of the popliteal lymph nodes of rats receiving an incision with and without treatments. Treatments are indicated at the bottom of each column. Color scale is a representation of the log₂ fold change in mRNA levels of various immune mediators compared with untreated baseline lymph nodes, ranging from bright red (upregulated to over log₂ 6-fold) to bright green (downregulated to under log₂ 2-fold). Black represents 0-fold change in mRNA expression. Data are presented as the mean level of mRNA expression in lymph nodes from three individual animals. (B) Fold change of mRNA expression in popliteal lymph nodes of rats receiving an incision with and without treatments. The data represent the log₂ fold change in mRNA expression for a panel of immune mediators postincision, with or without treatments, compared with untreated, baseline popliteal lymph nodes. Dotted lines indicate limits of 2-fold up- or downregulation from baseline. Data are presented as the mean level of mRNA expression in lymph nodes from three individual animals. **P* < 0.05 vs. baseline.

G-CSF, were significantly increased 1 hour postsurgery. Morphine alone had a minimal effect on reducing the increased levels of mRNA for the aforementioned chemokines and cytokines. However, the combination of morphine with the two CRAs reduced the mRNA for these elevated inflammatory mediators to baseline levels. Clark et al. (2007) reported that incision induced expression of some cytokines (TNF- α , IL-6, CXCL1/KC/IL-8, IL-1 β , and G-CSF) and neutrophil infiltration in paw tissues of mice following 30 minutes and 2 hours postsurgery, and that morphine reduced incision-induced local cytokine expression. Additionally, Carreira et al. (2013) showed that CXCL1/KC signaling is important for recruiting neutrophils to the incision, and neutrophil infiltration is one of the mediators of postincisional pain in mice. Peters and Eisenach (2010) showed that intrathecal administration of an antibody specific for CCL2 24 hours postincision reversed mechanical allodynia in rats. In the experiments reported here, the results correlating the levels of mRNA for chemokines after incision and treatment with chemokine receptor inhibitors were unexpected. The cognate chemokines, CCL5/RANTES and CXCL12/SDF-1 α , for maraviroc and AMD3100, respectively, were not upregulated by the incision. However, mRNAs for a panel of other unrelated chemokines and cytokines were upregulated, and the combination of morphine plus AMD3100 and maraviroc gave broad downregulation of these mRNAs. At present, we have no model to explain this finding.

Overall, for incisional pain, the evidence supports the conclusion that cytokines and chemokines are involved in the generation of pain stimuli. Since chemokines have also been shown to desensitize opioid receptors, blocking chemokine receptors in combination with opioids appears to be a novel and viable strategy in the field of pain treatment.

The results of the present studies show that maximal analgesia can be obtained by combining two chemokine receptor antagonists with subanalgesic doses of morphine. A strategy of pain relief that uses less morphine has many benefits. Among these are that it might diminish morphine's undesirable side effects. These include tolerance and dependence (Cowan and Macfarlane, 1975; Nozaki et al., 1975; Hasanein et al., 2015; Salmandadeh et al., 2017), inhibition of gastrointestinal motility (Niwa et al., 2002; Gallant and Meert, 2008), and respiratory depression (Emery et al., 2016; Whiteside et al., 2016). These preceding reports show that these adverse effects of morphine are dose dependent, and that the doses of morphine used in the present studies in combination with CRAs are sufficiently low that they would have the possibility of obviating the development of at least some of these adverse effects. Further testing of the effects of the CRAs in combination with morphine on undesirable side effects of the opioid is needed.

In conclusion, the addition of two CRAs, maraviroc and AMD3100 (having no analgesic activity themselves), to a subanalgesic dose of morphine significantly potentiated the analgesic activity of morphine to maximal levels, when tested on incisional pain in rats. The combination of two CRAs with morphine markedly reduced the dose of morphine required to obtain an equal analgesic effect. The results of the present study present an implementable strategy for reducing the dose of highly potent opioid analgesics, which could provide equivalent levels of analgesia and fewer and less serious side effects.

Acknowledgments

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Authorship Contributions

Participated in research design: Inan, Eisenstein, Chen, Tallarida, Geller, Meissler, Rawls, Cowan, Adler.

Conducted experiments: Inan, Watson, Doura, Meissler.

Performed data analysis: Tallarida, Inan.

Wrote or contributed the writing of the manuscript: Inan, Eisenstein, Geller, Meissler, Rawls, Cowan, Adler.

References

- Akgün E, Javed MI, Lunzer MM, Powers MD, Sham YY, Watanabe Y, and Portoghesi PS (2015) Inhibition of inflammatory and neuropathic pain by targeting a mu opioid receptor/chemokine receptor5 heteromer (MOR-CCR₅). *J Med Chem* **58**:8647–8657.
- Ali H, Richardson RM, Haribabu B, and Snyderman R (1999) Chemoattractant receptor cross-desensitization. *J Biol Chem* **274**:6027–6030.
- Benamar K, Geller EB, and Adler MW (2008) Elevated level of the proinflammatory chemokine, RANTES/CCL5, in the periaqueductal grey causes hyperalgesia in rats. *Eur J Pharmacol* **592**:93–95.
- Bidlack JM (2000) Detection and function of opioid receptors on cells from the immune system. *Clin Diagn Lab Immunol* **7**:719–723.
- Brennan TJ, Vandermeulen EP, and Gebhart GF (1996) Characterization of a rat model of incisional pain. *Pain* **64**:493–501.
- Carreira EU, Carregaro V, Teixeira MM, Moriconi A, Aramini A, Verri WA Jr, Ferreira SH, Cunha FQ, and Cunha TM (2013) Neutrophils recruited by CXCR1/2 signalling mediate post-incisional pain. *Eur J Pain* **17**:654–663.
- Chen C, Li J, Bot G, Szabo I, Rogers TJ, and Liu-Chen LY (2004) Heterodimerization and cross-desensitization between the μ -opioid receptor and the chemokine CCR5 receptor. *Eur J Pharmacol* **483**:175–186.
- Chen X, Geller EB, Rogers TJ, and Adler MW (2007a) Rapid heterologous desensitization of antinociceptive activity between mu or delta opioid receptors and chemokine receptors in rats. *Drug Alcohol Depend* **88**:36–41.
- Chen X, Geller EB, Rogers TJ, and Adler MW (2007b) The chemokine CX3CL1/fractalkine interferes with the antinociceptive effect induced by opioid agonists in the periaqueductal grey of rats. *Brain Res* **1153**:52–57.
- Chen X, Kirby LG, Palma J, Benamar K, Geller EB, Eisenstein TK, and Adler MW (2011) The effect of gp120 on morphine's antinociceptive and neurophysiological actions. *Brain Behav Immun* **25**:1434–1443.
- Chuang LF, Chuang TK, Killam KF Jr, Qiu Q, Wang XR, Lin JJ, Kung HF, Sheng W, Chao C, Yu L, et al. (1995) Expression of κ opioid receptors in human and monkey lymphocytes. *Biochem Biophys Res Commun* **209**:1003–1010.
- Clark JD, Shi X, Li X, Qiao Y, Liang D, Angst MS, and Yeomans DC (2007) Morphine reduces local cytokine expression and neutrophil infiltration after incision. *Mol Pain* **3**:28.
- Cowan A and Macfarlane IR (1975) Effect of propranolol on antinociceptive, tolerance- and dependence-producing properties of morphine in rodents and monkeys. *Eur J Pharmacol* **34**:87–94.
- Dansereau MA, Gosselin RD, Pohl M, Pommier B, Mechighel P, Mauborgne A, Rostene W, Kitabgi P, Beaudet N, Sarret P, et al. (2008) Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. *J Neurochem* **106**:757–769.
- Emery MJ, Groves CC, Kruse TN, Shi C, and Terman GW (2016) Ventilation and the response to hypercapnia after morphine in opioid-naïve and opioid-tolerant rats. *Anesthesiology* **124**:945–957.
- Fleishaker DL, Garcia Meijide JA, Petrov A, Kohen MD, Wang X, Menon S, Stock TC, Mebus CA, Goodrich JM, Mayer HB, et al. (2012) Maraviroc, a chemokine receptor-5 antagonist, fails to demonstrate efficacy in the treatment of patients with rheumatoid arthritis in a randomized, double-blind placebo-controlled trial. *Arthritis Res Ther* **14**:R11.
- Gallantine EL and Meert TF (2008) Antinociceptive and adverse effects of μ - and κ -opioid receptor agonists: a comparison of morphine and U50488-H. *Basic Clin Pharmacol Toxicol* **103**:419–427.
- Grimm MC, Ben-Baruch A, Taub DD, Howard OM, Resau JH, Wang JM, Ali H, Richardson R, Snyderman R, and Oppenheim JJ (1998) Opiates transactivate chemokine receptors: δ and μ opiate receptor-mediated heterologous desensitization. *J Exp Med* **188**:317–325.
- Guo CJ, Li Y, Tian S, Wang X, Douglas SD, and Ho WZ (2002) Morphine enhances HIV infection of human blood mononuclear phagocytes through modulation of β -chemokines and CCR5 receptor. *J Invest Med* **50**:435–442.
- Happel C, Steele AD, Finley MJ, Kutzler MA, and Rogers TJ (2008) DAMGO-induced expression of chemokines and chemokine receptors: the role of TGF- β 1. *J Leukoc Biol* **83**:956–963.
- Hasanein P, Teimuri Far M, and Emamjomeh A (2015) *Salvia officinalis* L. attenuates morphine analgesic tolerance and dependence in rats: possible analgesic and sedative mechanisms. *Am J Drug Alcohol Abuse* **41**:405–413.
- Heinisch S, Palma J, and Kirby LG (2011) Interactions between chemokine and mu-opioid receptors: anatomical findings and electrophysiological studies in the rat periaqueductal grey. *Brain Behav Immun* **25**:360–372.
- Huang W, Zheng W, Liu S, Zeng W, Levitt RC, Candiotti KA, Lubarsky DA, and Hao S (2014) HSV-mediated p55TNFSR reduces neuropathic pain induced by HIV gp120 in rats through CXCR4 activity. *Gene Ther* **21**:328–336.
- Kim CH and Broxmeyer HE (1999) Chemokines: signal lamps for trafficking of T and B cells for development and effector function. *J Leukoc Biol* **65**:6–15.
- Kwiatkowski K, Piotrowska A, Rojewski E, Makuch W, Jurga A, Słusarczyk J, Trojan E, Basta-Kaim A, and Mika J (2016) Beneficial properties of maraviroc on

