

JPET # 252890

**Co-Administration of Chemokine Receptor Antagonists with Morphine
Potentiates Morphine's Analgesic Effect on Incisional Pain in Rats**

Saadet Inan, Toby K Eisenstein, Mia N Watson, Menahem Doura, Joseph J
Meissler, Christopher S Tallarida, Xiaohong Chen, Ellen B Geller, Scott M Rawls,
Alan Cowan, and Martin W Adler

Center for Substance Abuse Research, Lewis Katz School of Medicine at Temple
University, 3500 North Broad Street, Philadelphia, PA 19140

Running title page

Co-administering chemokine receptor antagonist and morphine

Correspondence: Saadet Inan

Center for Substance Abuse Research, Lewis Katz School of Medicine at Temple

University, 3500 North Broad Street, Philadelphia, PA 19140

Email: sinan@temple.edu

Tel: (267)-872-7560

Fax: (215)-707-6661

Number of text pages: 45

Number of figures: 11

Number of Tables: 1

Number of references: 48

Number of words in the *Abstract*: 250

Number of words in the *Introduction*: 647

Number of words in the *Discussion*: 1339

Abbreviations: CCR5, CC chemokine receptor type 5; CRA, chemokine receptor antagonist; CRAs, chemokine receptor antagonists; CNS, central nervous system; CWTF, cold water tail flick; CXCR4, CXC chemokine receptor type 4; DMSO, dimethyl sulfoxide

Recommended section: Behavioral Pharmacology

Abstract

Cross-desensitization between opioid and chemokine receptors and involvement of chemokines in pain modulation are well established. We investigated if co-administration 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 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 2.3-fold and 1.8-fold, respectively. Co-administration 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; Chen et al., 2004; Zhang 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 (CNS), 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 CNS (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 CNS as well (Szabo et al., 2002; Chen et al., 2007a; Chen et al., 2007b). Szabo et al. (2002) showed that administering either

CXCL12/SDF-1 α or CCL5/RANTES into the periaqueductal gray (PAG) blocked the analgesic effect of the mu-selective opioid, DAMGO, given 30 min later into the PAG, as measured by the cold-water tail flick (CWTF) assay. Pretreatment of animals with an antibody against CCL5/ RANTES inhibited the hyperalgesia 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 knock-out (KO) mice compared to wild-type mice (Lee et al., 2013). Further, these researchers also reported that mu-opioid receptor immunoreactivity in the PAG area is higher in CCR5 KO mice than wild-type mice. Interestingly, gp120, an 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 cross-desensitization 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 FDA-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 co-administered with morphine in a rat assay of incisional pain (model for clinical post-surgical 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 (an hour each day).

Surgery

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, 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 min after the end of the surgery, and fully regained consciousness by the first testing time, which was 15 min post-surgery. Paw withdrawal thresholds were recorded at time points post-surgery (15, 30, 45, 60, 120, 240, and 360 min; 24, 48, and 72 hr) by a person blinded to the treatments. Morphine, chemokine receptor antagonists, or their vehicles were injected subcutaneously (s.c.) at $t = 25$ min post-surgery. 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 (from NIDA) was dissolved in sterile saline. Maraviroc and AMD3100 were purchased from Sigma-Aldrich (St. Louis, MO). Maraviroc was dissolved in 10% DMSO (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 (s.c.) at t = 25 min post-surgery. 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 5 mg/kg and AMD3100 6.7 mg/kg) were co-administered with different doses of morphine to establish dose-response curves. In the experiments for co-administration 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 co-administration 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. Percent reversal of mechanical allodynia was calculated using the formula:

$$\% \text{ reversal of mechanical allodynia} = \frac{[(\text{test threshold}) - (\text{pre-dose threshold}) / (\text{baseline threshold}) - (\text{pre-dose threshold})] \times 100}{}$$

*Test threshold: paw pressure response to the filament at testing time point

*Pre-dose threshold: response at 15 min after surgery before treatments

*Baseline threshold: baseline value before surgery

% reversal mechanical allodynia was calculated individually for each rat and then calculated mean, SD, and SEM for individual groups. In drug combination studies, percent reversal of mechanical allodynia at 60 min was used because morphine's effect was maximal at post-surgery 60 min.

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 Figure 7, but they were sacrificed at 1 hr post incision. 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 ng/μl. mRNAs from individual lymph nodes were used to generate cDNAs using the RT² First Strand[®] kit (Qiagen), which were then assayed using the RT² Profiler[®] PCR Array with the RT² SYBR[®] Green ROX[®] qPCR Mastermix (Qiagen). 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 PCR 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). 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 comparison tests.

To establish ED₅₀ values and 95% confidence limits for morphine and for the combination of morphine with a single CRA or with both CRAs, data from dose-response curves measuring percent reversal of mechanical allodynia at 60 min 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 \pm standard error of the mean (S.E.M.), and $p < 0.05$ was accepted as statistically significant. GraphPad Prism, version 7, was used for data analysis.

Statistics on the results of the RT² Profiler® Arrays were done by the Student t-test, using the online GeneGlobe Data Analysis Center (Qiagen).

