Title: A Kappa Opioid Receptor Agonist Blocks Bone Cancer Pain Without Altering Bone Loss, Tumor Size, or Cancer Cell Proliferation in a Mouse Model of Cancer-Induced Bone Pain

Authors: Katie A. Edwards[2], Joshua J. Havelin[2], Mary I. Mcintosh[1], Haley A. Ciccone[1], Kathlene Pangilinan[2], Ian Imbert[2], Tally M. Largent-Milnes[1], Tamara King[2], Todd W. Vanderah[1], and John M. Streicher[1]

Affiliation: [1]Department of Pharmacology, College of Medicine, University of Arizona, Tucson, AZ 85724 USA
[2]Department of Biomedical Sciences, College of Osteopathic Medicine, University of New England, Biddeford, ME 04005 USA

Corresponding Author:

John M. Streicher, Ph.D.

University of Arizona

College of Medicine, Department of Pharmacology

Life Sciences North 563, Box 245050

1501 N. Campbell Ave.

Tucson, AZ 85724

JStreicher@email.arizona.edu

520-626-7495

Running title: Kappa Opioids in Cancer Bone Pain

Disclosures: No authors have any conflicts of interest. Funded by a Pilot Grant from the Maine Cancer Foundation and a Pilot Project grant funded by an institutional COBRE grant (P20GM103643: PI: I. Meng). Institutional funds from the University of Arizona were also used. The Foundation had no role in study design and performance, manuscript writing, or submission.

Abstract

Breast cancer metastasizes to bone, diminishing quality of life of patients due to pain, fracture, and limited mobility. Cancer-induced bone pain (CIBP) is characterized as moderate to severe ongoing pain, primarily managed by Mu opioid agonists such as fentanyl. However, opioids are limited by escalating doses and serious side effects. One alternative may be kappa opioid receptor (KOR) agonists. There are few studies examining KOR efficacy on CIBP, while KOR agonists are efficacious in peripheral and inflammatory pain. We thus examined the effects of the KOR agonist U50,488 given 2x daily across 7 days to block CIBP, tumor-induced bone loss, and tumor burden. U50,488 dose-dependently blocked tumor-induced spontaneous flinching and impaired limb use, without changing tactile hypersensitivity, and was fully reversed by the KOR antagonist *nor*-binaltorphimine (norBNI). U50,488 treatment was higher in efficacy and duration of action at later time points. U50,488 blocked this pain without altering tumor-induced bone loss or tumor growth. Follow-up studies in human cancer cell lines confirmed that KOR agonists do not affect cancer cell proliferation. These studies suggest that KOR agonists could be a new target for cancer pain management that does not induce cancer cell proliferation or alter bone loss.

Perspective (50 words max)

This study demonstrates the efficacy of KOR agonists in the treatment of bone cancer-induced pain in mice, without changing tumor size or proliferation in cancer cell lines. This suggests that KOR agonists could be used to manage cancer pain without the drawbacks of Mu opioid agonists and without worsening disease progression.

Key Words

Kappa opioid; cancer; bone; pain; proliferation

Introduction

Tumor growth within bone is associated with moderate to severe pain. Pain is the most feared consequence of cancer and greatly diminishes the patient's quality of life.[5, 34] Mu opioid receptor (MOR)-mediated drugs such as morphine and fentanyl remain the gold standard for the treatment of moderate to severe cancer pain including ongoing and breakthrough cancer pain.[1] However, the severity of cancer pain generally requires high doses, and sustained treatment leads to tolerance, which leads to dose escalation. This feed-forward loop can lead to the development of severe side effects, including constipation, nausea, and dependence.[1] All of these stereotypical opioid side effects contribute to a diminishing patient quality of life. In addition to these adverse side effects, some clinical and preclinical studies suggest that opioids could be increasing the progression and severity of the cancer itself, including promotion of proliferation and metastasis and acceleration of bone loss and fracture.[3, 11,13,22]

The drawbacks of opioids described above indicate the importance of finding alternatives for pain management in patients with cancer-induced pain. One potential target is the kappa opioid receptor (KOR). KOR agonists do not cause side effects such as constipation, nausea and risk of addiction that are observed with the current clinically used opioids targeting the MOR.[23] KOR agonists produce anti-nociception,[39] and can decrease drug self-administration,[17, 24, 32] morphine-induced dependence,[39] and pruritus.[18] While these aspects are very promising for the treatment of pain, centrally-acting KOR agonists are limited most strongly by the development of dysphoria.[45] However, the use of peripherally restricted KOR agonists[10, 19, 40, 41] or functionally selective KOR agonists that show decreased dysphoria[12, 26, 35, 42] could allow for the clinical development of this class of compounds.

