

Heat Shock Protein 90 Inhibitors Block the Anti-Nociceptive Effects of Opioids in Mouse

Chemotherapy-Induced Neuropathy and Cancer Bone Pain Models

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Short Title: Hsp90 Inhibitors in Opioids and Cancer-Related Pain

Keywords: Heat Shock Protein 90 (Hsp90); Opioids; Cancer; Pain; Chemotherapy-Induced Peripheral Neuropathy (CIPN); Cancer-Induced Bone Pain (CIBP)

Number of Pages: 21

Number of Figures: 6

Summary: Heat shock protein 90 inhibitors, first developed to treat metastatic cancer, block the management of cancer-related pain by opioids in mouse models; these findings may impact the use of Hsp90 inhibitors to treat cancer in the clinic.

Abstract

Heat shock protein 90 (Hsp90) is a ubiquitous signal transduction regulator, and Hsp90 inhibitors are in clinical development as cancer therapeutics. However, there have been very few studies on the impact of Hsp90 inhibitors on pain or analgesia, a serious concern for cancer patients. We previously found that Hsp90 inhibitors injected into the brain block opioid-induced anti-nociception in tail flick, paw incision, and HIV neuropathy pain. This study extended from that initial work to test the cancer-related clinical impact of Hsp90 inhibitors on opioid anti-nociception in cancer-induced bone pain (CIBP) in female BALB/c mice and chemotherapy-induced peripheral neuropathy (CIPN) in male and female CD-1 mice. Mice were treated with Hsp90 inhibitors (17-AAG, KU-32) by the intracerebroventricular, intrathecal, or intraperitoneal routes, and after 24 hours pain behaviors were evaluated following analgesic drug treatment. Hsp90 inhibition in the brain or systemically completely blocked morphine and oxymorphone anti-nociception in CIPN; this effect was partly mediated by decreased ERK and JNK MAPK activation and by increased protein translation, was not altered by chronic treatment, and Hsp90 inhibition had no effect on gabapentin anti-nociception. We also found that the Hsp90 isoform Hsp90 α and the co-chaperone Cdc37 were responsible for the observed changes in opioid anti-nociception. In contrast, Hsp90 inhibition in the spinal cord or systemically partially reduced opioid anti-nociception in CIBP. These results demonstrate that Hsp90 inhibitors block opioid anti-nociception in cancer-related pain, suggesting that Hsp90 inhibitors for cancer therapy could decrease opioid treatment efficacy.

Introduction

Cancer-related pain is a prevalent symptom experienced by cancer patients, that can seriously impact patient quality-of-life [4]. Cancer-induced bone pain (CIBP), one of the most common and difficult to treat types of cancer pain, is exhibited by 33% of cancer patients who develop bone metastases [15]. Likewise, chemotherapy-induced peripheral neuropathy (CIPN) presents in 68% of patients undergoing chemotherapy treatments, and in some, CIPN can become permanent [34]. As a first approach, patients are typically treated with nonopioids like tricyclic antidepressants or gabapentinoids, but these can have limited efficacy, and only work for 42-76% of pain patients [19]. Consequently, patients with moderate to severe cancer pain are regularly prescribed opioids.

Separate from pain management, much of the focus in the cancer field has been on creating new therapeutics to treat malignancies. A new class of therapeutic that has sparked tremendous interest are Heat shock protein 90 (Hsp90) inhibitors. Hsp90 is a ubiquitous signaling regulator and chaperone protein [24]. While it has multiple mechanisms of action, it often acts by facilitating protein folding and encouraging proper conformation for activation. Several of its client proteins are oncoproteins, thus Hsp90 inhibitor treatment leads to oncoprotein degradation and cancer cell death, which has been used to develop novel tumor therapeutics [14]. While numerous Hsp90 inhibitors are in development, the impact of Hsp90 inhibitors on cancer patient pain management has not been studied.

In addition to Hsp90's role in tumor growth, Hsp90 has been recently identified as a regulator in several pain states and of opioid signal transduction. We previously found that Hsp90 regulates mu opioid receptor (MOR) signaling via promoting downstream ERK activation, where inhibition of Hsp90 in the brain was found to decrease morphine anti-nociception in tail flick, post-surgical paw-incision, and HIV neuropathic pain models [21]. Outside of our work, only a few papers have shown a role for Hsp90 in promoting inflammatory neuropathic pain [13; 23], and in promoting morphine dependence and withdrawal [1; 18]. This means that while there are many studies assessing the impact of Hsp90 inhibitors on tumor growth, there have been no studies of how Hsp90 inhibitors affect pain management in cancer patients. Based on our previous work, if Hsp90 inhibition had the same effect on opioid anti-nociception in cancer-related pain models that it does in the pain models we explored, then using Hsp90 inhibitors for cancer treatment could potentially suppress opioid analgesia [21]. This is a serious concern considering how many cancer patients experience pain; one study found that 67% of cancer

patients are affected by pain [7], while another found a pain incidence of 75% in patients being treated for advanced or metastatic cancer [41].

In this study, we thus evaluated the effects of Hsp90 inhibition on opioid anti-nociception *in vivo* in the cancer-related pain models of CIBP and CIPN. Our results suggest that Hsp90 inhibitors block opioid anti-nociception in cancer-related pain. They also suggest that the use of Hsp90 inhibitors as cancer therapeutics could have detrimental implications for patients who use opioids to manage their pain.

Methods

Drugs

KUNA115 [28], KUNB106 [27], KUNG65 (compound 30 in [8]), KU-32 (compound A4 in [2]), and KU177 (compound 12c in [43]) were synthesized by the Blagg laboratory using the cited protocols. The identity of the ligands was confirmed by high resolution mass spectrometry and nuclear magnetic resonance, while the purity of the compounds was confirmed to >95% by high performance liquid chromatography. Gedunin (#33-871-0), Celastrol (#32-031-0), 17-AAG (#AAJ66960MC), paclitaxel (#AAJ62734MC), gabapentin (#50-133-2934), U0126 (#11-445), SP600125 (#14-961-0), and Cycloheximide (#AC357420010) were obtained from Fisher Scientific. Morphine sulfate pentahydrate and oxymorphone were obtained from the NIDA Drug Supply Program.

All compounds except for morphine and oxymorphone were prepared as stock solutions, and diluted into a vehicle solution prior to injection. Morphine and oxymorphone were prepared as stock solutions in sterile USP saline, prepared fresh prior to each experiment. Matched vehicle controls were included for each drug injection. The vehicles used were: 2% DMSO and 98% sterile USP water for KUNA115, KUNB106, KUNG65, KU177, Cycloheximide; 1% DMSO and 99% sterile USP water for KU-32 and 17-AAG (10% DMSO and 90% USP saline for *ip* 17-AAG); 10% DMSO and 90% sterile USP water for gedunin, celastrol, and U0126; 11% DMSO and 89% sterile USP water for SP600125 and combined SP600125/U0126; 16.7% cremophor, 16.7% ethanol, and 66.6% USP saline for paclitaxel; and sterile USP saline for morphine, oxymorphone, and gabapentin. Drug powders were stored at -20°C under desiccation or as recommended by the manufacturer, and stock solutions were stored at -20°C.