Results

Morphine, but not maraviroc nor AMD3100, induces analgesia in a time- and dose-dependent manner

Figures 1, 2 and 3 show time- and dose-dependent % reversal of mechanical allodynia for rats receiving morphine alone, maraviroc alone, or AMD3100 alone, respectively. Baseline (pre-incision) paw withdrawal thresholds were similar for all rats in the different groups (33.91 ± 2.28 g), and they dropped abruptly 15 min after the incision in all groups (3.22 ± 0.20 g). Time 0 is the time surgery is completed; 15 min post-surgery is the time that baseline measurements were taken; and 25 min post-surgery 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 to saline ($p < 0.001$). The analgesic effect started at 45 min, reached a maximum at 60 min, lasted until 120 min, and disappeared by 240 min post-surgery. Morphine at 1 and 3 mg/kg did not produce analgesia (Fig.1, 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 hr, but no analgesia was observed at these time points (data not shown). ED_{50} 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 co-administration 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 of morphine. Maraviroc 2.5 mg/kg was co-administered with morphine at doses ranging from 1-5 mg/kg. Since the maximal effect with morphine was established at 60

min post-surgery, percent reversal of mechanical allodynia was calculated for all groups at this time point. As seen in Fig. 4A, co-administration of maraviroc at 2.5 mg/kg with morphine at 5 mg/kg significantly increased the analgesic effect compared to 10% DMSO plus morphine (5 mg/kg) ($p < 0.05$; 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 $49.8 \pm 13.1\%$, addition of maraviroc 2.5 mg/kg increased this effect to $100 \pm 0\%$ ($p < 0.01$). The ED_{50} value for the combination of morphine and maraviroc 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 0.5-5 mg/kg. (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 ED_{50} for this combination was calculated as 1.01 mg/kg (95% CL: 0.60-1.56, Table 1.). Co-administration 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 mg/kg and 0.5 to 3 mg/kg, respectively). Results for co-administration of AMD3100 2.5 mg/kg with morphine at 5 mg/kg (Two-way ANOVA revealed a significant effect of dose [$F(4,61)= 9.776$, $p < 0.0001$] but not for 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 (Two-way ANOVA revealed a significant effect of dose [$F(4,69)=20.27$, $p < 0.0001$] but not for 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. 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 (Table1). Co-administration 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).

Co-administration of both maraviroc and AMD3100 with morphine significantly enhances the potency of morphine

As 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, co-administration of both CRAs with morphine significantly potentiated morphine's analgesic effect (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 1/5th of its maximal effective dose, reversed mechanical allodynia $74.5 \pm 11.1\%$ (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 the methods section. The objective was to screen a large number of immune mediators to assess how the surgery and treatments with morphine, with CRAs, or with 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, Figures 8 A and B only presents the results for the ones of interest or with significant changes. 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-1 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.

Discussion

The present study tested the hypothesis that simultaneous administration of one or two CRAs with a sub-analgesic 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 sub-analgesic dose of morphine, a modest enhancement in analgesia was observed. 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 mg/kg to 1.01 mg/kg 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 to 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 post-surgery condition, and is a more stringent paradigm than a prophylactic experimental design. Further, this 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 (i.t.) before chronic constrictive nerve injury, and then continued by i.t. 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 i.t. before remifentanyl and found that pretreatment with maraviroc inhibited remifentanyl-induced thermal and mechanical allodynia. Akgun et. al. (2015) showed that i.t. 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 to 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, 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. Further, single neurons in the PAG 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 chemokine receptor antagonist with morphine can potentiate the activity of morphine, is that the CRA is preventing cross-

desensitization 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 cross-desensitization 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. Further, the effect on incisional pain was evaluated using the drugs therapeutically, rather than prophylactically. Finally, maraviroc increased morphine's analgesic effect on post-surgery 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 G-CSF, were significantly increased 1 hr post-surgery. Morphine alone had a minimal effect on reducing the increased levels of mRNA for chemokines and cytokines mentioned above. 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 min and 2 hr post-surgery, 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 post-incisional pain in mice. Peters and Eisenach (2010) showed that i.t. administration of an antibody specific for CCL2 24 hr post-incision 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 sub-analgesic 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; Hasnein et al., 2015; Nozaki et al., 1975; Salmanzadeh et al., 2017), inhibition of gastrointestinal motility (Niwa et al., 2002; Gallantine 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, addition of two CRAs, maraviroc and AMD3100 (having no analgesic activity themselves), to a sub-analgesic 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

The authors thank to Miss Emily Dziedowiec for technical support.

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

- Akgun E, Javed MI, Lunzer MM, Powers MD, Sham YY, Watanabe Y, and Portoghese 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, Carregora V, Teixeira MM, Moriconi A, Aramini A, Verri Jr WA, Ferreira SH, Cunha FQ, and Cunha TM (2013) Neutrophils recruited by CXCR1/2 signaling 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 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 interface 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 gp 120 on morphine's antinociceptive and neurophysiological actions. *Brain Behav Immun* **25**:1434-1443.

Chuang LF, Chuang TK, Killam Jr KF, Qiu Q, Wang XR, Lin JJ, Kung HF, Sheng W, Chao C, and Yu L. (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 DY, 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, and Melik-Parsadaniantz S (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, Meijide JAG, Petrov A, Kohen MD, Wang X, Menon S, Stock TC, Mebus CA, Goodrich JM, Mayer HB, and Zeiher BG (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 U-50488-H. *Basic Clin Pharmacol Toxicol* **103**: 419-427.

Grimm MC, Ben-Baruch A, Taub DD, Howard OMZ, Resau JH, Wang JM, Ali H, Richardson R, Snyderman R, and Oppenheim JJ (1998) Opiates transdeactivate 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 mononuclear phagocytes through modulation of β -chemokines and CCR5 receptor. *J Investing Med* **50**: 435-442.

Happel H, 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 Leuk Bio* **83**: 956-963.

Hasanein P, Far MT, 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, Lewitt 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, Rojewska E, Makuch W, Jurga A, Slusarczyk

J, Trojan E, Basta-Kaim A, and Mika J (2016) Beneficial properties of maraviroc on neuropathic pain development and opioid effectiveness in rats. *Prog Neuropsychopharmacol Biol Psychiatry* **39** :2245-2251.

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 to chemical or inflammatory stimuli. *Neuropharmacol* **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, Freitag 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*

Biothechnol Biochem **66**: 1233-1240.