Previous investigations into the effects of KOR agonists on cancer pain determined the effects of KOR agonist administration locally to the tumor or into the spinal cord.[2, 6, 20] These studies indicate that KOR agonists acting at peripheral and spinal sites can block tumor-induced bone pain. However, these studies only examined acute anti-nociception in response to a single administration of KOR agonist. The efficacy of KOR agonists in cancer pain has not been tested in a model consisting of repeated systemic administration over time as would be used in a clinical setting for the management of CIBP. Such a study is important due to potential effects that

prolonged administration of an opioid agonist may have on aspects of disease progression such as tumor growth or tumor-induced bone remodeling.

We tested the hypothesis that repeated systemic administration of a KOR agonist blocks tumor-induced bone pain (measured as nocifensive behaviors) without altering tumor growth or tumor-induced bone remodeling in a mouse model of CIBP. The effects of administration of the KOR agonist U50,488 on tumor-induced pain behavior, bone degradation and tumor burden were examined. The ability of the KOR agonist to alter the proliferation of multiple human cancer cell lines was also examined *in vitro*, to further evaluate the safety of KOR agonists for cancer pain treatment.

Methods

Subjects.

Female adult naïve Balb/cfC3H mice, weighing 20-25 g (Charles River, Willington, MA), were chosen for histocompatibility with the 66.1 adenocarcinoma cell line and because breast cancer mainly affects female patients.[28] All mice were housed with a maximum of three per cage with a 12 hour light/dark cycle and food and water available *ad libitum*, and used in strict accordance with the NIH Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee (IACUC) at the University of New England Animal Care Unit and at the University of Arizona.

Cancer Implantation and Treatment

Baseline radiograph digital images (Carestream Health, Inc Woodbridge CT) of the right femurs were obtained prior to surgery for the single injection groups. Mice were anesthetized with isoflurane and an arthrotomy was performed, exposing the condyles of the distal femur as previously described.[37] A hole was drilled into the distal femur and a needle inserted into the intramedullary space for injection of the cancer cells. Needle placement inside the intramedullary space of the femur was verified using radiograph images. Mice received injections of 5 µl of serum-free minimal essential medium (MEM) containing approximately 5 x 10^5 66.1 cells (culture details below) into the intramedullary space of the right femur.[27] Sham-treated mice had the same procedure performed, but culture media without cells was injected into the femur. The injection site was sealed

with dental cement (Simplex).

Pain Assays and *In Vivo* Imaging

Experimental Design

Each mouse was tested for impaired limb use, spontaneous flinching episodes, limb guarding behavior, and tactile hypersensitivity prior to surgery (pre-surgery baselines), 7 days after surgery, and prior to and after the morning drug injection on Days 7, 10, and 14. Mice were first tested for limb use, flinching, and guarding by allowing them to walk in an empty mouse pan for 2 minutes. They were then placed in von Frey testing chambers for assessment of evoked tactile hypersensitivity using von Frey filaments and the up-down method.[8] The experimenter, who was blinded to the treatments with animals randomized to treatment groups, conducted all pain measurements. Starting day 7 post-surgery, mice received 2 daily injections of (±)-U50,488 hydrochloride (Tocris, U50,488) (10 or 40 mg/kg, s.c.) or distilled water-vehicle (s.c.) 12 hours apart. In a separate set of experiments, mice received a single daily injection of *nor*-Binaltorphimine (Tocris, norBNI) (10 mg/kg, s.c.) or vehicle 1 hour prior to the first U50,488 injection of the day for the 7 day treatment period. No behavioral differences could be observed between the single and double injection sets as described above, so the data sets were combined for analysis in **Figure 3**. Tumor-induced pain behaviors were then measured at 7, 10, and 14 days post-surgery, corresponding to day 1, 3, and 7 of injections. All testing occurred at either a time course (0.5 – 8 hrs), or 4 hours after the morning injection of U50,488.