Mice

Female BALB/cfC3H and male and female CD-1 (a.k.a. ICR) mice were all obtained from Charles River Laboratories, and used over 5-8 weeks of age. Mice were randomized to treatment group in age-matched controlled cohorts. Mice were recovered for at least 5 days after shipping prior to use in experiments. All mice were maintained with standard lab chow and water available *ad libitum* in a temperature and humidity controlled vivarium on a 12 hour light:dark cycle. The University of Arizona vivarium is AAALAC-accredited. All experiments were approved by the University of Arizona IACUC, and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals handbook.

Behavioral Experiments

All mice were brought up to the testing room for at least 30 minutes prior to any experiment for acclimation. The experimenter was blinded to treatment group by the delivery of coded drug vials, which would be decoded after the acquisition of all data. All behavioral experiments were performed at the same approximate time of day (~10 AM), and care was taken to minimize noises, scents, and similar. Intracerebroventricular (*icv*) or intrathecal (*it*) injections were performed as reported in our previous studies [21; 22]. Mechanical thresholds were measured using calibrated Von Frey filaments using the up-down method, as reported in our previous studies [11; 21; 22]. The spontaneous pain behaviors of flinching and guarding were measured by observation and grading over a 2 minute observation window, also as reported in our previous studies [11].

Chemotherapy-Induced Peripheral Neuropathy

CIPN was induced by *ip* injection of 2 mg/kg paclitaxel on days 1, 3, 5, and 7. Baseline mechanical threshold measurements were taken on days 1, 7, and 8 (prior to opioid injection). Hsp90 inhibitors were generally administered on day 7 concurrent with the final paclitaxel injection, with a 24 hour treatment window. Short term inhibitors such as U0126 or cycloheximide were injected on day 8. Opioid injection and behavioral measurements were performed beginning on day 8 over a time course.

Cancer-Induced Bone Pain

CIBP was induced by injection of 66.1 mouse adenocarcinoma breast cancer cells into the medullary space of the femur, as reported in our previous work [11]. Female BALB/cfC3H mice were used for this experiment due to histocompatibility with the cells, permitting the use of intact mice with functional immune systems (i.e. not nude mice or similar). The bone tumors and the resultant pain syndrome was allowed to develop for 14 days. Behavioral measurement baselines were performed on days 0, 7, and 13, with Hsp90 inhibitor injection on day 13. Inhibitor injection was followed by a 24 hour recovery, with baseline behavior recorded on day 14, followed by drug injection and behavioral measurement over a time course.

Statistical Analysis

All data reported as the mean \pm SEM, with the sample sizes and technical replicates reported in the Figure Legends. The data is reported raw, without normalization or other changes. Statistical analysis was performed in all cases by 2 Way Repeated Measures (RM) ANOVA; for experiments with 2 treatment groups, a Sidak's *post hoc* test was used, and for experiments with more than 2 treatment groups, a Tukey's *post hoc* test was used. In all cases, a result was considered significantly different with $p < 0.05$.

Results

Hsp90 Inhibition Fully Blocks Morphine Anti-Nociception in CIPN

We first tested the impact of Hsp90 inhibition on opioid anti-nociception in CIPN. We injected the Hsp90-selective pan-isoform inhibitor 17-AAG or Vehicle control by the *icv* (0.5 nmol), *it* (0.5 nmol), or systemic *ip* (50 mg/kg) routes. These doses were established after a dose/response experiment in our earlier work [21] or from the literature [16]. We treated the mice for 24 hours, to match our earlier work, and to model a more clinically-relevant chronic treatment model. We then injected morphine (10 mg/kg) by the subcutaneous (*sc*) route. We found that brain inhibition of Hsp90 completely blocked morphine anti-nociception in CIPN (**Figure 1A**), consistent with our findings in other pain models [21]. Interestingly, spinal cord Hsp90 inhibition had no impact on anti-nociception (**Figure 1B**). Systemic Hsp90 inhibition on the other hand gave the same result as brain inhibition, resulting in complete loss of morphine anti-nociception (**Figure 1C**); this is notable since cancer patients taking Hsp90 inhibitors would be taking them by the systemic route, suggesting our results could be

clinically relevant. We also performed a control experiment with the Hsp90-selective pan-isoform inhibitor KU-32; this ligand binds to a different site than 17-AAG, and is also chemically dissimilar to 17-AAG, making it very unlikely that the two drugs would share any off-target interactions [5]. Treatment with KU-32 resulted in the same loss of anti-nociception as 17-AAG, suggesting that our results are specific to Hsp90 and not due to off-target interactions (**Figure 1D**). We also found no effect of Hsp90 inhibition on baseline pain responses in any experiment. In our earlier work we also tested 17-AAG for confounding motor effects, and found none, suggesting these results are due to a *bona fide* impact on the opioid system [21].

Hsp90 Inhibition Partially Blocks Morphine Anti-Nociception in CIBP

We next tested the impact of Hsp90 inhibition on an alternate and more severe cancer pain model, CIBP. This model is induced by injection of cancer cells into the medullary space of the femur, and progresses over 14 days, resulting in strong evoked (mechanical allodynia) and spontaneous (flinching, guarding) pain behaviors. 17-AAG (0.5 nmol for *icv* or *it*, 50 mg/kg for *ip*) or vehicle was injected on day 13, with morphine (10 mg/kg, *sc*) and pain measurement on day 14. We found that brain Hsp90 inhibition, in contrast to the results in CIPN, had essentially no impact on morphine anti-nociception in evoked or spontaneous pain (**Figure 2A**). However, spinal cord Hsp90 inhibition did have an impact; opioid anti-nociception for evoked mechanical allodynia was reduced by ~66%, while spontaneous flinching and guarding was unaffected (**Figure 2B**). When given systemically by the *ip* route, 17-AAG caused a similar reduction in mechanical allodynia anti-nociception but not spontaneous pain, very similar to *it* treatment (**Figure 2C**). These results show how central nervous system (CNS) region and pain type strongly impact the results of Hsp90 inhibition on opioid anti-nociception.

Hsp90 Inhibition Blocks Oxymorphone but not Gabapentin Anti-Nociception in CIPN and CIBP

Morphine is a moderately potent and efficacious partial agonist, so we next sought to determine if the high potency and efficacy agonist oxymorphone would be similarly impacted by Hsp90 inhibition [30]. For these studies we used CIPN and CIBP as above, with *ip* injection (to maximize translatability) of 17-AAG (50 mg/kg) or Vehicle for 24 hours, followed by 0.1 mg/kg oxymorphone *sc*. In male and female mice with CIPN, the anti-nociceptive impact of oxymorphone was completely blocked, similar to the morphine results above (**Figure 3A**). In mice with CIBP, we also found similar results to those above, with mechanical allodynia anti-nociception by

oxymorphone partially blocked while spontaneous pain anti-nociception was unaffected (**Figure 3B**). We also tested the non-opioid analgesic gabapentin (30 mg/kg, sc) in the same models, and found that Hsp90 inhibition had no impact on anti-nociception with this drug in CIPN (**Figure 3C**) or CIBP (**Figure 3D**). One caveat however is that gabapentin had weak to modest efficacy in the CIBP model, which may have obscured potential differences (**Figure 3D**). These results suggest that Hsp90 inhibition is not specific to morphine, and similarly blocks even the high potency and efficacy opioid agonist oxymorphone; on the other hand, the effect is not universal, since the non-opioid analgesic gabapentin was unaffected.