Nozaki M, Akera T, Lee CY, and Brody TM (1975) The effects of age on the

development of the 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 potentially blocks CCR2- and CCR5-mediated monocyte chemotaxis

and inflammation. *Pain* **153**: 95-106.

Peters CM, and Eisenach JC (2010) Contribution of the chemokine CCL2 to

mechanical hypersensitivity following 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 Semnanian 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*

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. Chemokine receptors: signposts to brain development

and disease. *Nat Rev Neurosci* **4**: 444-455.

Wetzel MA, Steele LD, Eisenstein TK, Adler MW, Henderson EE, and Rogers TJ.

(2000) μ -opioid induction of monocyte chemokine 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 Y, and Yue Y (2016) Early repeated administration of CXCR4 antagonist AMD 3100 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, Rogers TJ, Caterina M, and Oppenheim JJ (2004) Proinflammatory

JPET # 252890

chemokines, such as c-c chemokine ligand 3, desensitize μ -opioid receptors on dorsal root ganglia neurons. *J Immunol* **173**: 594-599.

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 TRPV 1. *Proc Natl Acad Sci*

102: 4536-4541.

Footnotes

This work was supported by the Department of Defense and U.S. Army [Grant W81XWH-15-1-0252]; and the National Institute of Health/National Institute on Drug Abuse [P30 Grant DA 013429].

This work was presented, in part, International Narcotics Research Conference, 2017, Chicago, IL; and Mid-Atlantic Pharmacology Society, 2017, Philadelphia, PA.

Figure Legends

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

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

Fig.3. AMD3100 time- and dose-response 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 (s.c.) at $t = 25$ min and paw withdrawal thresholds were measured at 30, 60, 120, 240, and 360 min post-surgery. Percent reversal of mechanical allodynia was calculated. No analgesia was observed with AMD3100. Two-way ANOVA followed by Tukey's multiple comparison revealed no significant differences ($n=8-10$).

Fig. 4. Effect of the co-administration of maraviroc with morphine on incisional pain. Determination of baseline values and surgery are the same as in Fig.1. Animals were injected at $t = 25$ min with: Panel A: 10% DMSO + morphine (s.c.), or maraviroc 2.5 mg/kg + morphine (s.c.) or Panel B 10% DMSO + morphine (s.c.), or maraviroc 5 mg/kg + morphine (s.c.). Paw withdrawal thresholds were measured from 30 through 360 min. Percent reversal of mechanical allodynia was calculated using data at 60 min. Co-administration of maraviroc 2.5 mg/kg with morphine 5 mg/kg significantly increased morphine's analgesic effect (Panel A). Two-way ANOVA followed by Sidak's multiple comparison test showed $**p < 0.01$ ($n=8-10$).

Fig.5. Effect of co-administration of AMD3100 with morphine on incisional pain. Determination of baseline values and surgery are the same as in Fig.1. Animals were injected at $t = 25$ min with: Panel A: water + morphine (s.c.) or AMD3100 2.5 mg/kg + morphine (s.c.) or Panel B: water + morphine (s.c.) or AMD3100 6.7 mg/kg + morphine (s.c.). Paw withdrawal thresholds were measured at time points between 30 and 360

min. Percent reversal of mechanical allodynia was calculated using data at 60 min. Co-administration of AMD3100 2.5 mg/kg with morphine 5 mg/kg (Panel A) and co-administration of AMD3100 6.7 mg/kg with morphine (3 mg/kg) (Panel B) show a trend to increase analgesia (Two-way ANOVA revealed significance only for dose) (n=8-10).

Fig. 6. Effect of co-administration 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 min animals were injected with either 10% 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. Percent reversal of mechanical allodynia was calculated using data at 60 min. Combining 2 CRAs with sub-analgesic doses of morphine (0.5, 1, and 2 mg/kg) significantly increased morphine's analgesic effects. Two-way ANOVA followed by Tukey's multiple comparison showed, *p< 0.05, **p< 0.01 (n=6-10).

Fig.7. 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.8. 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.

JPET # 252890

Table.1. Calculated ED₅₀ values plus 95% confidence limits for morphine in combination with fixed doses of maraviroc or AMD3100, or a combination of morphine plus maraviroc and AMD3100.

| Treatment | Calculated ED₅₀ of Morphine | 95% Confidence Interval |
|-----------------------------------|---|--------------------------------|
| Morphine alone | 2.34 mg/kg | 1.69-3.17 |
| Morphine + Maraviroc | 1.01 mg/kg | 0.60-1.56 |
| Morphine + AMD3100 | 1.32 mg/kg | 0.85-1.89 |
| Morphine + Maraviroc + AMD3100 | 0.71 mg/kg | 0.42-1.14 |