Spontaneous Flinching

All mice were placed in an empty mouse pan and allowed to acclimate for 30 min. The number of flinching episodes in 2 minutes was then measured for each mouse. All spontaneous behaviors were measured in the same 2 minute window.

Limb Use

Each mouse was placed in an empty mouse pan and observed while walking across the pan in a continuous motion. Limping and/or guarding behavior of the right (cancer-treated) hind limb was rated on the following scale: 0 = complete lack of use, 1 = partial non-use, 2 = limping and guarding, 3 = limping, 4 = normal walking.

Limb Guarding

During the 2 minute observation period as above, the mice were recorded for the number of seconds they kept their right hind limb elevated in a fully retracted position (i.e., guarding).

Tactile Hypersensitivity

Paw withdrawal thresholds in response to probing with calibrated Von Frey filaments were determined using the **"**up-down**"** method described by Chaplan and colleagues.[8] Mice were kept in suspended cages with wire grid floors and the von Frey filament applied perpendicularly to the plantar surface of the ipsilateral paw until it buckled. A positive response was indicated by a sharp withdrawal of the paw. An initial probe equivalent to 0.4 g was applied and if the response was negative, the stimulus was incrementally increased until a positive response was obtained, then decreased until a negative result was obtained. This up-down method was repeated until three changes in behavior were determined, and the pattern of positive and negative responses was tabulated.[9]

Determination of Bone Remodeling and Tumor Burden

Radiographic Analysis

Digital radiographs were taken after behavioral testing for the single injection set using a Carestream bioimaging machine, and images captured by a digital camera. Bone loss was rated by an experimenter blinded to treatment according to a 3 point scale: 0 = normal, 1 = osteolytic/osteoblastic bone remodeling, and 2 = cortical fracture.

Bone Histology

On day 14, following the behavioral testing and radiographic imaging, the 40 mg/kg U50,488 or vehicle treated mice received an overdose of pentobarbital (30 mg/kg, i.p.) and were perfused transcardially with physiological heparinized saline followed by 10% neutral-buffered formalin (Sigma, St. Louis, MO, USA). Femurs were collected from the right (cancer-treated) leg and postfixed overnight in 10% neutral buffered formalin. Femurs were rinsed in water to remove formalin and then placed in an EDTA solution for 2 weeks to achieve decalcification, which was verified using radiographic analysis. Samples were dehydrated in graded ethanol solutions before embedding in paraffin. They were oriented so that the entire length of the femur could be

longitudinally sectioned at 5 μm thickness. Sections were stained with hematoxylin and eosin (H&E) to visualize normal marrow elements and cancer cells under bright field microscopy. For image analysis, 6 sections per femur that contained the marrow space were randomly selected for analysis using Image J (NIH). Femurs from 4 vehicle treated and 4 U50,488 treated mice were analyzed. Area occupied by tumor was presented as percent of the total area of the intramedullary space within the bone, calculated as: (tumor area/total area)*100.

Cell Culture

Murine cell line 66.1 derived from spontaneously occurring mammary adenocarcinoma was maintained in MEM (Cellgro) containing 10% fetal bovine serum (FBS), 1X penicillin/streptomycin (P/S), and 1X insulin-transferrin-selenium (ITS) (Cellgro) at 37°C and in a 5% CO2 atmosphere. Cells that were cultured for injection into animals were cultured without ITS. The cells were used between passages 12 and 21.

Five human cell lines were obtained from ATCC and were maintained at 37˚C in a 5% CO_2 atmosphere. MCF7 (HTB-22) is a mammary gland adenocarcinoma and was maintained in MEM (Cellgro) containing 10% FBS, 1X P/S, and 1X ITS (Cellgro). MDA-MB-231 (HTB-26) is a mammary gland adenocarcinoma and was maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro) containing 10% FBS and P/S. SK-BR-3 (HTB-30) is a mammary gland adenocarcinoma and was maintained in McCoy's 5A (Cellgro) containing 10% FBS and P/S. A549 (CCL-185) is a lung carcinoma and was maintained in DMEM with 10% FBS and P/S. LNCaP clone FGC (CRL-1740) is a prostate carcinoma and was maintained in RPMI 1640 (Cellgro) containing 10% FBS and P/S.