Hsp90 Blockade of Anti-Nociception in CIPN is Unaltered by Repeated Treatment

The above results do suggest that Hsp90 inhibition can block opioid anti-nociception in cancer-related pain, but all studies were carried out with acute treatment. Patients in the clinic would be treated with both chronic opioids and chronic Hsp90 inhibitors in the course of cancer treatment. We thus tested the impact of repeated systemic 17-AAG (50 mg/kg, ip) or Vehicle treatment on daily morphine (10 mg/kg, sc) anti-nociception in CIPN. Over the course of the 4 day treatment, the Vehicle-treated mice displayed morphine anti-nociception that gradually lowered with time as tolerance developed; however, 17-AAG-treated mice had absolutely no anti-nociceptive response to morphine at any point during the treatment period (**Figure 4**). These results suggest that the anti-nociceptive impact of Hsp90 inhibition will not decrease with chronic treatment.

Hsp90 Regulation of CIPN Anti-Nociception Requires ERK/JNK MAPK and Protein Translation

In our earlier work, we found that Hsp90 inhibition in the brain resulted in the loss of opioid activation of ERK and JNK MAPK; we further found that the loss of ERK signaling was likely responsible for the loss of opioid anti-nociception with Hsp90 inhibition [21]. We thus sought to determine whether ERK and JNK MAPK were involved in opioid anti-nociception in the brain in CIPN. We first injected the MEK/ERK inhibitor U0126 (5 µg, icv) or Vehicle 15 minutes prior to morphine (10 mg/kg, sc) on day 8 of CIPN in male mice. We found that ERK inhibition partially (~60%) reduced morphine anti-nociception in CIPN (**Figure 5A**), suggesting that ERK promotes part of the anti-nociceptive response, in contrast to the full effect observed in our earlier studies [21]. We thus expanded these studies to include both ERK and JNK inhibitors in male and female mice. In male mice, we found that ERK (U0126) or JNK (SP600125, 5 nmol) inhibitors injected as above produced the same ~60%

decrease in morphine anti-nociception, and that combining the 2 inhibitors together did not cause further inhibition (**Figure 5B**). In female mice, both ERK and JNK inhibitors caused a statistically similar inhibition, and again combining ERK and JNK inhibitors did not have an additive effect (**Figure 5C**). These results suggest that ERK and JNK MAPK both promote morphine anti-nociception in CIPN, and likely in series rather than in parallel due to the lack of additivity.

We also sought to determine if active protein translation was involved in the impact of Hsp90 inhibition on morphine anti-nociception in CIPN. We treated mice with 17-AAG (0.5 nmol, *icv*) or Vehicle on day 7 of CIPN as for earlier studies; on day 8 we injected the protein translation inhibitor cycloheximide (85 nmol, *icv*) or Vehicle 30 minutes before morphine injection. We found that cycloheximide treatment completely restored morphine anti-nociception in 17-AAG-treated mice back to Vehicle-treated levels, while cycloheximide treatment in Vehicle-treated mice had no impact on anti-nociception (**Figure 5D**). These results strongly suggest that Hsp90 inhibition results in the active translation of a short half-life protein that is responsible for suppressing morphine anti-nociception in CIPN. These results may help to narrow down the mechanism by which Hsp90 blocks opioid anti-nociception.

The Isoform Hsp90 α and the Co-Chaperone Cdc37 Regulate Opioid Anti-Nociception in CIPN

Recent effort in the Hsp90 field has focused on isoform- and co-chaperone-selective inhibitors as a means to treat cancer while reducing potential side effects [8; 25; 27; 28]. If only certain isoforms or co-chaperones regulate opioid anti-nociception, then targeting other isoforms or co-chaperones could permit the use of Hsp90 inhibitors to treat cancer without impacting pain management with opioids. We thus screened 3 isoform-selective and 3 co-chaperone-selective inhibitors in morphine anti-nociception in CIPN. Citations for each inhibitor can be found in the Methods, and our validation of the doses and selectivity of each ligand can be found in our earlier work [20]. Each inhibitor was injected by the *icv* route on day 7 of CIPN as above, followed by 10 mg/kg *sc* morphine on day 8. We found that the Hsp90 α -selective inhibitor KUNA115 fully blocked morphine anti-nociception in CIPN, very similarly to 17-AAG and KU-32 above (**Figure 6A**). The Hsp90 β -selective inhibitor KUNB106 and the Grp94-selective inhibitor KUNG65 had no impact on morphine anti-nociception (**Figures 6B-C**). Similarly, the co-chaperone Cdc37-selective inhibitor celastrol strongly blocked morphine anti-nociception in CIPN (**Figure 6D**), while the p23-selective inhibitor gedunin and the Aha1-selective

inhibitor KU177 had no impact (**Figure 6E-F**). These results thus suggest that Hsp90 α and Cdc37 selectively regulate CIPN anti-nociception in the brain, while the other isoforms and co-chaperones tested are not involved.

Discussion

In this study, we found that Hsp90 inhibition in the CNS had a strong but selective impact on opioid anti-nociception in cancer-related pain models. The effect on anti-nociception in CIPN was complete, fully blocking even a high dose of morphine or oxymorphone, while the effect in CIBP was moderate. This impact did not change with repeated treatment, relied on ERK/JNK MAPK signaling and protein translation, and was evoked through specific isoforms and co-chaperones of Hsp90. These findings have strong implications for the management of cancer pain, as Hsp90 inhibitors are being developed as novel cancer therapeutics [35]. These findings suggest that Hsp90 inhibitors utilized to treat cancers could compromise patient pain management with opioids; this is a serious concern since opioids are a key tool for the management of moderate to severe cancer pain [32; 36]. Our findings did show that gabapentin anti-nociception was unaltered, however, gabapentin has limited efficacy in strong pain (apparent in **Figure 3D**) and further only works for a subpopulation of pain patients [19]. This question has also not been addressed to our knowledge in the literature. Only a very small number of papers have studied how Hsp90 and pain interact, and none of the Hsp90 inhibitor clinical trials addressed pain as a primary endpoint. Of the published literature, ours is the first to directly study the impact of Hsp90 inhibitors on cancer pain management. In previous work, Hsp90 inhibitors were shown to decrease inflammatory pain by directly impacting the inflammatory process [13; 23], and decrease diabetic neuropathic pain by promoting neuronal survival [39; 40]; models not directly relevant to cancer. Our work is also the first to study the impact of Hsp90 inhibition on opioid anti-nociception [21], as the only previous papers studied the impact of Hsp90 inhibition on opioid dependence and withdrawal [1; 18]. Together our findings suggest that the impact of Hsp90 inhibitors on cancer patient pain management should be closely studied.