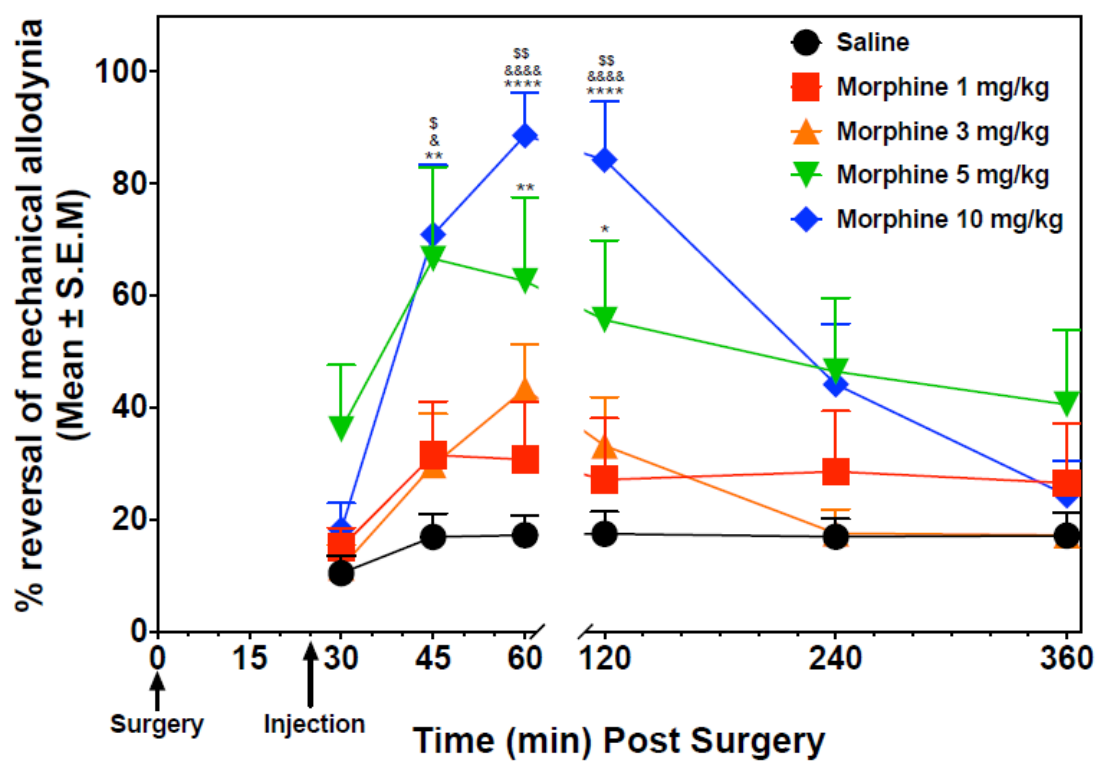


Fig.1

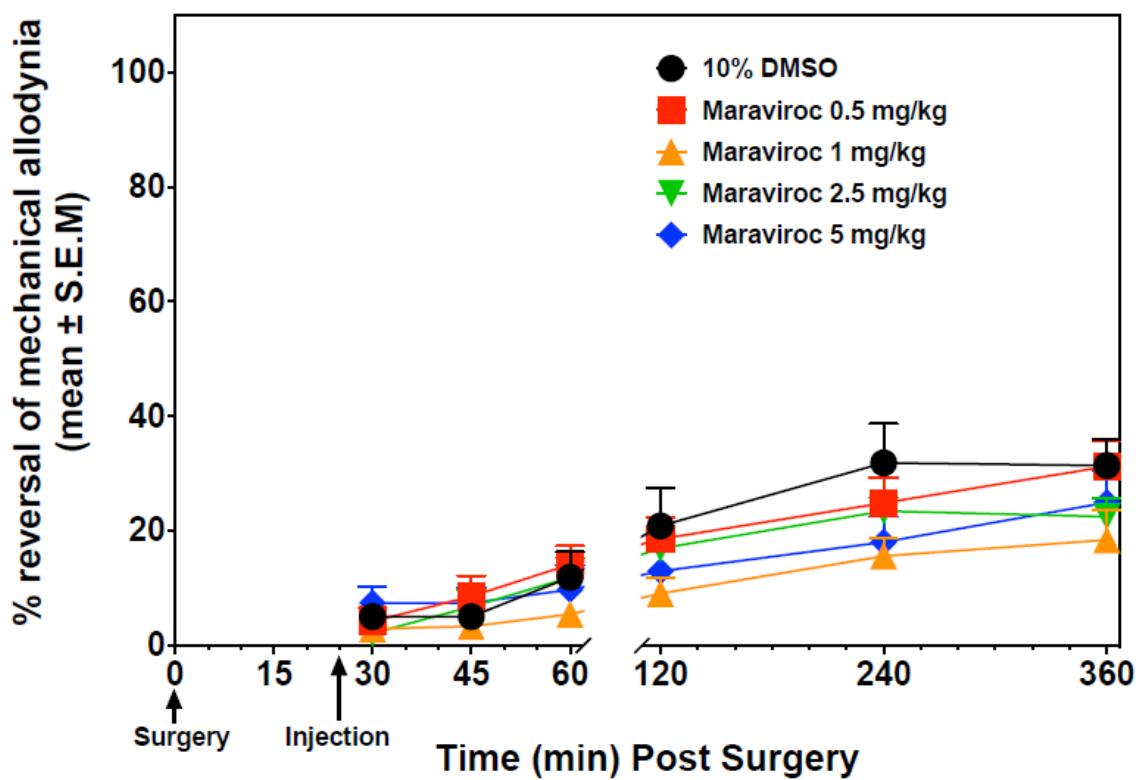


Fig.2