- neuropathic pain development and opioid effectiveness in rats. *Prog Neuro-psychopharmacol Biol Psychiatry* **64**:68–78.
- Lee YK, Choi DY, Jung YY, Yun YW, Lee BJ, Han SB, and Hong JT (2013) Decreased pain responses of C–C chemokine receptor 5 knockout mice to chemical or inflammatory stimuli. *Neuropharmacology* **67**:57–65.
- Li N, Zhang L, Shu R, Ding L, Wang Z, Wang H, Yu Y, and Wang G (2016) Involvement of CCL3/CCR5 signaling in dorsal root ganglion in remifentanyl-induced hyperalgesia in rats. *Clin J Pain* **32**:702–710.
- Luo X, Tai WL, Sun L, Pan Z, Xia Z, Chung SK, and Cheung CW (2016) Crosstalk between astrocytic CXCL12 and microglial CXCR4 contributes to the development of neuropathic pain. *Mol Pain* **12**:1–15.
- Mahajan SD, Schwartz SA, Shanahan TC, Chawda RP, and Nair MPN (2002) Morphine regulates gene expression of α - and β -chemokines and their receptors on astroglial cells via the opioid μ receptor. *J Immunol* **169**:3589–3599.
- Menichella DM, Abdelhak B, Ren D, Shum A, Frietag C, and Miller RJ (2014) CXCR4 chemokine receptor signaling mediates pain in diabetic neuropathy. *Mol Pain* **10**: 42.
- Niwa T, Nakao M, Hoshi S, Yamada K, Inagaki K, Nishida M, and Nabeshima T (2002) Effect of dietary fiber on morphine-induced constipation in rats. *Biosci Biotechnol Biochem* **66**:1233–1240.
- Nozaki M, Akera T, Lee CY, and Brody TM (1975) The effects of age on the development of tolerance to and physical dependence on morphine in rats. *J Pharmacol Exp Ther* **192**:506–512.
- Padi SS, Shi XQ, Zhao YQ, Ruff MR, Baichoo N, Pert CB, and Zhang J (2012) Attenuation of rodent neuropathic pain by an orally active peptide, RAP-103, which potently blocks CCR2- and CCR5-mediated monocyte chemotaxis and inflammation. *Pain* **153**:95–106.
- Peters CM and Eisenach JC (2010) Contribution of the chemokine (C–C motif) ligand 2 (CCL2) to mechanical hypersensitivity after surgical incision in rats. *Anesthesiology* **112**:1250–1258.
- Piotrowska A, Kwiatkowski K, Rojewska E, Makuch W, and Mika J (2016) Maraviroc reduces neuropathic pain through polarization of microglia and astroglia—evidence from in vivo and in vitro studies. *Neuropharmacology* **108**:207–219.
- Ragozzino D (2002) CXC chemokine receptors in the central nervous system: role in cerebellar neuromodulation and development. *J Neurovirol* **8**:559–572.
- Salmanzadeh H, Azizi H, and Semnani S (2017) Adolescent chronic escalating morphine administration induces long lasting changes in tolerance and dependence to morphine in rats. *Physiol Behav* **174**:191–196.
- Szabo I, Chen XH, Xin L, Adler MW, Howard OMZ, Oppenheim JJ, and Rogers TJ (2002) Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. *Proc Natl Acad Sci USA* **99**: 10276–10281.
- Tallarida CS, Bires K, Avershal J, Tallarida RJ, Seo S, and Rawls SM (2014) Ethanol and cocaine: environmental place conditioning, stereotypy, and synergism in planarians. *Alcohol* **48**:579–586.
- Tanaka T, Minami M, Nakagawa T, and Satoh M (2004) Enhanced production of monocyte chemoattractant protein-1 in the dorsal root ganglia in a rat model of neuropathic pain: possible involvement in the development of neuropathic pain. *Neurosci Res* **48**:463–469.
- Tran PB and Miller RJ (2003) Chemokine receptors: signposts to brain development and disease. *Nat Rev Neurosci* **4**:444–455.
- Wetzel MA, Steele AD, Eisenstein TK, Adler MW, Henderson EE, and Rogers TJ (2000) μ -Opioid induction of monocyte chemoattractant protein-1, RANTES, and IFN- γ -inducible protein-10 expression in human peripheral blood mononuclear cells. *J Immunol* **165**:6519–6524.
- Whiteside GT, Hummel M, Boulet J, Beyenhof JD, Strenkowski B, John JD, Knappenberger T, Maselli H, and Koetzner L (2016) Robustness of arterial blood gas analysis for assessment of respiratory safety pharmacology in rats. *J Pharmacol Toxicol Methods* **78**:32–41.
- Wick MJ, Minnerath SR, Roy S, Ramakrishnan S, and Loh HH (1996) Differential expression of opioid receptor genes in human lymphoid cell lines and peripheral blood lymphocytes. *J Neuroimmunol* **64**:29–36.
- Xie F, Wang Y, Li X, Chao YC, and Yue Y (2016) Early repeated administration of CXCR4 antagonist AMD3100 dose-dependently improves neuropathic pain in rats after L5 spinal nerve ligation. *Neurochem Res* **41**:2289–2299.
- Zhang N, Hodge D, Rogers TJ, and Oppenheim JJ (2003) Ca^{2+} -independent protein kinase Cs mediate heterologous desensitization of leukocyte chemokine receptors by opioid receptors. *J Biol Chem* **278**:12729–12736.
- Zhang N, Inan S, Cowan A, Sun R, Wang JM, Rogers TJ, Caterina M, and Oppenheim JJ (2005) A proinflammatory chemokine, CCL3, sensitizes the heat- and capsaicin-gated ion channel TRPV1 [published correction appears in *Proc Natl Acad Sci USA* (2005) **102**:7050]. *Proc Natl Acad Sci USA* **102**:4536–4541.
- Zhang N, Rogers TJ, Caterina M, and Oppenheim JJ (2004) Proinflammatory chemokines, such as C–C chemokine ligand 3, desensitize μ -opioid receptors on dorsal root ganglia neurons. *J Immunol* **173**:594–599.