Cell Proliferation Assay

The effect of KOR agonists on cell growth was measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Cells were seeded at 5000 cells/well (A549, MDA-MB-231 and SK-BR-3: 4000 cells/well) into clear-bottomed 96-well plates in 50 μL of colorless complete growth medium (described above). Immediately after plating, 50 μL of ten concentrations of U50,488 yielding final concentrations ranging from 1 μM to 1 mM or vehicle (complete growth medium) were added to the cells to give a final volume of 100 μL and centrifuged for 1 minute at 1000 rpm. Cells were incubated with drug at 37˚C in a 5% CO_2 atmosphere for 24, 48 or 72 hours following treatment. 20 μL of CellTiter 96 Aqueous One Solution (Promega) was added to each well and incubated for two hours, after which absorbance was recorded

at 490 nm using an ELx808 Ultra Absorbance Microplate Reader (BioTek). In individual experiments, compound concentrations were run in replicates of four that were blank-subtracted, averaged and normalized to the Day 1 vehicle absorbance to generate a concentration-response curve (CRC) using GraphPad Prism software. Curves were fit using a variable slope nonlinear regression analysis. IC_{50} values and Hill Slopes were determined for each individual curve for at least three independent experiments and reported as the mean ± SEM.

To assess whether U50,488-mediated cell death was occurring through a KOR-mediated mechanism, cells were plated as described above and treated with vehicle, 100 μM U50,488, 100 μM norBNI or 100 μM U50,488 and 100 μM norBNI. To evaluate growth with an alternative KOR agonist, GR 89696 fumarate (Tocris, GR89696), cells were plated as described above and treated with vehicle, 10 μM or 100 μM GR89696. Proliferation was evaluated as described above at 24, 48 and 72 hours. To evaluate whether daily replacement of medium and compound would alter growth, medium was removed and replaced with fresh compound daily for 24, 48 or 72 hours in a two-point concentration assay with U50,488.

Statistical Analysis

Statistical comparisons for the behavioral analysis between treatment groups were done using 2-way analysis of variance (ANOVA). Post-hoc comparisons between groups were done using the Bonferroni post-hoc test. For all analysis, significance was set at $p < 0.05$. For the *in vitro* experiments, the data were compared across days and drug treatment groups using a 2-Way ANOVA. Post-hoc comparisons were performed using a Fisher's Least Significant Difference test. For all analysis, significance was set at $p < 0.05$.

Results

KOR agonist blocks spontaneous pain, guarding, and impaired limb use without altering tactile hypersensitivity

Tumor-bearing mice developed pain behaviors indicative of spontaneous pain, impaired limb use, limb guarding, and referred tactile hypersensitivity at 7-14 days post cancer cell implantation (Pre-injection baselines on Days 7, 10, and 14 in **Figures 1-2**; vehicle treated mice on Day 14 in **Figure 3**). We began our studies with twice-daily injections of U50,488 (10 mg/kg, s.c.) or vehicle. A dose of 10 mg/kg is a moderate to high dose to

demonstrate anti-nociception in mice, which can be blocked with a KOR-selective antagonist.[30] We found that the first acute administration of U50,488 on Day 7 only had a small effect; limb use scores were returned to sham-treated levels and tactile hypersensitivity was slightly improved at 1.5-2 h post-injection while flinching and guarding was unchanged (**Figure 1**). This changed on Day 10, where U50,488 significantly improved flinching, limb use, and guarding scores vs. vehicle treated controls for up to 8 hours post-injection. Notably, U50,488 treatment had no effect on tactile hypersensitivity on Day 10 or 14. On Day 14, the pre-injection baselines of the U50,488-treated CIBP group were significantly improved vs. the vehicle injected CIBP animals for flinching, limb use, and limb guarding (**Figure 1**). This suggests a long-lasting anti-nociceptive benefit of repeated U50,488 injection. U50,488 injection on Day 14 only appeared to transiently improve these scores vs. the pre-injection baseline; however the pre-injection baseline and the entire 8 hour time course all showed sustained improvement vs. the vehicle injected CIBP group. Tactile hypersensitivity was not changed by U50,488 injection on Day 14, and the sham controls showed no signs of pain or effects of U50,488 injection except for a brief effect on Day 7 tactile hypersensitivity (**Figure 1**).