These findings build on our earlier work, which found that Hsp90 inhibition in the brain (spinal cord not tested) strongly blocked paw incision and HIV neuropathy opioid anti-nociception but had a very mild effect on tail flick pain, explained by a loss of ERK MAPK signaling [21]. In this study we show a very strong effect on CIPN that can be localized to the brain, while the impact on CIBP was moderate and localized to the spinal cord. We also show a very strong effect on opioids but no effect on the non-opioid gabapentin. These comparative

results show how the role of Hsp90 in regulating anti-nociception is strongly dependent on the pain type, which can further differ by class of analgesic drug and region of the CNS inhibited. Further differences include the presence of a tumor in the CIBP model which is not present in CIPN; this could also impact Hsp90-regulated anti-nociception. As explained above, Hsp90 has been very lightly studied in pain and anti-nociception, and indeed, has been poorly studied in neuroscience in general. The literature thus offers few clues at this time to explain these striking differences in pain type, drug type, and CNS region. Different pain types can be conveyed to the CNS and processed in the spinal cord and brain by different mechanisms including different classes of neuron [17]; thus Hsp90 could be organized differently in different neuron types, leading to differential impacts on pain models. We've also shown that Hsp90 inhibition is acting through specific signal transduction changes, so these signaling molecules could be differently organized in different neuron types, brain regions, etc. These specific molecular changes by Hsp90 could also explain the specific drug class impacts; Hsp90 could be impacting the signaling cascades of the MOR, the target of drugs like morphine and oxycodone [30], without impacting the targets of other drugs like gabapentin. These possibilities will need to be explored to explain how Hsp90 has specific impacts on different pain models (including cancer pain), which could help predict which pain types will be impacted by Hsp90 inhibitor treatment.

Similar to the specificity discussed above, we observed that Hsp90 inhibition impacted anti-nociception of measures of evoked pain (mechanical hypersensitivity) but not spontaneous pain (flinching, guarding) in CIBP. This divergence between evoked and spontaneous pain has been observed before, where Sukhtankar and colleagues found that p38 MAPK inhibition blocked spontaneous but not evoked pain in CIBP [37]. This finding shows how different responses of the same pain model have different mechanisms, down to specific signaling kinases involved in specific aspects of the overall response. It is thus not that surprising that we observed a similar divergence in CIBP with Hsp90 inhibition. Similar to the discussion above, Hsp90 may regulate the specific neurons or signaling molecules responsible for evoked pain but not spontaneous pain in CIBP. These results also suggest that even within a single pain model, we should investigate more than one type of pain response to determine the full picture of how Hsp90 inhibition alters opioid anti-nociception in a specific pain type. Notably, we only used reflexive measures (Von Frey) in our CIPN experiments, which is a limitation of our study. Non-reflexive measures in CIPN in future studies could reveal further complexities of this model.

We also found that ERK and JNK MAPK signaling as well as active protein translation was required for Hsp90 regulation of CIPN anti-nociception. This finding was presaged by our previous results showing that loss of ERK signaling was involved in Hsp90 regulation of paw incision and HIV neuropathy anti-nociception [21], and that active protein translation was involved in Hsp90 regulation of paw incision anti-nociception [20]. These results begin to define a molecular mechanism for Hsp90, and could provide an explanation for the model, brain region, and drug selectivity differences discussed above. ERK has been shown to promote different aspects of the pain response (anxiety, etc.) [9; 38], as well as regulate different opioid side effects [6; 26]; our own work shows that ERK promotes acute opioid anti-nociception (above and [21]). This pleiotropy shows that understanding how ERK is organized downstream of different receptors in different tissues and neurons will be key to deciphering how ERK can evoke very different specific roles. Likewise, JNK has been implicated in diverse roles, including promoting morphine tolerance [6] and promoting opioid hyperalgesia [33]; we are the first to our knowledge to show that JNK mediates acute opioid anti-nociception. Future study should focus on the downstream effectors by which JNK and ERK link to anti-nociception (ion channels? other targets?) which will be key to deciphering their mechanism in opioid anti-nociception.

We have taken the first step in this investigation by our finding that active protein translation is required for Hsp90 inhibition to alter opioid anti-nociception. This finding is not unprecedented, as shown by the work of Ted Price and colleagues, who in a series of studies have found a crucial role for eIF4E-induced protein translation in regulating the onset of pain states [29]. Others have linked protein translation to the development of chronic opioid side effects [42]. To our knowledge, we are the first to link protein translation to acute anti-nociceptive changes, although importantly, these changes only manifest after Hsp90 inhibitor treatment; cycloheximide had no effect in vehicle-treated animals in our model. It is thus not clear at this time what are the likely factors being translated that impact anti-nociception, although they must have a short protein half-life, as we gave the cycloheximide only 30 minutes prior to opioid treatment. Future protein profiling studies, such as quantitative proteomics, could reveal the identity of the proteins translated after Hsp90 inhibition.

Lastly we identified the isoform Hsp90 α and the co-chaperone Cdc37 in the brain as critical for promoting opioid anti-nociception in CIPN, while the isoforms Hsp90 β /Grp94 and the co-chaperones p23/Aha1 had no impact. These are similar to an earlier study of ours in paw incision pain, with the exception that p23 promoted anti-nociception in this non-cancer pain model [20]. These findings echo the themes discussed above, in that

very specific isoforms and co-chaperones of Hsp90 are involved in regulating opioid anti-nociception in CIPN. These molecules can also differ between pain states, as shown for p23. Knowing the specific molecules involved will also begin to identify molecular mechanisms. For instance, Cdc37 has a canonical role in promoting the maturation and activation of signaling kinases; Cdc37 could thus act in our model by promoting the activation of ERK and JNK [12; 24; 31]. The four Hsp90 isoforms also have distinct subcellular locations, roles, and client protein assemblages, which will provide further insight into the molecular mechanisms involved in regulating opioid anti-nociception [10]. Understanding the specific isoforms and co-chaperones involved could also provide a path to develop Hsp90 cancer therapeutics that won't impact opioid anti-nociception. Using selective inhibitors to target Hsp90 β /Grp94/p23/Aha1 could target cancers without altering cancer pain management by opioids, an approach suggested by work with Hsp70 [3]. Our findings thus offer a caution as to the potential impacts of Hsp90 therapy on pain relief, but also offer a potential means to circumvent this difficulty.

Acknowledgments

We would like to acknowledge the assistance of Dr. Todd Vanderah from the University of Arizona for technical training on the CIBP model. We also acknowledge the support of an Arizona Biomedical Research Commission New Investigator Award #ADHS18-198875 and institutional funds from the University of Arizona to JMS. The authors have no other relevant conflicts of interest to declare.

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Figure Legends

Figure 1: Brain Hsp90 Inhibition Blocks Morphine Anti-Nociception in CIPN. Male and female CD-1 mice used, sexes noted in each experiment. CIPN induced as in the Methods, with inhibitor injection on day 7 with testing on day 8, 24 hours later, using 10 mg/kg morphine sc with testing for mechanical threshold on a time course. Data reported as the mean \pm SEM with sample sizes of mice/group noted in each graph. Two technical replicates were performed for each experiment. *, **, ***, **** = $p < 0.05, 0.01, 0.001, 0.0001$ vs. same time point 17-AAG group by 2 Way RM ANOVA with Sidak's *post hoc* test. **A)** 17-AAG (0.5 nmol) or Vehicle injected by the *icv* route, male mice. **B)** 17-AAG (0.5 nmol) or Vehicle injected by the *it* route, male mice. **C)** 17-AAG (50 mg/kg) or Vehicle injected by the *ip* route, male mice. **D)** KU-32 (0.1 nmol) or Vehicle injected by the *icv* route, male and female mice (equal numbers).