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Chemokine receptor antagonists enhance the antinociceptive activity of oxycodone and meperidine on incisional pain in rats

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Editor—Opioid and chemokine receptors are members of the G_i protein-coupled seven-membrane receptor family. There is strong evidence for cross desensitisation between opioid and chemokine receptors in both the immune and the neural systems.^{1–4} Peripheral or central administration of chemokines induces hyperalgesia in rodents.^{5–7} Furthermore, we have shown that central administration of chemokines reduces the analgesic activity of opioids in rats.^{8,9} Also, electrophysiological studies of periaqueductal gray neurons showed that CXCL12 and CXCL1 inhibit morphine-induced hyperpolarisation and reduction of input resistance, which provides evidence for crosstalk between mu opioid receptors and chemokine receptors on a cellular level.⁴

Based on these observations, we hypothesised that blocking chemokine receptors using antagonists would prevent desensitisation of opioid receptors by chemokines and increase analgesic activity of opioids. We have previously shown that co-administration of the chemokine receptor antagonists (CRAs) AMD3100 (a CXCR4 antagonist) and maraviroc (a CCR5 antagonist) with morphine potentiated morphine's analgesic effect on incisional pain in rats.¹⁰ Maraviroc and AMD3100 were chosen as the test CRAs because both compounds are US Food and Drug Administration-approved for human use. We investigated whether co-administration of these two CRAs with either oxycodone or meperidine, which are commonly prescribed as analgesics, would similarly increase the antinociceptive effects of these opioids on incisional pain in rats.

Animal care and experimental procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee of Temple University (protocol number 4486). Male Sprague–Dawley rats (200–240 g; Taconic Biosciences, Germantown, NY, USA) were used ($n=8–10$). Rats were acclimated for 30 min, and then their individual baseline values for paw withdrawal threshold were measured using von Frey filaments (North Coast Medical, Inc., Gilroy, CA, USA). The filaments were applied to the plantar side of each hind paw in an ascending manner. Each filament was tested five consecutive times a few seconds apart. A positive response was defined as quick withdrawal or paw flinching after application of a filament.

Surgery was performed as described¹¹ under isoflurane anaesthesia with aseptic conditions. The time when surgeries were completed was designated as time 0. Animals awoke in 5–8 min, and fully regained consciousness by the first testing time after surgery (15 min). Paw withdrawal thresholds were recorded 15–360 min after surgery by a person blinded to the treatments. Since we have previously reported¹⁰ dose–response curves for AMD3100 (1–10 mg kg^{−1}) and maraviroc (0.5–5 mg kg^{−1}), these experiments were not repeated. They had no effect by themselves. For combination experiments, fixed doses (based on our previous results) of maraviroc (5 mg kg^{−1}), AMD3100 (6.7 mg kg^{−1}), or both were co-administered s.c. with different doses of oxycodone (0.25–5 mg kg^{−1}) or meperidine (3–40 mg kg^{−1}) to establish dose–response curves. For co-administration of one CRA with oxycodone or meperidine, rats were injected

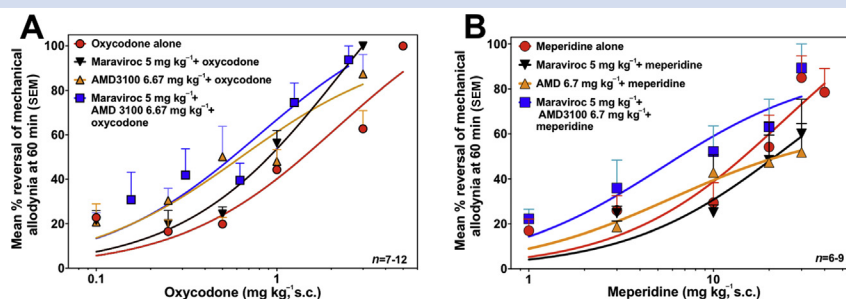


Fig 1. Dose-response curves for opioids alone and with chemokine receptor antagonists. (a) Combination of oxycodone with two chemokine receptor antagonists shifts the oxycodone dose–response curve to the left. Percent reversal of mechanical allodynia was calculated using data at 60 min after incision. (b) The combination of meperidine with two chemokine receptor antagonists shifts the meperidine dose–response curve to the left. Percent reversal of mechanical allodynia was calculated using data at 60 min after incision.

(s.c.) with either vehicle plus oxycodone or meperidine, a fixed dose of the CRA plus oxycodone or meperidine, the CRA alone plus saline, or one of the two vehicles. For co-administration of two CRAs with oxycodone or meperidine, animals received either two vehicles plus oxycodone or meperidine, CRAs plus oxycodone or meperidine, CRAs plus saline, or three vehicles. Reversal of mechanical allodynia at 60 min (time when the effect of both compounds was maximal) was calculated for each rat:

$$\% \text{ reversal of mechanical allodynia} = \frac{[\text{test threshold at 60 min}] - [\text{pre-dose threshold at 15 min}]/[\text{baseline threshold before surgery}] - [\text{pre-dose threshold at 15 min}]}{[\text{pre-dose threshold at 15 min}]} \times 100$$

ED₅₀ values and 95% confidence limits (CL) were established using nonlinear regression analysis. Dose–response curves for oxycodone or meperidine alone and in combination with CRAs were compared using paired Student's *t*-test.¹² Data are expressed as mean (standard error of the mean, SEM), with *P* < 0.05 accepted as statistically significant, and GraphPad Prism, ver. 7 (GraphPad Prism Inc., San Diego, CA, USA), was used for analysis.

Figure 1 shows the dose–response curves for oxycodone and meperidine. ED₅₀ values were oxycodone alone, 1.26 mg kg^{−1} (95% CL, 0.91–1.76); oxycodone with maraviroc, 0.84 mg kg^{−1} (95% CL, 0.64–1.11); oxycodone with AMD3100, 0.63 mg kg^{−1} (95% CL, 0.39–1.01); and oxycodone with both CRAs, 0.66 mg kg^{−1} (95% CL, 0.48–0.91). Combining AMD3100 with oxycodone shifted the oxycodone dose–response 2.0-fold to the left (**P* = 0.042). The combination of two CRAs with oxycodone shifted the oxycodone dose–response 1.9-fold to the left, but it was not statistically significant. ED₅₀ values with meperidine alone were 15.4 mg kg^{−1} (95% CL, 9.3–21.7); meperidine with maraviroc, 19.2 mg kg^{−1} (95% CL, 10.5–30.0); meperidine with AMD3100, 18.5 mg kg^{−1} (95% CL, 8.8–31.1); and meperidine with both CRAs, 6.6 mg kg^{−1} (95% CL, 3.1–11.6). In contrast to oxycodone, combining meperidine with either CRA alone did not shift the meperidine dose–response curve. However, the combination of meperidine with both CRAs shifted the meperidine dose–response 2.3-fold to the left (**P* = 0.014). In our previous study,¹⁰ combining morphine with maraviroc, AMD3100 alone, or both CRAs, shifted the morphine dose–response curve to the left 2.3-, 1.8-, and 3.3-fold, respectively. The present study shows that simultaneous administration of one or two CRAs with oxycodone or meperidine enhances their analgesic potency on incisional pain in a manner similar to that reported for morphine. The differences between results obtained with morphine in contrast to oxycodone and meperidine might be attributable to affinity differences to mu opioid receptors, since oxycodone and meperidine have lower affinity for mu receptors than morphine,^{13,14} the effect of CRAs may be less robust. As all of these opioids are used in the clinical setting, the extension of the work to show that the combination of sub-analgesic doses of any of these opioids with CRAs results in enhanced analgesia, supports the viability of the approach. These studies provide a potential pain treatment strategy that is opioid sparing, permitting maximal analgesia with lower doses of opioids.