The time course data from **Figure 1** is summarized in **Figure 2**. For each behavioral measurement, we display the effects at 4 h post-injection, a consensus peak effect time across the behaviors and time courses. We also show the whole time course area under the curve (AUC) measurement, incorporating each day and post-injection time point. These data demonstrate that chronic U50,488 injection significantly but partially improves the spontaneous pain measures of flinching, guarding, and limb use without altering the evoked pain measure of tactile hypersensitivity. This effect is not apparent with acute administration on Day 7, but is clear on Days 10 and 14 (**Figure 2**).

While 10 mg/kg U50,488 did have beneficial anti-nociceptive effects, the efficacy was only partial. To determine if higher doses could achieve full efficacy, we performed CIBP experiments as above using 40 mg/kg twice daily injections of U50,488. Behavior was measured only on day 14 at 4 hours post-injection, to match the peak effect of the 10 mg/kg experiments above (**Figures 1-2**). In this experiment, tumor-bearing mice treated with distilled water (vehicle) demonstrated a significant number of flinching episodes (**Figure 3A,** **** $p<0.0001$ vs Day 0 Vehicle) that was fully reversed by twice daily injections of U50,488 (40 mg/kg, s.c.) beginning on day 7 after tumor implantation (**Figure 3A**, #### $p<0.0001$ vs. Day 14 all other groups). This is a high dose of

U50,488, increasing the chances of off-target effects, thus the mice were also pre-treated with the KOR-selective antagonist norBNI. Daily pre-treatment with norBNI (10 mg/kg, s.c., 1x/day) alone had no effect on pain behavior when compared with vehicle treatment, but reversed the decrease in flinching caused by U50,488 back to vehicle treated levels (**Figure 3A**). Tumor-bearing mice treated with vehicle demonstrated impaired limb use 14 days post-cancer cell injections (**Figure 3B**, **** $p<0.0001$ vs. Day 0 Vehicle) that was blocked by twice daily injections of U50,488 (40 mg/kg, s.c.) starting 7 days post cancer cell injections (**Figure 3B**, #### $p<0.0001$ vs. Day 14 all other groups). Daily pre-treatment with norBNI also had no effect on its own, but fully reversed the limb use improvement caused by U50,488 treatment back to vehicle-treated levels (**Figure 3B**). Tumor-bearing mice treated with vehicle also developed tactile hypersensitivity 14 days post cancer cell injection (**Figure 3C**, **** $p<0.0001$ vs. Day 0 Vehicle). Notably, the tumor-induced tactile hypersensitivity was not reversed by the U50,488 treatment, and norBNI pre-treatment on its own or prior to U50,488 treatment also had no effect (**Figure 3C**, $p > 0.05$). These differential results with spontaneous vs. evoked pain align with the results with 10 mg/kg U50,488 in **Figures 1-2**. These results suggest that higher doses of U50,488 are completely efficacious in treating bone cancer-induced ongoing pain and impaired limb use, but not referred tactile hypersensitivity. These results also suggest that the U50,488 is exerting this effect via the KOR even at the very high dose of 40 mg/kg, as a selective dose of the KOR antagonist norBNI (10 mg/kg[30]) fully reversed the anti-nociceptive effects of U50,488 treatment. In addition, the 40 mg/kg dose of U50,488 had the same behavioral effects as 10 mg/kg, just with greater efficacy.