Figure 2: Spinal Cord Hsp90 Inhibition Moderately Blocks Morphine Anti-Nociception of Evoked Pain in CIBP. CIBP induced in female BALB/c mice as in the Methods, with baseline testing on day 7, *icv* (0.5 nmol, **A**), *it* (0.5 nmol, **B**), or *ip* (50 mg/kg, **C**) injection of 17-AAG or Vehicle on day 13, and 10 mg/kg morphine sc 24 hours later on day 14. Evoked (mechanical threshold) or spontaneous (flinching, guarding) pain measured at baseline points and in a time course post-morphine. Data reported as the mean \pm SEM with the sample sizes of mice/group noted in the graphs. Experiments performed in 2 (**A**), 3 (**B**), and 2 (**C**) technical replicates. *, **, *** = $p < 0.05, 0.01, 0.001$ vs. same time point 17-AAG group by 2 Way RM ANOVA with Sidak's *post hoc* test.

Figure 3: Hsp90 Inhibition Blocks Oxymorphone but not Gabapentin Anti-Nociception in CIPN and CIBP. Male and female CD-1 mice had CIPN induced with the same testing paradigm as for **Figure 1**, with 50 mg/kg 17-AAG or Vehicle injected *ip* on day 7. Female BALB/c mice had CIBP induced with the same testing paradigm as for **Figure 2**, with 50 mg/kg 17-AAG or Vehicle injected *ip* on day 13. Data reported as the mean \pm SEM with sample sizes of mice/group noted in the graphs. **** = $p < 0.0001$ vs. same time point 17-AAG group by 2 Way RM ANOVA with Sidak's *post hoc* test. **A)** Male and female CIPN mice injected with 0.1 mg/kg oxymorphone or Vehicle sc on day 8 as above. Three technical replicates. **B)** Female CIBP mice injected with 0.1 mg/kg oxymorphone or Vehicle on day 14 as above. Two technical replicates. **C)** Male and female CIPN mice injected

with 30 mg/kg gabapentin or Vehicle sc on day 8 as above. Two technical replicates. **D)** Female CIBP mice injected with 30 mg/kg gabapentin or Vehicle sc on day 14 as above. Two technical replicates.

Figure 4: The Impact of Hsp90 Inhibition on CIPN Anti-Nociception is not Altered by Repeated Treatment.

Male and female CD-1 mice had CIPN induced as above. Beginning on day 7, mice injected daily with 50 mg/kg 17-AAG or Vehicle *ip* (days 7-10). Beginning on day 8, pre-opioid baseline measured, then mice injected daily with 10 mg/kg morphine sc (days 8-11). Mechanical thresholds measured after each opioid injection in a time course. Data reported as the mean \pm SEM with the sample size of mice/group noted in the graphs. Two technical replicates. **, ***, **** = $p < 0.01, 0.001, 0.0001$ vs. same time point 17-AAG group by 2 Way RM ANOVA with Sidak's *post hoc* test.

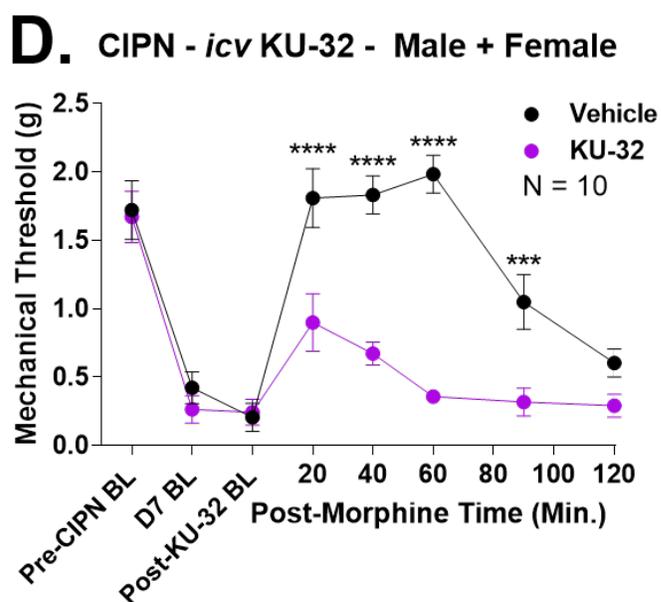
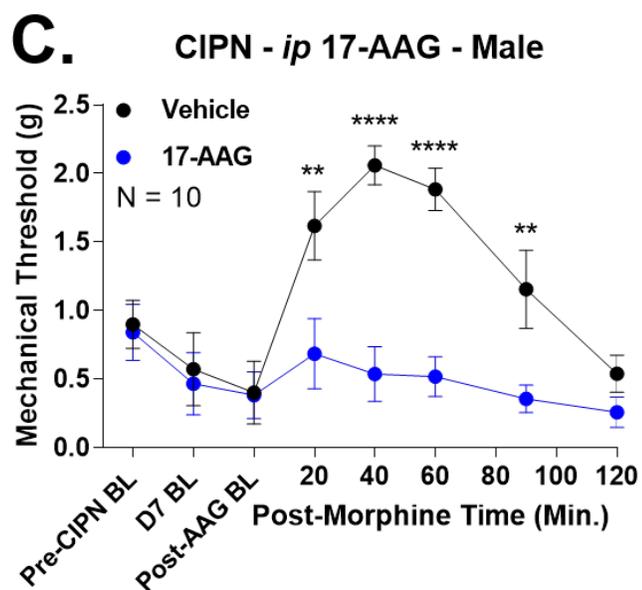
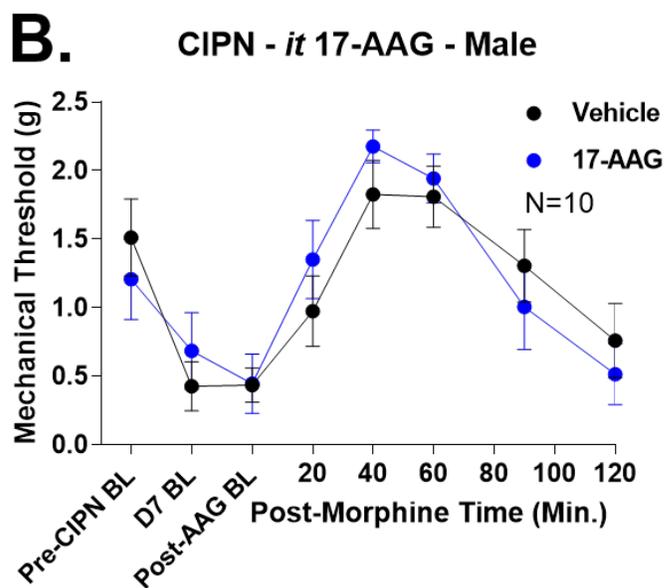
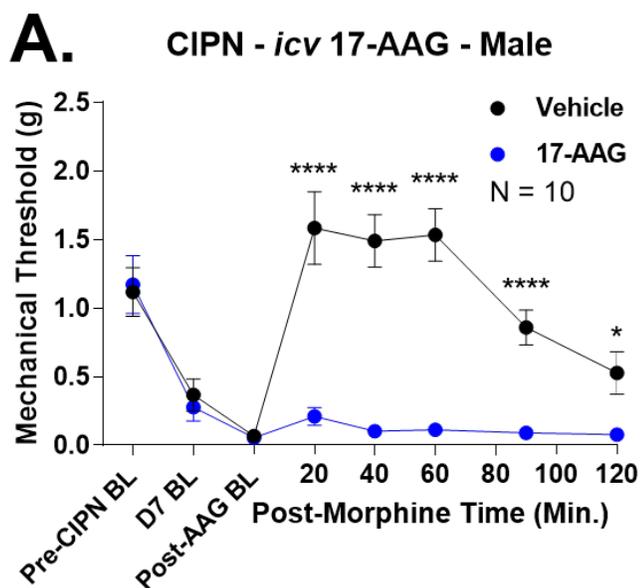
Figure 5: Hsp90 Regulation of CIPN Anti-Nociception Requires ERK/JNK MAPK and Protein Translation.