Author's contributions

Participated in research design: SI, JJM, XC, CST, EBG, SMR, AC, TKE, MWA.

Conducted experiments: SI, MNW, MD, JJM.

Performed data analysis: CST, SI, JJM.

Wrote or contributed to writing of the paper: SI, JJM, EBG, SMR, AC, TKE, MWA.

All authors accept responsibility for the contents of the paper.

Declaration of interest

Authors declare that they have no conflicts of interest.

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References

1. Zhang N, Hodge D, Rogers TJ, Oppenheim JJ. Ca²⁺-independent protein kinase Cs mediate heterologous desensitization of leukocyte chemokine receptors by opioid receptors. *J Biol Chem* 2003; **278**: 12729–36
2. Chen C, Li J, Bot G, Szabo I, Rogers TJ, Liu-Chen LY. Heterodimerization and cross-desensitization between the μ and the chemokine CCR5 receptor. *Eur J Pharmacol* 2004; **483**: 175–86
3. Zhang N, Rogers TJ, Caterina M, Oppenheim JJ. Proinflammatory chemokines, such as c-c chemokine ligand 3, desensitize μ -opioid receptor on dorsal root ganglia neurons. *J Immunol* 2004; **173**: 594–9
4. Heinisch S, Palma J, Kirby LG. Interactions between chemokine and mu-opioid receptors: anatomical findings and electrophysiological studies in the rat periaqueductal grey. *Brain Behav Immun* 2011; **25**: 360–72
5. Szabo I, Chen XH, Xin L, et al. Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. *Proc Natl Acad Sci* 2002; **99**: 10276–81
6. Zhang N, Inan S, Cowan A, et al. A proinflammatory chemokine, CCL3, sensitizes the heat- and capsaicin-gated ion channel TRPV 1. *Proc Natl Acad Sci* 2005; **102**: 4536–41
7. Benamar K, Geller EB, Adler MW. Elevated level of the proinflammatory chemokine, RANTES/CCL5, in the periaqueductal gray causes hyperalgesia in rats. *Eur J Pharmacol* 2008; **592**: 93–5
8. Chen X, Geller EB, Rogers TJ, Adler MW. Rapid heterologous desensitization of antinociceptive activity between mu or delta opioid receptors and chemokine receptors in rats. *Drug Alcohol Depend* 2007; **88**: 36–41
9. Chen X, Geller EB, Rogers TJ, Adler MW. The chemokine CX3CL1/fractalkine interface with the antinociceptive effect induced by opioid agonists in the periaqueductal gray of rats. *Brain Res* 2007; **1153**: 52–7
10. Inan S, Eisenstein TK, Watson MN, et al. Coadministration of chemokine receptor antagonists with morphine potentiates morphine's analgesic effect on incisional pain in rats. *J Pharmacol Exp Ther* 2018; **367**: 433–41
11. Brennan TJ, Vandermeulen EP, Gebhart GF. Characterization of a rat model of incisional pain. *Pain* 1996; **64**: 493–501
12. Tallarida CS, Bires K, Avershal J, Tallarida RJ, Seo S, Rawls SM. Ethanol and cocaine: environmental place conditioning, stereotypy, and synergism in planarians. *Alcohol* 2014; **48**: 579–86

13. Peckham EM, Traynor JR. Comparison of the antinociceptive response to morphine and morphine-like compounds in male and female Sprague–Dawley rats. *J Pharmacol Exp Ther* 2006; **16**: 1195–201

14. Gourlay GK. Advances in opioid pharmacology. *Support Care Cancer* 2005; **13**: 153–9

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Opioid stewardship: a need for opioid discharge guidance. Comment on Br J Anaesth 2019; 122: e198–e208

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Editor—We read with interest the special and insightful article by Soffin and colleagues¹ on the prescription opioid crisis. They carefully examine many of the drivers for subsequent opioid dependence after surgery and provide suggestions on how practice can and should be improved. As proponents of enhanced recovery programmes, we acknowledge that with reduced postoperative length of stay, and with the increased utilisation of ambulatory/day surgery, patients are no longer being fully weaned off their analgesics by the time of hospital discharge. Thus, surgery is now a risk factor for subsequent opioid dependence, which may be as high as 6.5% in previously opioid naïve patients.² We too are concerned with the dearth of robust data to guide the optimal type and duration of discharge analgesia medication, effective deprescribing strategies, and safe disposal of excess opioid medication with the aim of preventing opioid dependence both in the patient and others through illicit diversion. This lack of clarity further fuels the problem of prescribed postoperative opioid dependence.

At the 6th World Congress of the Enhanced Recovery After Surgery Society in May 2018, we asked delegates to complete an online survey on postoperative opioid stewardship at their institution. Of the 585 international and multidisciplinary delegates, 199 returned the forms. Whilst 47% of the respondents felt that opioids were overprescribed in their country, 76% of respondents' hospitals still measured pain purely by pain intensity scales (e.g. the numerical pain score [NPS]). Only 30% of delegates reported that their patients were explicitly told to avoid repeat/refill prescriptions and only 29% of the respondents' hospitals promoted safe opioid disposal on discharge. The use of simple analgesics on discharge was variable, as was the duration of the discharge opioid prescription. Encouragingly, 36% of the respondents' hospitals had a protocolised pathway for prescribing discharge medication, however, some delegates' institutions advocated

routine use of modified/sustained-release opioid preparations on discharge.

Our data further demonstrate the unwarranted variation in practice. In order to reduce the burden of the prescribed opioid crisis, we concur with the stance of the article¹ that the durations of opioid prescriptions need to be limited, as there is substantial evidence that this is a risk factor for subsequent opioid dependence.^{3,4} We also concur that postoperative use of simple analgesics needs to be encouraged, both for inpatients and after discharge from hospital. This is because there is considerable evidence that the use of multimodal analgesia improves outcomes and has the dual effect of promoting functional return and simultaneously limits opioid consumption and their side-effects.⁵

In addition to the strategies identified by Soffin and colleagues,¹ we recognise that the sole use of the NPS drives opioid administration such that functional activity scores should be used to monitor pain and guide opioid administration.⁶ As sustained/modified-release opioid preparations have been identified as the major risk factor for subsequent opioid dependence,⁴ several North American and Antipodean organisations have now recommended that these preparations should not be used in the management of acute pain.⁷ Thus, there is no place for their routine use in the surgical pathway, and especially as part of discharge medications.⁷

As there is now irrefutable evidence that once an opioid becomes a repeat/refill prescription the risk of subsequent opioid dependence increases,^{3,4} it is essential that all opioid naïve patients are explicitly counselled to avoid repeat/refill opioid prescriptions after discharge. If there is ongoing pain, alternative diagnosis and strategies must be sought and utilised.