KOR agonist does not alter tumor-induced bone remodeling

To determine whether treatment with the KOR agonist blocks bone remodeling, *in vivo* bone imaging was done following behavioral testing D14 post tumor-implantation. The imaging and histology experiments were only performed with 40 mg/kg U50,488 (or vehicle) treated mice as bone remodeling should be maximized at the higher dose. Radiographs demonstrated that U50,488 treatment had no effect on the tumor-induced bone remodeling (**Figure 4A**). Bone ratings by an experimenter blinded to treatment groups further indicate failure of the KOR agonist treatment to alter tumor-induced bone remodeling (**Figure 4B**).

KOR agonist does not alter tumor burden D14 post implantation.

To determine whether treatment with the KOR agonist blocks tumor burden within the intramedullary space, H&E stained sections (**Figure 5A**) were analyzed for tumor burden. The U50,488 treated mice demonstrated an equivalent tumor burden compared to distilled water-treated mice (**Figure 5B**).

KOR agonist alters cell proliferation *in vitro* through a non-KOR mechanism

In order to further confirm that KOR agonists do not promote (or inhibit) cancer cell growth and to help establish safety for human use, we tested the ability of U50,488 to alter cancer cell proliferation *in vitro* with multiple human cancer cell lines. We chose relevant human cancer cell lines from several tissues and cancer types, some of which metastasize to bone – 66.1 (mouse mammary, used in our *in vivo* study), MCF7 (breast adenocarcinoma), LNCaP (prostate carcinoma), MDA-MB-231 (breast adenocarcinoma), A549 (lung carcinoma), and SK-BR-3 (breast adenocarcinoma).

We then measured the proliferation of these cell lines over 3 days in the presence of CRCs of U50,488 using an MTS assay. We found that the cell lines grew over the 3 day treatment period, with CRC baselines that match with vehicle-treated controls (**Figure 6**). U50,488 did decrease proliferation and/or result in cell death with high efficacy on all 3 days, driving down the measurable level of cells near to 0 in all cases. However, the measured IC_{50} concentrations required for this anti-proliferative effect were extremely high, and ranged from 36.1 µM for 66.1 cells, Day 3 at the lowest, to 268.6 µM for SK-BR-3 cells, Day 1 at the highest (**Figure 6**, **Table 1**). U50,488 is a high potency full agonist, with an affinity for the KOR in the low nanomolar range (K_i of 3.1 ± 1.5 nM vs. [^{3}H]-Diprenorphine in KOR-CHO cells, graphs not shown, n=3 independent experiments). Thus U50,488 should have near-complete receptor occupancy in the range of 100 nM or less. The high micromolar results found here suggest a non-KOR, off-target or non-pharmacological mechanism, such as chemical toxicity or blockade of sodium channels,[36] to be responsible for these effects. In addition, we measured very high Hill Slopes above 2.0 in nearly all cases, further suggesting that the mechanism of action is not well described by a typical receptor binding model, which has a Hill Slope approximating 1.0 (**Table 1**).

One explanation for the extremely low potencies observed for U50,488 could be degradation of the drug over the 3 day treatment period. To help rule out this possibility, we performed a set of experiments with MDA-MB-231 cells with daily replacement of the media and U50,488. We found that while 100 µM U50,488 did efficaciously result in cell death over the treatment period, 10 µM U50,488 was indistinguishable from vehicle-

treated controls (**Figure 7**). This result shows that daily replacement of drug did not improve the potency of U50,488, suggesting that drug degradation is not the explanation for our results.

To further confirm that the anti-proliferative efficacy of U50,488 at high concentrations is non-KOR mediated, we co-incubated all human cell lines with 100 µM U50,488 and 100 µM norBNI, a moderately selective KOR antagonist with a very long duration of action.[7] We found that norBNI alone was indistinguishable from vehicle treatment, while U50,488 plus norBNI treatment was indistinguishable from U50,488 alone (**Figure 8**). These results further confirm that the low potency anti-proliferative efficacy of U50,488 on cancer cells does not occur through the KOR.