Male and female CD-1 mice had CIPN induced, with treatment on day 7 or 8 as noted below. Data reported as the mean \pm SEM with the sample sizes of mice/group noted in the graphs. **A)** Male mice were injected on day 8 of CIPN with 5 μ g of U0126 or Vehicle by the *icv* route, 15 minutes, followed by 10 mg/kg morphine sc. Two technical replicates. **, **** = $p < 0.01, 0.0001$ vs. same time point U0126 group by 2 Way RM ANOVA with Sidak's *post hoc* test. **B)** Male mice were injected on day 8 of CIPN with U0126 (5 μ g), SP600125 (5 nmol), SP600125 + U0126, or Vehicle by the *icv* route 15 minutes prior to the injection of 10 mg/kg morphine, sc. Four technical replicates. *, ** = $p < 0.05, 0.01$ vs. same time point SP600125, U0126, or SP600125 + U0126 group by 2 Way RM ANOVA with Tukey's *post hoc* test. **C)** Female mice treated and analyzed as in **B**. **D)** Female mice were injected with 0.5 nmol 17-AAG or Vehicle by the *icv* route on day 7 of CIPN, with a 24 hour recovery. On day 8, the mice were injected again with 85 nmol cycloheximide or Vehicle *icv*, 30 minutes, followed by 10 mg/kg morphine sc. Three technical replicates. **** = $p < 0.0001$ vs. same time point 17-AAG/Veh group by 2 Way RM ANOVA with Tukey's *post hoc* test.

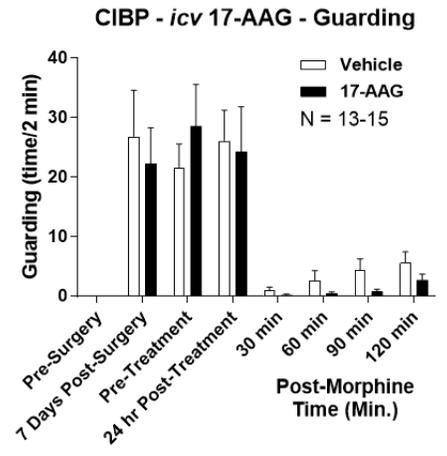
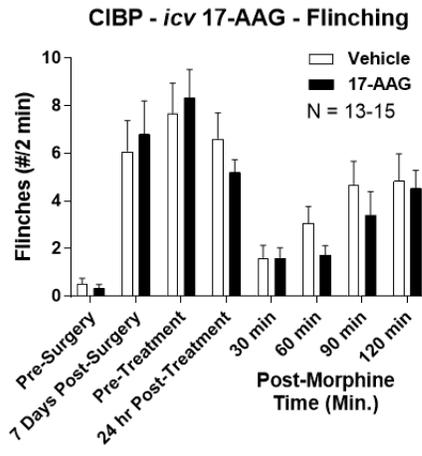
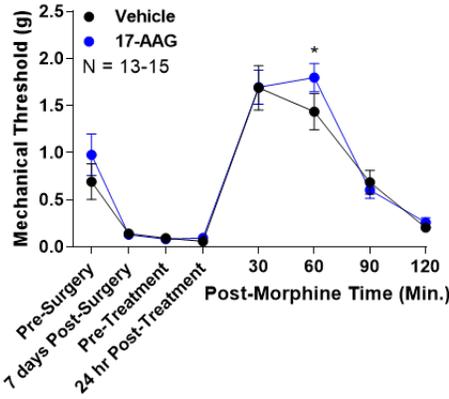
Figure 6: The Isoform Hsp90 α and the Co-Chaperone Cdc37 Promote Opioid Anti-Nociception in CIPN.

Male and female CD-1 mice had CIPN induced. On day 7, mice were injected with inhibitor (doses below) or Vehicle by the *icv* route with a 24 hour recovery, followed by 10 mg/kg morphine sc. Data reported as the mean

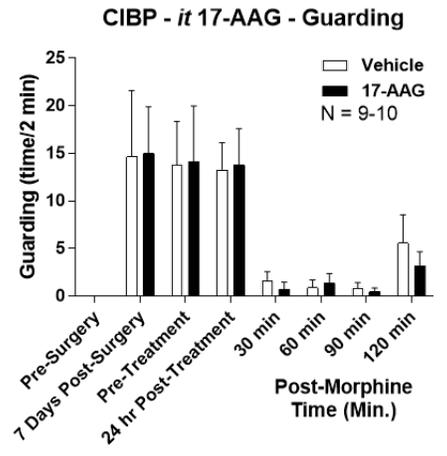
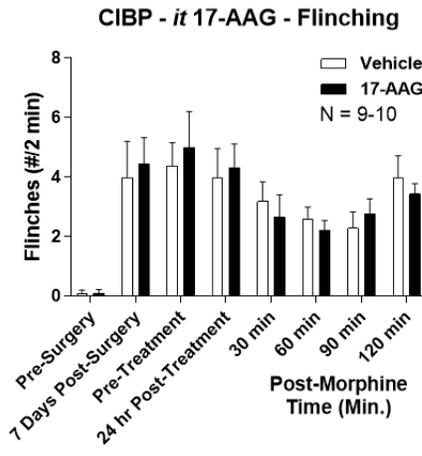
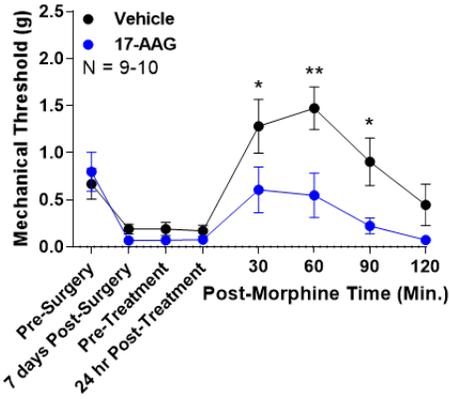
± SEM with the sample sizes of mice/group noted in the graphs. Two technical replicates for each experiment. **, ***, **** = $p < 0.01$, 0.001 , 0.0001 vs. same time point Inhibitor group by 2 Way RM ANOVA with Sidak's *post hoc* test. **A)** KUNA115 (0.1 nmol, Hsp90 α -selective) used. **B)** KUNB106 (0.1 nmol, Hsp90 β -selective) used. **C)** KUNG65 (0.1 nmol, Grp94-selective) used. Female only. **D)** Celastrol (10 nmol, Cdc37-selective) used. Female only. **E)** Gedunin (10 nmol, p23-selective) used. Female only. **F)** KU177 (0.1 nmol, Aha1-selective) used.