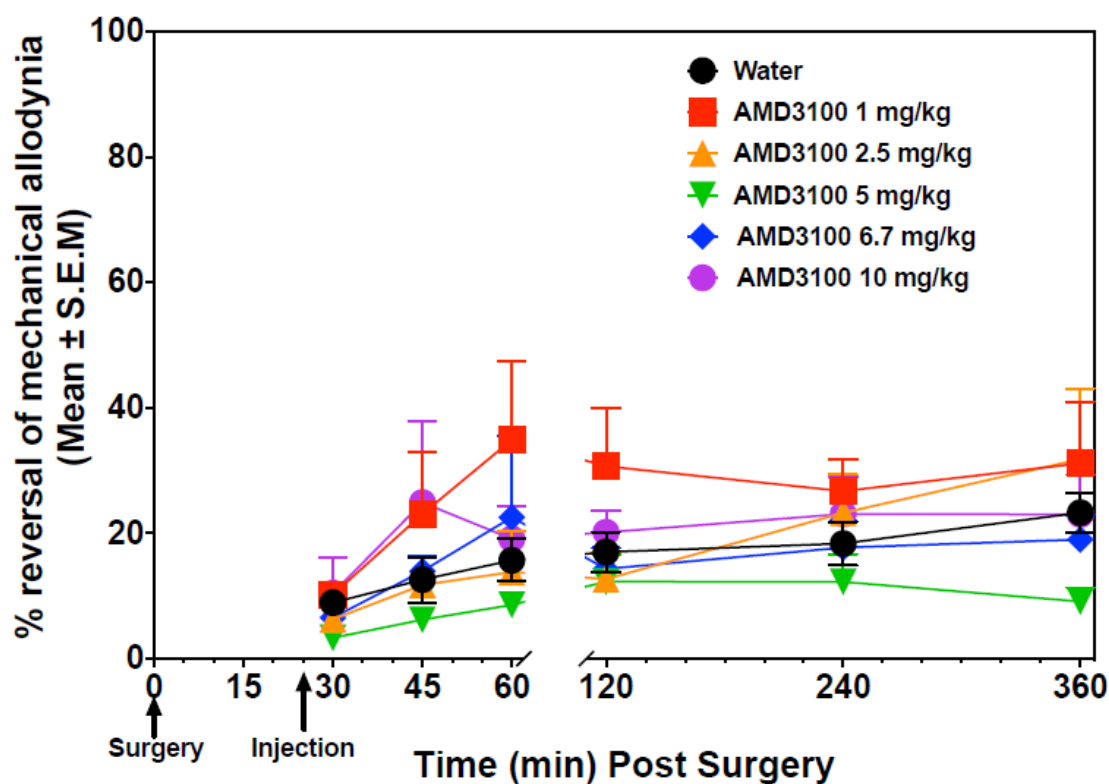


Fig.3

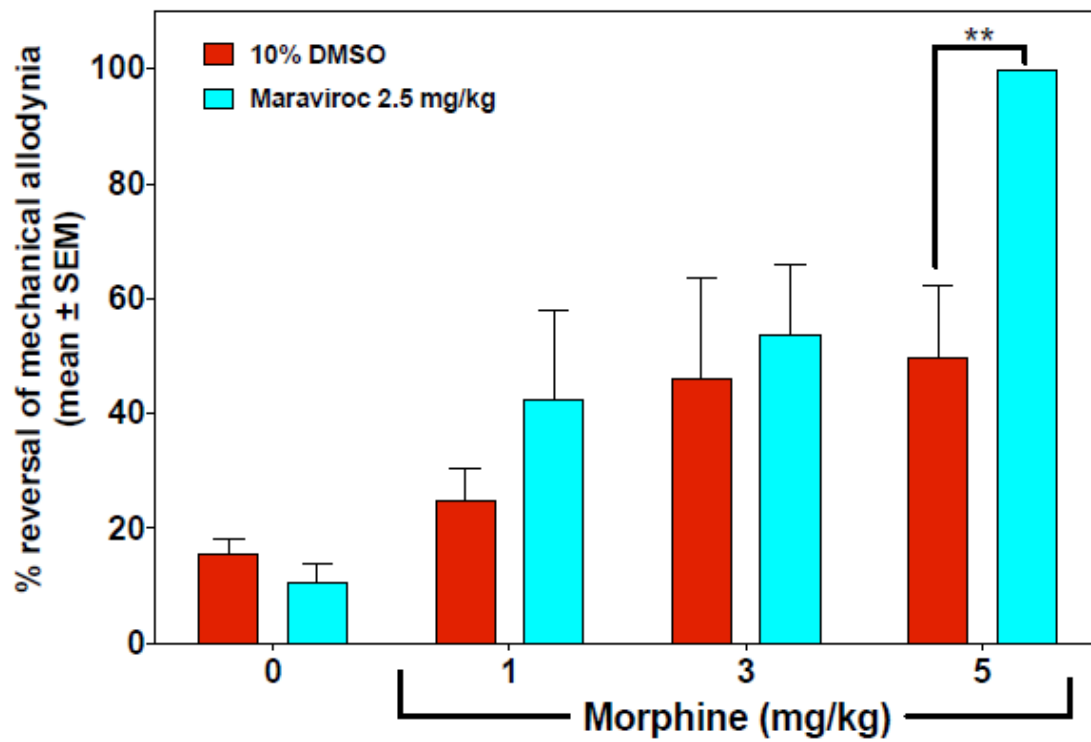


Fig.4A

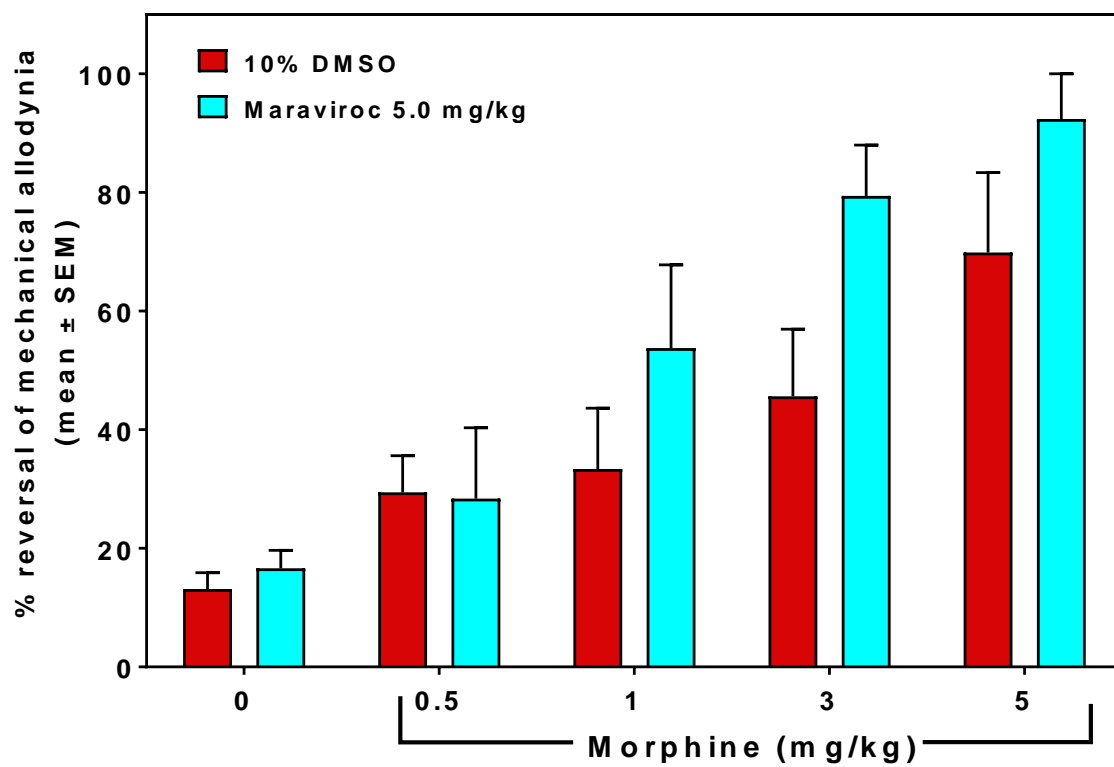