Recent evidence from the UK highlights that weak opioids (e.g. codeine, co-codamol) are not innocuous; the risk of subsequent addiction in some instances is higher with weak opioids than with strong opioids.⁸ It is therefore essential that patients are counselled about the increased risks when using these drugs, and medical practitioners need to be aware of the increased risks too. This study⁸ provides further evidence that

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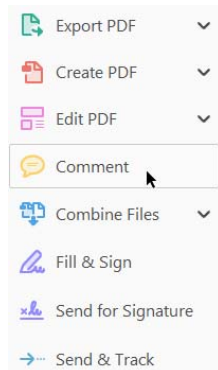
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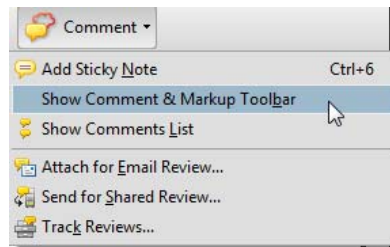


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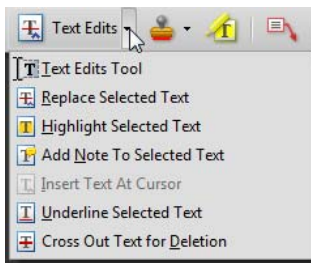


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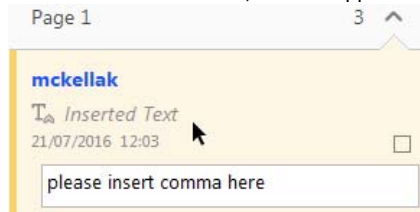


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Chemokine Receptor Antagonists in Combination with Morphine as a Novel Strategy for Opioid Dose Reduction in Pain Management



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ABSTRACT Introduction: Although opioids are widely prescribed for pain, in many circumstances, they have only modest efficacy. Preclinical studies have shown that chemokines, immune mediators released during tissue injury and inflammation, can desensitize opioid receptors and block opioid analgesia by a process termed “heterologous desensitization.” The present studies tested the hypothesis that in evoked pain, certain chemokine receptor antagonists (CRAs), given with a submaximal dose of morphine, would result in enhanced morphine potency. Methods: Three rodent pain assays were used: incisional pain in rats, the cold-water tail flick test in rats, and the formalin test in mice. The FDA-approved, commercially available CRAs, maraviroc and AMD3100 were used. They block the chemokine receptors and ligands, CCR5/CCL5 (RANTES) and CXCR4/CXCL4 (SDF1- α), respectively. Results: In the incisional pain assay, it was found that the combination of a single CRA, or of both CRAs, with morphine significantly shifted the morphine dose-response curve to the left, as much as 3.3-fold. In the cold-water tail flick and formalin tests, significant increases of the antinociceptive effects of morphine were also observed when combined with CRAs. Conclusions: These results support the potential of a new “opioid-sparing” approach for pain treatment, which combines CRAs with reduced doses of morphine.

INTRODUCTION

Pain management continues to be a major medical problem for treatment of personnel in the armed forces, as well as in the civilian population. Overuse of opioids is currently a major problem of epidemic proportion, with 47,600 deaths in 2017.¹ According to a 2017 Substance Abuse and Mental Health Services Administration report, 63% of people who misused opioids gave relief of pain as the major reason for taking the drugs.² It is well recognized that opioids are less effective in chronic pain, which usually involves an underlying inflammatory component.³ Chemokines are a family of 41 small protein molecules released by a variety of immune cells that increase inflammatory responses by attracting subsets of leukocytes that express the cognate chemokine receptor, which leads to a process of directed cell migration to the site of inflammation. Receptors for opioids and chemokines are members of the G-protein-coupled seven-transmembrane family. Several studies have established an interaction between chemokines and

opioids via heterologous cross-desensitization of their receptors. Grimm et al⁴ showed that the directed migration of human peripheral blood mononuclear cells, exposed to a chemokine in vitro, was blocked by prior exposure to the opioid peptide, met-enkephalin. Further, chemokines were shown to desensitize mu opioid receptors on dorsal root ganglia neurons.⁵ Our laboratories have also provided evidence that chemokines can desensitize opioid receptors in the neural system. Rats, cannulated in the pain-sensing periaqueductal gray (PAG) region of the brain, responded to infusion of morphine by keeping their tails immersed in ice-cold water for a longer time (cold-water tail flick [CWTF] test). Infusion of a chemokine up to half an hour prior to giving morphine blocked morphine-induced analgesia.⁶ Further, single neurons of the PAG express both chemokine receptors and opioid receptors.⁷ Our group has also shown that transfected cells can form heterodimers between opioid and chemokine receptors, and the fact that both receptors can be detected on single neurons makes the potential for interaction between these two classes of receptors feasible in the nervous system.⁸ Electrophysiological tracings of PAG neurons have also demonstrated an interaction between chemokines and morphine.⁷ These studies provide a mechanism to explain why in many pain conditions opioids are less effective. If chemokines are released as part of the inflammatory process, they could desensitize opioid receptors, making them less responsive to morphine and other opioids. Based on these observations, the hypothesis was formulated that chemokine receptor antagonists (CRAs), by blocking the signaling ability of chemokines at their receptors, would augment opioid potency and provide greater analgesia for a given dose of

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CRA in Combination with Morphine

an opioid. In these studies, a submaximal dose of morphine was tested in combination with a CRA, or two CRAs, in several pain assays. This strategy was found to be effective in enhancing the potency of morphine in such assays.

METHODS

Animals

Male Sprague-Dawley rats (Taconic Biosciences, Germantown, New York), weighing 175–200 g, were housed in groups of 2, and Swiss-Webster female mice (Taconic Biosciences), weighing 15–20 g, were housed in groups of 5. All animals were acclimated for at least 1 week in an animal room maintained at $22 \pm 1^\circ\text{C}$ and approximately $50 \pm 5\%$ relative humidity. Lighting was on a 12/12 h light/dark cycle. All animals were allowed free access to food and water. Animal care and experimental procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee of Temple University.

Drugs

Morphine sulfate was obtained through the NIDA Drug Supply Program and was dissolved in 0.9% saline. The chemokine antagonists, AMD3100 (dissolved in sterile, pyrogen-free water) and maraviroc (dissolved in 5% DMSO for mice or 10% DMSO for rats) were obtained from Sigma-Aldrich, Inc. (St. Louis, Missouri). AMD3100 ~~specifically~~ binds to the chemokine receptor, CXCR4, and blocks the chemokine, CXCL12/SDF-1.⁹ Maraviroc selectively binds to CCR5 and blocks the chemokine, CCL5/RANTES.^{10,11}

Pain Assessment Assays

Three pain models were used in the present study to test the analgesic potency of morphine alone, or in combination with CRAs.

Incisional Pain Assay in Rats

In this assay, a 1.0-cm longitudinal incision was made with a scalpel through the skin and fascia of the plantar surface of the rat left hind paw, and the post incisional sensitivity to mechanical allodynia was measured in control and treated animals using von Frey filaments. The details of this assay are as follows: 2 days before surgery and behavioral testing, rats were acclimated in individual transparent cubicles with a wire mesh floor for an hour each day. On the day of surgery, rats were acclimated for 30 min and then their individual baseline values for paw withdrawal threshold were measured using a series of von Frey filaments (North Coast Medical, Inc., Gilroy, California), with gradually increasing equal logarithmic bending forces (spanning forces of 2–60 g). The filaments were applied to the plantar side of each hind paw in an ascending manner. Each filament was tested five consecutive times a few seconds apart. A positive response was defined as

quick withdrawal or paw flinching after the application of a filament. Surgery was performed as previously described by Brennan et al.¹² under isoflurane anesthesia (4% isoflurane for induction and 2.5% isoflurane for maintenance of anesthesia) in aseptic conditions. The time when surgeries were completed was designated as time 0. The animals awoke on the average 5–8 min after the end of the surgery, and fully regained consciousness by the first testing time, which was 15 min post surgery. Morphine, CRAs, or their vehicles were injected subcutaneously (s.c.) at $t = 25$ min post surgery into separate sites on the right or left flank, as well as behind the neck area if needed for a third injection. Paw withdrawal thresholds were recorded at various time points (15–360 min) post surgery by a person blinded to the treatments. Percent reversal of mechanical allodynia was calculated at the 60 min time point using the formula:

$$\begin{aligned} \% \text{reversal of mechanical allodynia} = & \left[\frac{(\text{test threshold}) - (\text{predose threshold})}{(\text{baseline threshold}) - (\text{predose threshold})} \right] \times 100 \end{aligned}$$

The test threshold represents paw pressure response to the filament at testing time point; the predose threshold represents the response at 15 min after surgery before treatments; and the baseline threshold represents the baseline value before surgery. The percentage reversal of mechanical allodynia was calculated individually for each rat and then the mean, SD, and SEM values were calculated for individual groups.