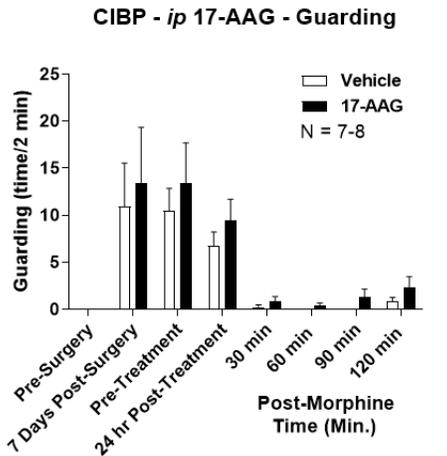
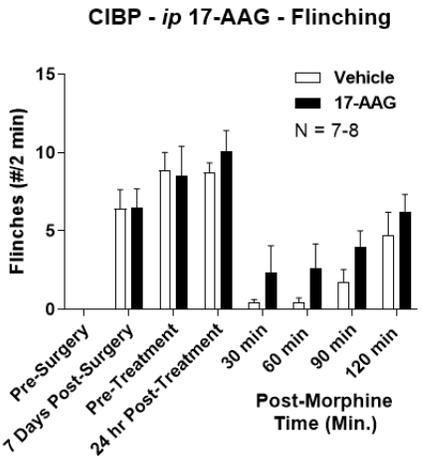
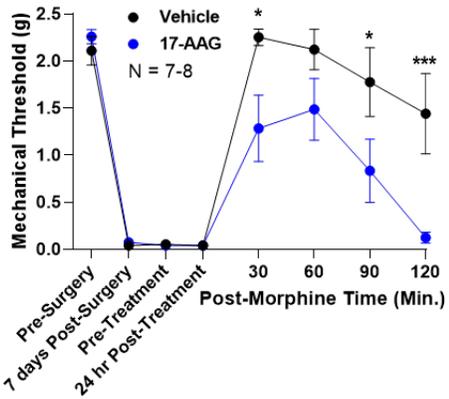
A. CIBP - *icv* 17-AAG - Von Frey - Female

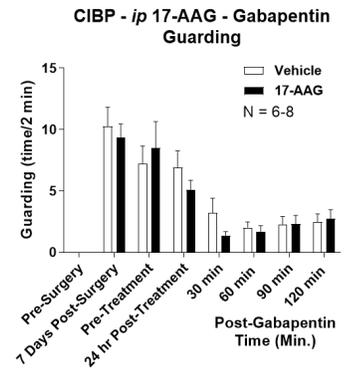
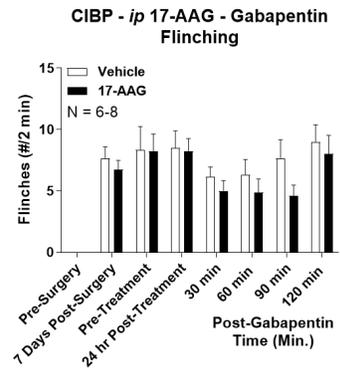
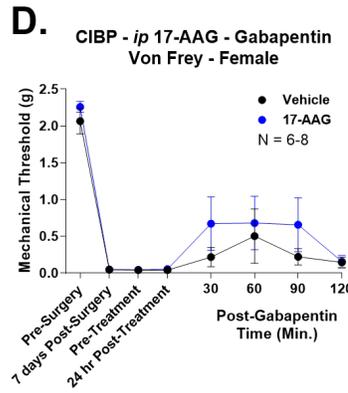
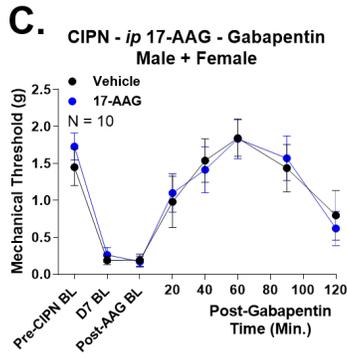
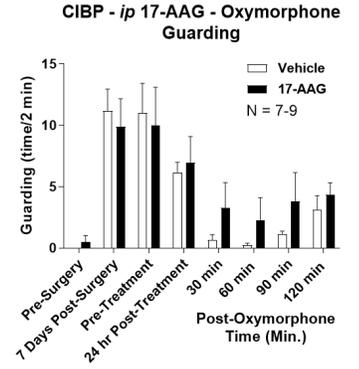
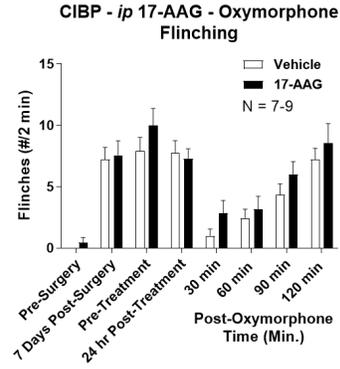
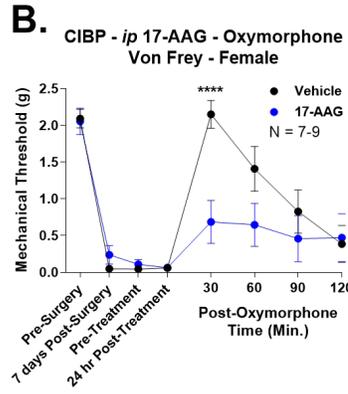
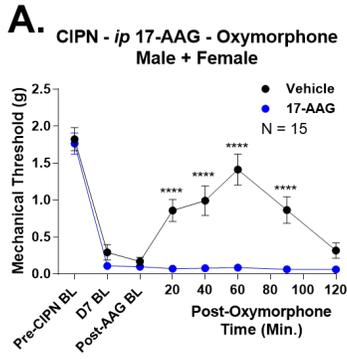


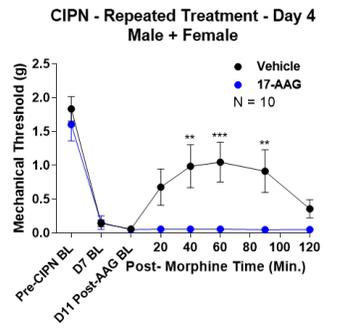
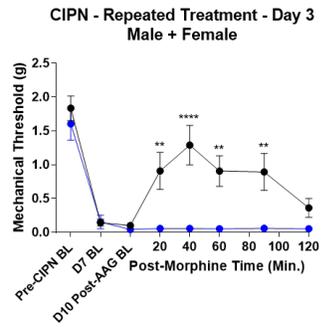
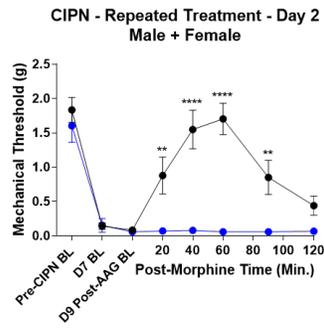
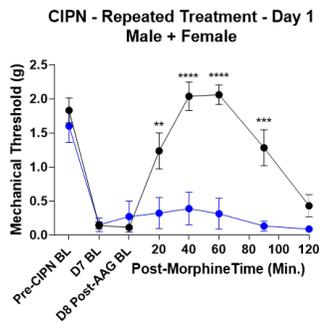
B. CIBP - *it* 17-AAG - Von Frey - Female