Fig.4B

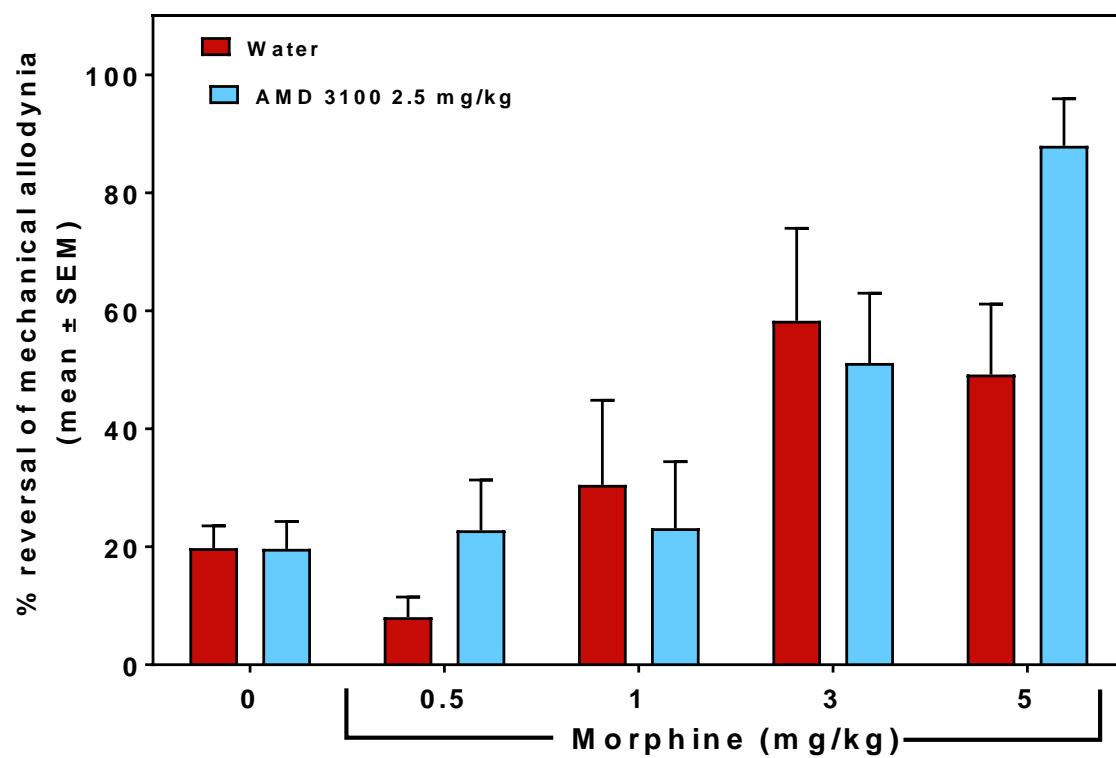


Fig.5A

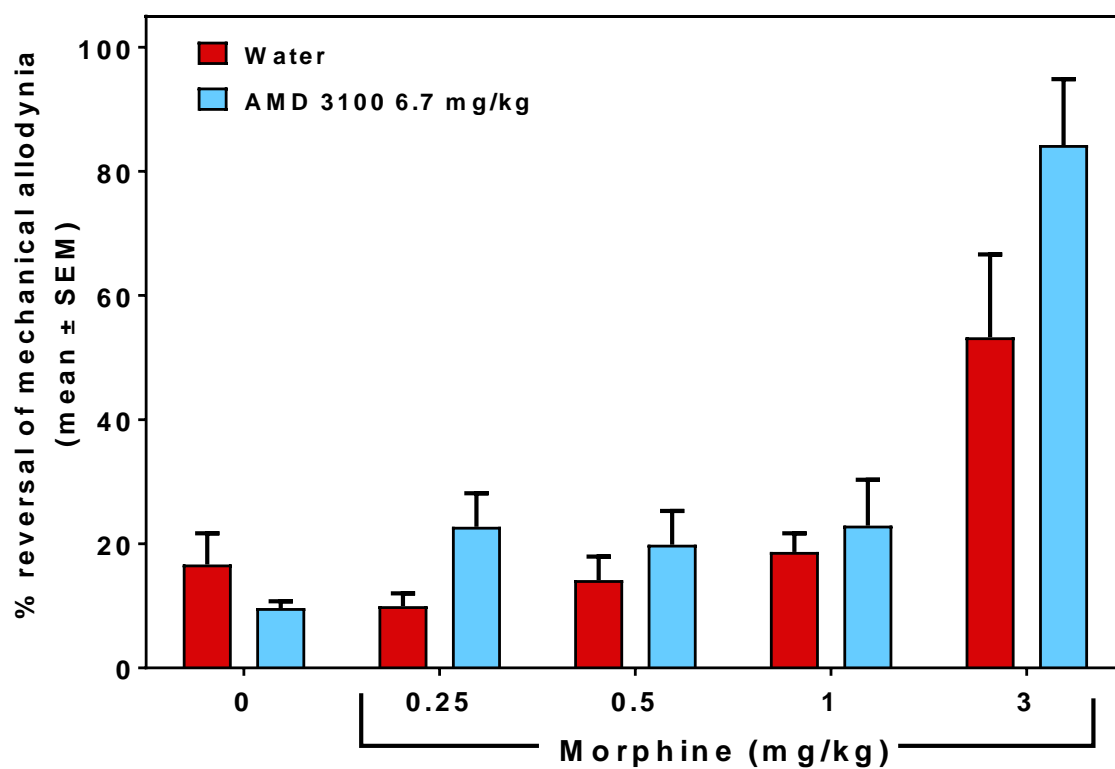