Cold-Water Tail Flick Test in Rats

The latency to flick the tail in cold water was used as the antinociceptive index, according to a standard protocol.¹³ A 1:1 mix of ethylene glycol:water was maintained at -3°C in a circulating water bath. Rats were held over the bath with their tails submerged approximately halfway into the solution. All animals were tested at 60, 15, and 0 min before drug injection. For each animal, the first reading was discarded and the mean of the second and third readings was taken as the baseline value. Latencies to tail flick after injection of drugs were expressed as percentage change from baseline. Measurements were taken at 30, 45, and 60 min after injection of vehicle or drugs. A cutoff limit of 60 sec was set to avoid damage to the tail. The percent of maximal possible antinociception (%MPA) for each animal at each time was calculated using the following formula: $\% \text{MPA} = \left[\frac{(\text{test latency} - \text{baseline latency})}{(60 - \text{baseline latency})} \right] \times 100$. To test the effect of morphine, with or without administration of CRAs, the following protocol was used. At $t = 0$, rats were injected s.c. with either morphine or vehicle on one dorsal flank. On the opposite flank, they received a s.c. injection of vehicle or a CRA. If both AMD3100 and maraviroc were used in the same experiment, the two CRAs were injected on the opposite flanks. Control animals received only vehicle injections. Tail

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withdrawal determinations were recorded by a person blinded to the treatments.

Formalin Pain Assay in Mice

The standard formalin paw injection assay was used.¹⁴ A solution of 5% formalin prepared in 0.9% saline was injected into the dorsal surface of the right hind paw of Swiss-Webster, female mice (25–35 g) in a volume of 20 μ L. Morphine, CRAs, or vehicle was injected into separate s.c. sites on the right and left flanks. Mice were then placed in a transparent box for observing the licking response. At $t = +20$ min post formalin injection, the duration time of formalin-injected paw licking was scored for the next 15 min (licking that occurred between 20 and 35 min post formalin injection). Licking was scored by a person blinded to the treatment.

Quantitation of mRNA for Immune Mediators

For the experiments using incisional pain, the effect of morphine alone, CRAs alone, or the combination of morphine plus the CRAs, on levels of mRNA of selected immune mediators, was determined using the TaqMan Gene Expression Assays (Applied Biosystems, ThermoFisher, Foster City, California). Paw tissue samples were collected from baseline animals (no surgery), animals given the incision surgery with no treatment, and rats given surgery plus morphine alone, the CRAs alone, or morphine plus the CRAs. Paw tissues were collected 1 hr post incision by excision and flash freezing to -80°C . Tissues were processed by the Qiagen RNeasy Microarray Tissue Mini Kit (Qiagen, Germantown, Maryland) following the manufacturer's protocols. Individual mRNA preparations were quantitated by a Nanodrop 2000 spectrophotometer (ThermoScientific, Waltham, Massachusetts) to determine mRNA concentrations in ng/ μ L. mRNAs from paw tissue of three individual rats from each treatment group were used to generate cDNAs using the SuperScript III First-Strand Synthesis System for RT-PCR (inVitrogen, ThermoFisher, Carlsbad, California), which were then assayed using the TaqMan Gene Expression Assays for rat CXCL1, CXCL2/MIP-2, CCL2/MCP-1, CCL5/RANTES, CXCL12/SDF-1 α , and β 2-microglobulin. Each gene expression assay was tested on all three samples from each treatment group. The reactions were performed using TaqMan Fast Advanced Master Mix. The RT-PCR array was run on an Applied Biosystems StepOne Plus RT-PCR thermocycler, using the cycling conditions for TaqMan reagents. Results obtained are expressed as the mean $\log_2$ -fold difference in mRNA expression in the treated groups compared with the level of mRNA expression in the baseline untreated control. Data were processed by the DataAssist v3.01 software (Applied Biosystems).

Statistical Analysis

The size of groups of animals used in the pain tests varied from 7 to 10. Power analysis determined 6 animals/group to be sufficient. Where the power is 80%, alpha is 0.05, beta is

0.2, incidence for group 1 is 10% and for group 2 is 90%. Data for the results of pain tests are expressed as the mean $\pm$ the standard error of the mean. Differences between groups were assessed using either one- or two-way analysis of variance (ANOVA) depending on the number of variables. The Sidak's multiple comparison test was applied if the ANOVA was $p < 0.05$ in order to test whether morphine alone vs morphine plus a CRA was significant. The data were analyzed using Prism Graphpad software version 7. In all cases, $p < 0.05$ was taken as the significant level of difference.

RESULTS

Incisional Pain Test in the Rat

These experiments sought to test whether the combination of one or two CRAs with a submaximal dose of morphine could result in maximal analgesia. Neither CRA produced significant analgesia by itself in the incisional pain test.¹⁵ The analgesic activity of the combination of various doses of morphine plus either AMD3100 (6.7 mg/kg) or maraviroc (5.0 mg/kg) and of morphine in combination with both CRAs was tested. The composite results of von Frey measurements taken at 60 min are shown in Figure 1. The combination of morphine with a single CRA significantly shifted the morphine dose-response curve to the left (ED_{50} for morphine alone = 2.34 mg/kg; ED_{50} for morphine + AMD3100 = 0.132 mg/kg, a 1.8-fold shift; ED_{50} for morphine + maraviroc = 1.01, a 2.3-fold shift). The ED_{50} of the combination of morphine plus both CRAs was 0.71 mg/kg, a 3.3-fold shift to the left compared to morphine alone.

mRNA Levels in Paw Tissue in the Incisional Pain Test

As shown in Figure 2, mRNAs for the 5 chemokines analyzed were detected in paw tissue 1 hr after incision. mRNA for CCL5 and CXCL12, the chemokines that are blocked by maraviroc and AMD3100, respectively, did not show an elevation as a result of the incision. The other 3 chemokines, which are involved in acute inflammatory responses, CXCL1, CXCL2 and CCL2, were higher than baseline, but their increase was only a little over 2-fold. Although none of the results were statistically significant for these 3 chemokines, the CRAs alone, or the combination of the CRAs with the suboptimal dose of morphine, shifted mRNA levels to below baseline.

Cold-Water Tail Flick Test in the Rat

A similar series of experiments was carried out for the CWTF test as was done for the incisional pain test described above. Individual dose-response and time curves were obtained for AMD3100 and maraviroc, each tested individually, for analgesic activity. Neither CRA had any intrinsic antinociceptive activity in this test (data not shown). Morphine alone gave a

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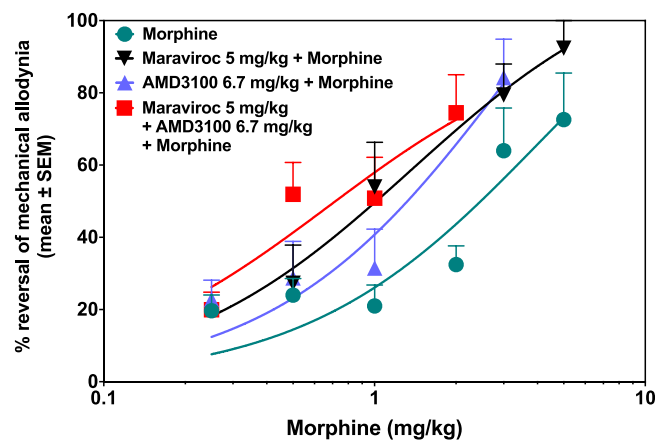


FIGURE 1. Combination of morphine with 2 CRAs in the incisional pain test shifts the morphine dose-response curve to the left. Rats were injected with morphine alone or with the CRAs, into separate s.c. dorsal sites 25 min after completion of the surgery. Percent reversal of mechanical allodynia was calculated using data at 60 min post incision. The combination of morphine with either AMD3100 or maraviroc, individually, as well as the combination of morphine with both CRAs, significantly shifted the morphine dose-response curve to the left. The paired Student t-test showed $*p < 0.05$, ($n = 6-10$). (From: S Inan, TK Eisenstein, MN Watson, M Doura, JJ Meissler, CS Tallarida, X Chen, EB Geller, SM Rawls, A Cowan, and MW Adler (2018). Co-administration of Chemokine Receptor Antagonists with Morphine Potentiates Morphine's Analgesic Effect on Incisional Pain in Rats. *J Pharmacol Exp Ther.* 367 (3): 433–44. [doi:10.1093/jpet/kxy035](#) Used with permission).