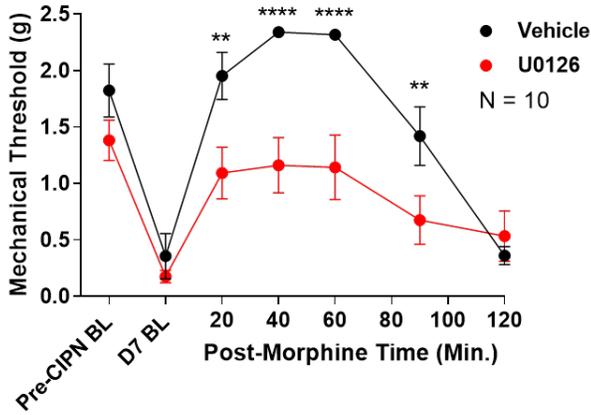
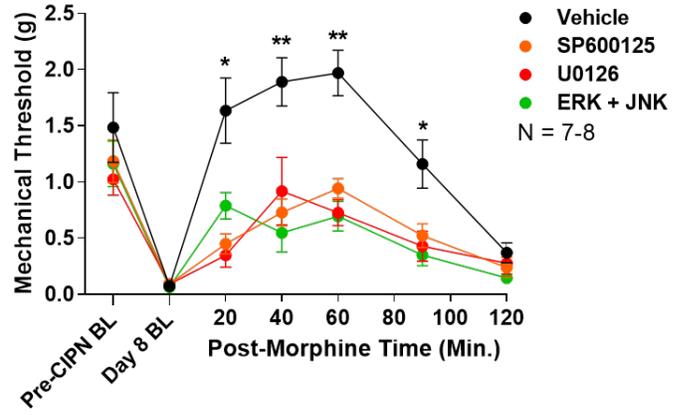
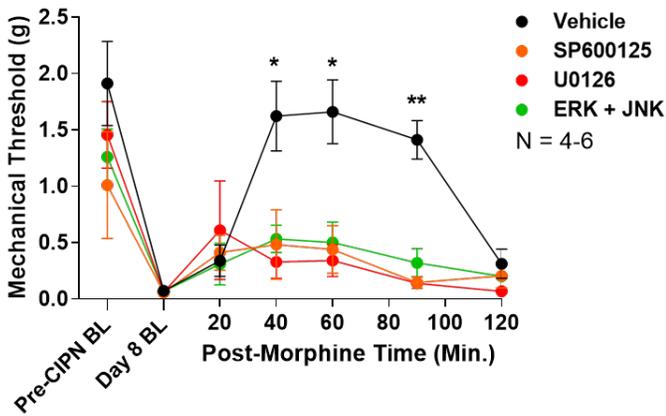
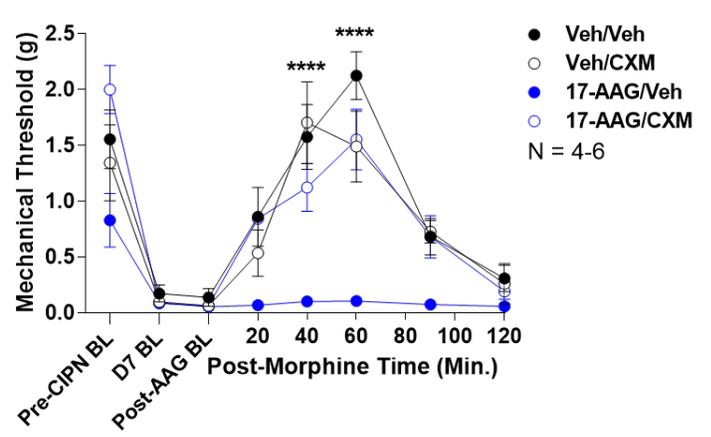


C. CIBP - *ip* 17-AAG - Von Frey - Female

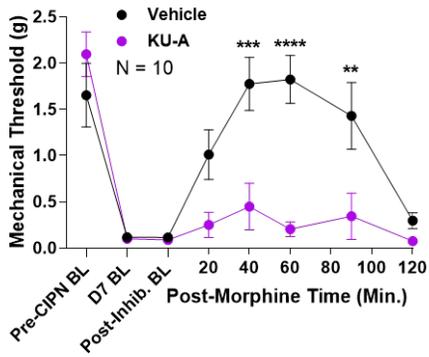




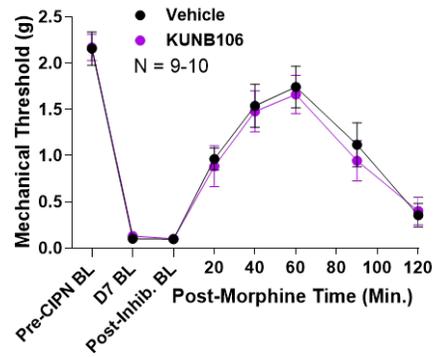


A.**CIPN - icv ERK Inhibitor - Male****B.****CIPN - icv ERK & JNK Inhibitors Male****C.****CIPN - icv ERK & JNK Inhibitors Female****D.****CIPN - icv 17-AAG + Cycloheximide Female**

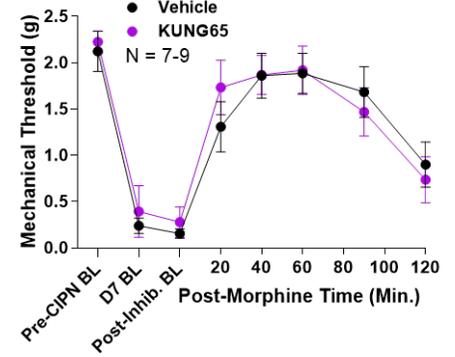
A. CIPN - *icv* KUNA115 (Hsp90 α)
Male + Female



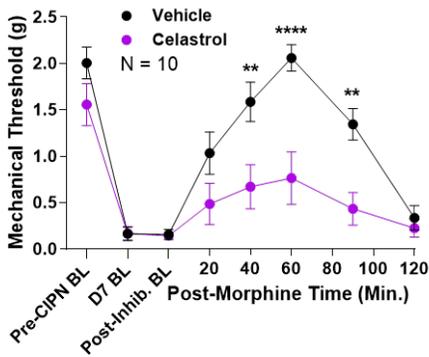
B. CIPN - *icv* KUNB106 (Hsp90 β)
Male + Female



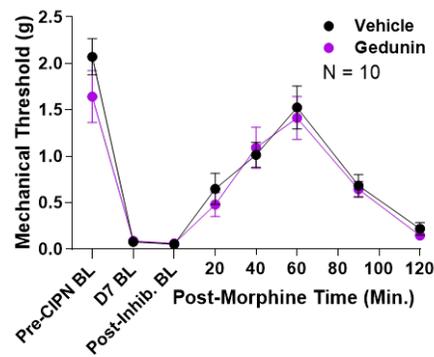
C. CIPN - *icv* KUNG65 (Grp94)
Female



D. CIPN - *icv* Celastrol (Cdc37)
Female



E. CIPN - *icv* Gedunin (p23)
Female



F. CIPN - *icv* KU177 (Aha1)
Male + Female