Fig.5B

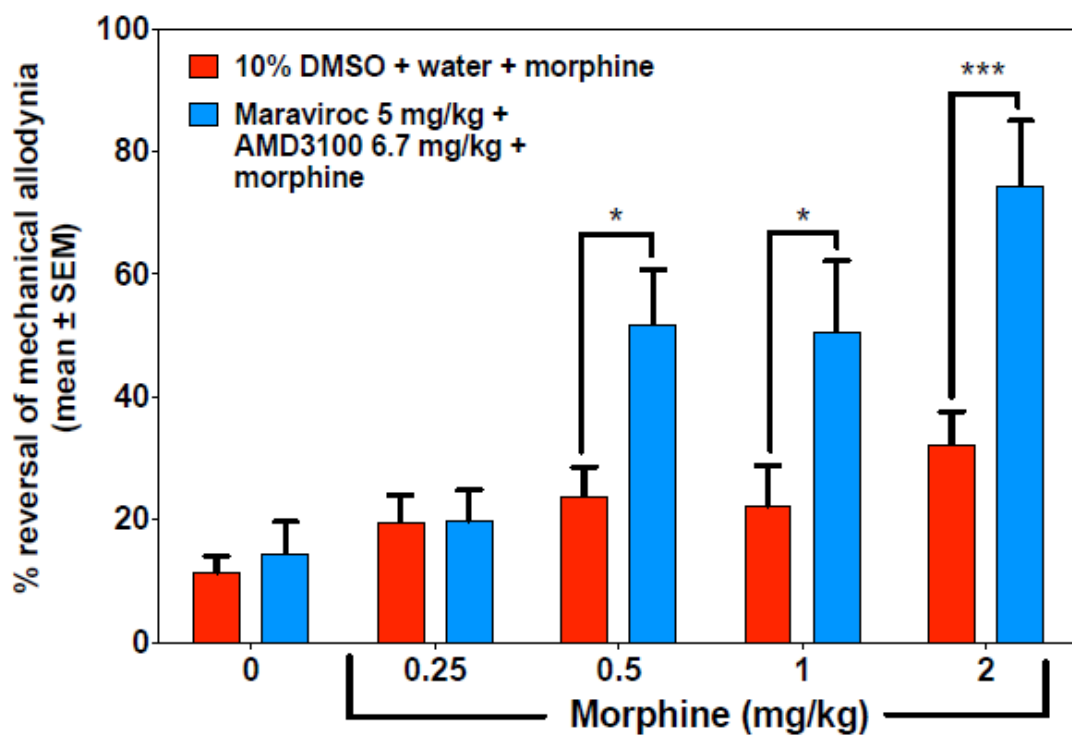


Fig.6

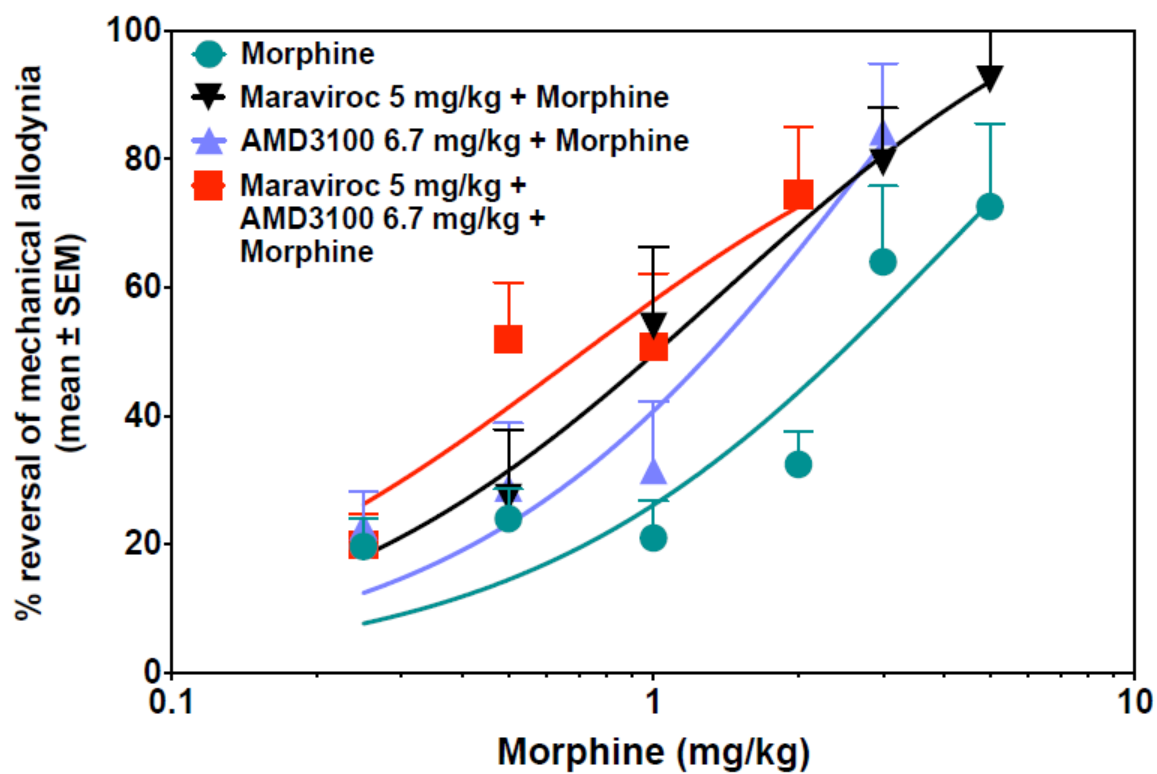