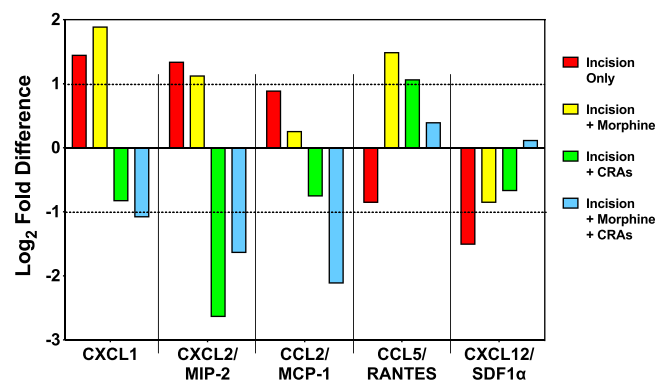


FIGURE 2. Fold difference of gene expression in rat paw tissue, from animals receiving incision and morphine plus CRAs. Data are expressed as log2-fold difference in expression compared with untreated control rat samples. Data are presented as the mean level of mRNA expression in paw tissue from 3 individual animals. Dotted lines indicate limits of 2-fold up- or down-regulation from baseline.

typical dose-response curve with significant analgesia (83% of the MPA) observed at all time points for the 10 mg/kg dose, and lesser analgesia observed with lower doses. Pain thresholds were measured at 30, 45, and 60 min, but only the results that showed maximal statistical significance are presented in Figure 3, which was at 30 min for maraviroc and 45 min for AMD3100. When the 5 mg/kg dose of morphine was combined with maraviroc (5 mg/kg), significant enhancement of analgesia was observed compared to morphine alone at the 30-minute time point (Fig. 3A). The combination of morphine (3 mg/kg) and AMD3100 (7.5 mg/kg) produced a

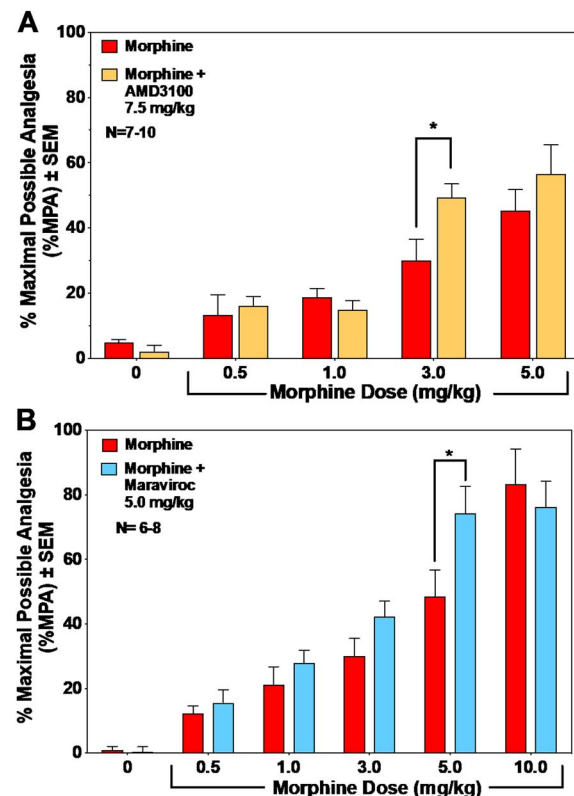


FIGURE 3. Effects of the combination of a submaximal dose of morphine plus a chemokine in the cold-water tail flick test. Panel A: Percent maximal possible analgesia of different doses of morphine in combination with AMD3100. Measurement at 30 min post drug or vehicle injection. Panel B: Percent maximal possible analgesia of different doses of morphine in combination with maraviroc. Measurement at 45 min post drug or vehicle injection. Two-way ANOVA followed by Sidak's multiple comparison test showed significant differences between morphine alone vs. morphine plus a CRA. $*p < 0.05$.

significant enhancement of the MPA compared to morphine alone at the 45 min time point (Fig. 3B).

Formalin Test in the Mouse

As in the other two pain tests described above, neither AMD3100 nor maraviroc alone displayed analgesic activity (data not shown). When a submaximal dose of morphine (1 mg/kg) was combined with either AMD3100 (5 mg/kg) or maraviroc (5 mg/kg), significantly enhanced analgesia was observed compared to morphine alone, as evidenced by decreased paw-licking time (Fig. 4A and B).

DISCUSSION

The relationship of various chemokines to several pain states has been reported.^{16–18} Our laboratories reported that CCL5 induced hyperalgesia in the CWTF test, and an antibody to this chemokine blocked the effect.¹⁹ Various pain stimuli have been shown to be reduced in CCR5 knockout mice.^{20,21} Pain associated with administration of antiretroviral drugs that induce toxic neuropathy, alone or in combination

CRAs in Combination with Morphine

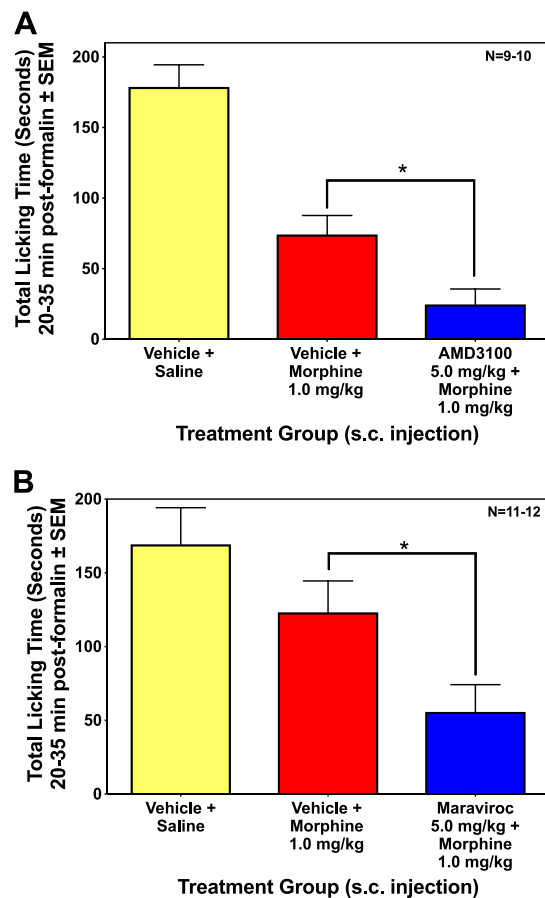


FIGURE 4. Effect of combinations of morphine with AMD3100 (panel A) or maraviroc (panel B) on analgesia in the mouse formalin test. One-way ANOVA followed by Sidak's multiple comparison test showed significant differences between morphine alone and either morphine plus AMD3100 (panel A) or morphine plus maraviroc (panel B). * $p < 0.05$.

with HIV gp120, has been shown to be mediated by up-regulation of CXCL12, and blocked by treatment with a CRA, AMD3100.^{22,23} A synthetic dual CRA, RAP-103, that targets CCR5 and CCR2, attenuated neuropathic pain in rats.²¹ A bivalent ligand that is a mu opioid agonist and a CCR5 antagonist produced nociception.²⁴ The concept of using a CRA in conjunction with reduced doses of morphine for pain control emanated from experiments that had been conducted in our laboratories, and confirmed by others, showing that selective chemokines blocked morphine analgesia via a process termed “heterologous desensitization.”^{5,6} These observations led to the formulation of the hypothesis that inhibition of chemokines could augment the potency of morphine. In this article, data are presented using three different pain assays in rodents to support this hypothesis. It was shown in all three tests that submaximal doses of morphine, when combined with a single CRA, produced analgesia that was significantly greater than that observed with morphine alone. In the incisional test, the combination of morphine plus 2 CRAs was tested and found to be superior

to that of morphine plus a single CRA. Results were most robust in the incisional pain assay where the compounds were given therapeutically after surgery. Morphine plus AMD3100 and maraviroc shifted the morphine dose-response curve an impressive 3.3-fold to the left. In the CWTF test and the formalin pain test, significant increases in antinociception were also found using selected submaximal doses of morphine plus a CRA. The mechanism by which the CRAs are exerting this additive effect with morphine is hypothesized to be by blocking the heterologous desensitization of morphine receptors by the chemokines. Based on this theory, it was expected that the combination treatment would lower the levels of CCL5 and CXCL12. The results in Figure 2 show that mRNA for these chemokines, that are the cognate ligands of the CRA inhibitors that were used, were not elevated. These individual PCR results corroborate data obtained using an mRNA array.¹⁵ The mechanism by which the morphine plus CRA therapy lowers mRNA for chemokines that are not part of the combination administered is not currently understood.