Fig.7

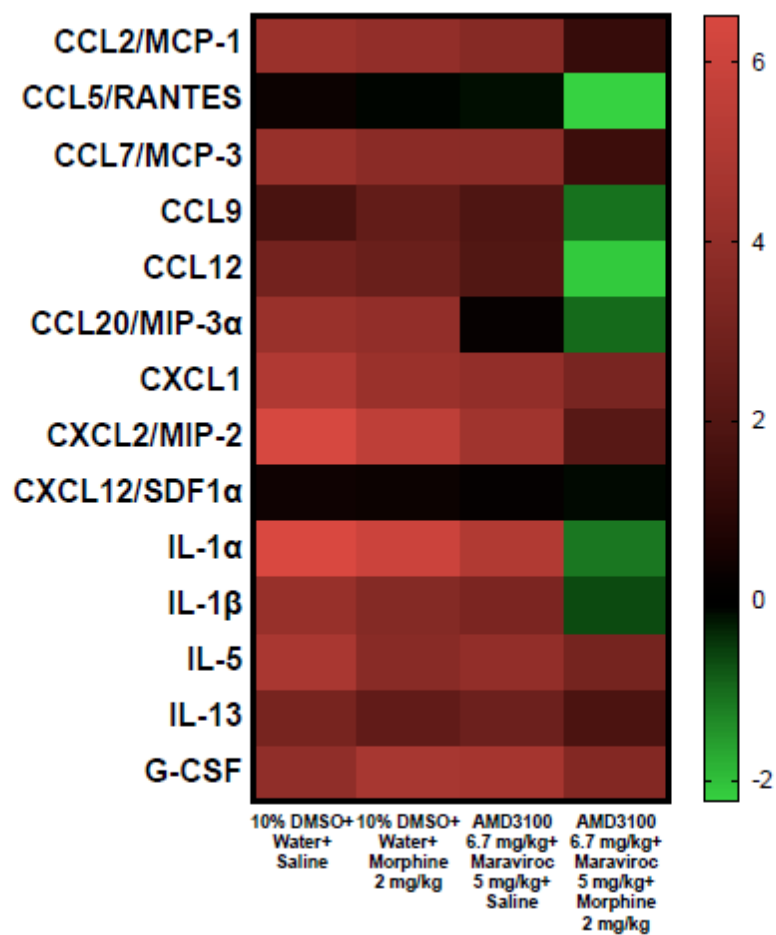


Fig.8A

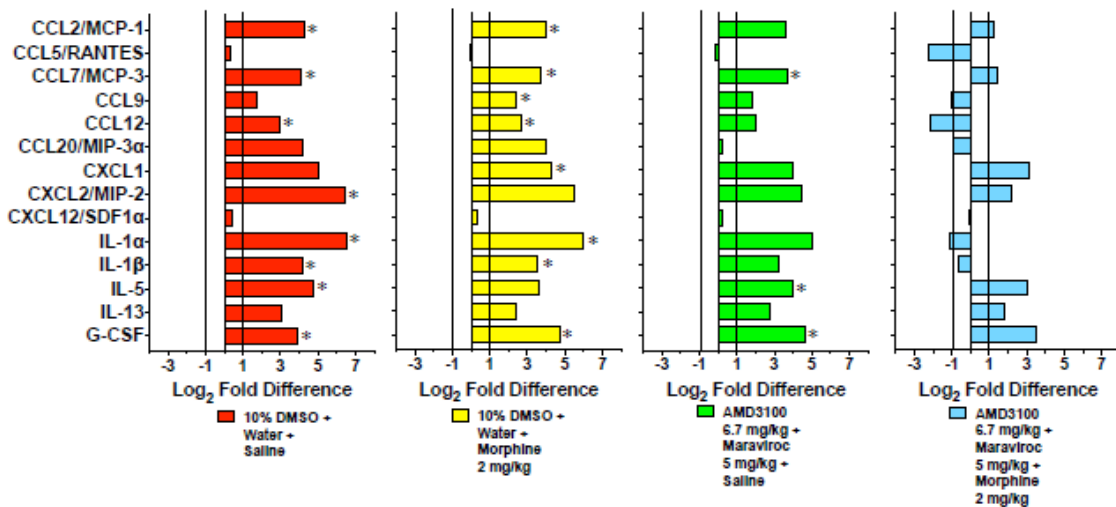


Fig.8B