Of the three pain assays presented in this article, two were carried out in male rats, the incisional pain test and cold-water tail flick test. The results for the formalin test were carried out in female mice. This article does not attempt to make a conclusion about differences between sexes in response to the pain assays or treatment. The only assay where both male and female animals were used was the formalin assay. Because the results in male animals were more variable than what was observed in female mice, only the results for the females are presented in this manuscript.

As part of these studies, the paradigm of combining a suboptimal dose of morphine with one or more CRAs was also tried in a carrageenan rat pain model (resulting in both pain and edema) where the substance is injected into the paw, and paw withdrawal thresholds tested using a heat beam, and in a rat paclitaxel model of chemotherapy-induced neuropathic pain assessed by mechanical allodynia using von Frey filaments. The combination therapy of morphine plus CRAs was not effective in either of these pain tests. The choice of preclinical pain tests to best simulate human pain conditions is complex, and different mechanisms may be involved in different types of pain.³

In the present study, the combination therapy of a submaximal dose of morphine in combination with one or more CRAs was efficacious in three different pain assays. These results represent a new approach to pain treatment that has potential for use in a variety of acute and chronic pain conditions resulting from surgery or injury. This opioid-sparing strategy might have the additional advantage of reducing opioid-induced side effects such as respiratory depression and inhibition of gastrointestinal transit, as morphine could be used in markedly lower doses. Experiments are in progress to explore if these morphine-CRA combinations do in fact result in diminished undesirable effects of opioids.

*CRAs in Combination with Morphine***ACKNOWLEDGMENTS**

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REFERENCES

- Scholl L, Seth P, Kariisa M, Wilson N: Drug and opioid-involved overdose deaths—United States, 2013–2017. *Morb Mortal Wkly Rep* 2019; 67(51-52): 1419–27.
- Ahrnsbrak R, Bose J, Hedden SL, Lipari RN, Park-Lee E: Key Substance Use and Mental Health Indicators in the United States: Results from the 2016 National Survey on Drug Use and Health (NSDUH). Washington, D.C., Substance Abuse and Mental Health Services Administration, DHHS, 2017. Available at <https://store.samhsa.gov/system/files/sma17-5044.pdf>; accessed March 19, 2019.
- Martinez-Navarro M, Maldonado R, Banos JE: Why mu-opioid agonists have less analgesic efficacy in neuropathic pain? *Eur J Pain* 2019; 23: 435–54.
- Grimm MC, Ben-Baruch A, Taub DD, Howard OMZ, Wang JM, Oppenheim JJ: Opiate inhibition of chemokine-induced chemotaxis. *Ann NY Acad Sci* 1998; 840: 9–20.
- Zhang N, Rogers TJ, Caterina M, Oppenheim JJ: Proinflammatory chemokines, such as C-C chemokine ligand 3, desensitize μ -opioid receptors on dorsal root ganglia neurons. *J Immunol* 2004; 173: 594–9.
- Szabo I, Chen XH, Xin L et al: Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. *Proc Natl Acad Sci USA* 2002; 99: 10276–81.
- Heinisch S, Palma J, Kirby LG: Interactions between chemokine and mu-opioid receptors: anatomical findings and electrophysiological studies in the rat periaqueductal gray. *Brain Behav Immun* 2011; 25: 360–72.
- Chen C, Li J, Bot G, Szabo I, Rogers TJ, Liu-Chen LY: Heterodimerization and cross-desensitization between the μ -opioid receptor and the chemokine CCR5 receptor. *Eur J Pharmacol* 2004; 483: 175–86.
- Fricker SP, Anastassov V, Cox J et al: Characterization of the molecular pharmacology of AMD3100: a specific antagonist of the G-protein coupled chemokine receptor, CXCR4. *Biochem Pharmacol* 2006; 72: 588–96.
- Dorr P, Westby M, Dobbs S et al: Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrob Agents Chemo* 2005; 49: 4721–32.
- Woollard SM, Kanmogne GD: Maraviroc: a review of its use in HIV infection and beyond. *Drug Disc Devel Ther* 2015; 9: 5447–68.
- Brennan TJ, Vandermeulen EP, Gebhart GF: Characterization of a rat model of incisional pain. *Pain* 1996; 64: 493–501.
- Pizziketti RJ, Pressman NS, Geller EB, Cowan A, Adler MW: Rat cold water tail-flick: a novel analgesic test that distinguished opioid agonists from mixed agonist-antagonists. *Eur J Pharmacol* 1985; 119: 23–9.
- Hunskar S, Hole K: The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* 1987; 30: 103–14.
- Inan S, Eisenstein TK, Watson MN et al: Coadministration of chemokine receptor antagonists with morphine potentiates morphine's analgesic effect on incisional pain in rats. *J Pharmacol Exp Ther* 2018; 367: 433–41.
- Dawes JM, McMahon SB: Chemokines as peripheral pain mediators. *Neurosci Lett* 2013; 557: 1–8.
- Miller RJ, Jung H, Bhangoo SK, White FA: Cytokine and chemokine regulation of sensory neuron function. In: *Handbook of Experimental Pharmacology*. Edited by Canning BJ, Spina D. Berlin Heidelberg, Springer-Verlag, 2009.
- Abbadie C, Bhangoo S, De Y, Malcangio M, Melik-Parsadaniantz S, White FA: Chemokines and pain mechanisms. *Brain Res Rev* 2009; 60: 125–34.
- Benamar K, Geller EB, Adler MW: Elevated level of the proinflammatory chemokine, RANTES/CCL5, in the periaqueductal grey causes hyperalgesia in rats. *Eur J Pharmacol* 2008; 592: 93–5.
- Lee YK, Choi DY, Jung YY et al: Decreased pain responses of C-C chemokine receptor 5 knockout mice to chemical or inflammatory stimuli. *Neuropharmacol* 2013; 67: 57–65.
- Padi SSV, Shi XQ, Zhao YQ et al: Attenuation of rodent neuropathic pain by an orally active peptide, RAP-103, which potently blocks CCR2- and CCR5-mediated monocyte chemotaxis and inflammation. *Pain* 2012; 153: 95–106.
- Bhangoo SK, Ren D, Miller RJ et al: CXCR4 chemokine receptor signaling mediates pain hypersensitivity in association with antiretroviral toxic neuropathy. *Brain Behav Imm* 2007; 21: 581–91.
- Bhangoo SK, Ripsch MS, Buchanan DJ, Miller RJ, White FA: Increased chemokine signaling in a model of HIV 1-associated peripheral neuropathy. *Mol Pain* 2009; 5: 48–65.
- Akgün E, Javed MI, Lunzer MM et al: Inhibition of inflammatory and neuropathic pain by targeting a mu opioid receptor/chemokine receptor 5 heteromer (MOR-CCR5). *J Med Chem* 2015; 58: 8647–57.