Lastly, we treated all human cell lines with 10 µM and 100 µM of GR89696, a KOR agonist with higher selectivity and potency than U50,488 (IC_{50} = 0.04 nM in KOR tissue bioassay).[31] GR89696 had a small but significant anti-proliferative effect vs. vehicle treatment only on Day 3 of the assay, only at the 100 µM concentration, and only in A549, MDA-MB-231, and SK-BR-3 cells (**Figure 9**). These results contrast with the U50,488 results (**Figures 6-8, Table 1**) which at 100 µM strongly affects all cell lines at Day 2 and 3, and all but SK-BR-3 on Day 1. U50,488 has these much stronger effects despite a lower affinity and selectivity for the KOR than GR89696. These results further confirm that U50,488's anti-proliferative effect in cancer cells is via a non-KOR mechanism, potentially through blockade of sodium channels.[36]

Discussion

Our data demonstrates that repeated administration of the KOR agonist U50,488 across 7 days reversed spontaneous flinching, guarding, and limb use, without altering tumor-induced tactile hypersensitivity. This anti-nociceptive effect was dose-dependent, as 10 mg/kg partially reversed signs of spontaneous pain, while 40 mg/kg fully reversed spontaneous pain. We also observe a time-dependent effect of U50,488 treatment, as the anti-nociceptive effects of U50,488 were greater on days 10 and 14 than day 7. As CIBP is a progressive pain state, it is not yet clear whether this represents increased pain the drug can affect on days 10 and 14, or a potentiating effect of chronic anti-nociceptive treatment. Along these lines, on day 14 the pre-injection baselines for spontaneous pain are already significantly improved vs. vehicle treatment. This suggests a benefit of repeated U50,488 administration, and suggests the animals do not become tolerant to repeated dosing. Further

experiments will be needed to explore this day 14 effect, including whether drug is accumulating systemically, along with an investigation as to whether drug tolerance is occurring.

Our data also demonstrates that this anti-nociceptive effect occurred through the KOR, as pre-treatment with a selective dose of the KOR antagonist norBNI completely reversed all effects of the high dose (40 mg/kg) of U50,488. Notably, these anti-nociceptive effects were observed in the absence of any effects of the KOR agonist on disease progression as measured by tumor-induced bone remodeling or tumor growth within the bone. These findings suggest that KOR agonists may not alter tumor growth or cancer cell proliferation *in vivo*. To further investigate this, we performed extensive *in vitro* studies on the effects of KOR agonists on human cancer cell line growth. We found that administration of U50,488 directly on cancer cells resulted in cell death only at extremely high concentrations, suggesting a non-KOR-mediated mechanism. Confirming this hypothesis, we found that daily drug replacement and co-incubation of the high potency, long duration KOR antagonist norBNI had no effect on U50,488 anti-proliferative potency and efficacy. We also found that an alternate KOR agonist (GR89696) with higher potency and selectivity than U50,488 for the KOR had significantly reduced anti-proliferative cancer cell effects. Taken together, these results warrant further investigation of the effects of KOR agonists on CIBP, and bone cancer disease progression, with the potential that KOR agonists could be efficacious and safe for clinical cancer pain management. With more research, perhaps KOR agonists could be used with confidence that they will not risk increasing tumor growth or cancer cell proliferation, a current possibility with MOR agonists used clinically.[11, 13] These findings suggest potential benefits of further research into peripherally restricted or functionally selective KOR agonists with diminished dysphoric effects as a potential novel strategy for pain management of cancer pain patients.

One striking result was the finding that at a high dose U50,488 treatment completely reversed the non-evoked pain measures of spontaneous flinching and impaired limb use, without altering evoked tactile hypersensitivity. This dichotomy between the efficacy of U50,488 on non-evoked vs. evoked measures of pain may represent different mechanisms underlying CIBP, with KOR agonists more effective against ongoing pain. These observations are consistent with earlier studies demonstrating differential effects of the administration of p38 MAPK inhibitors on cancer-induced pain.[37, 38] Administration of a p38 MAPK inhibitor effectively blocked aspects of cancer pain such as flinching and guarding, but failed to block tactile hypersensitivity of the ipsilateral

hindpaw, a measure of referred pain. Notably, previous studies using acute peripheral administration of the KOR agonist U50,488 blocked tumor-induced thermal hypersensitivity in a rat model of CIBP.[2] No observations of tactile hypersensitivity or impaired limb use were reported. However, these results indicate that the effects of KOR agonists may not differ along the lines of evoked and spontaneous pain, but rather along modalities. Of interest, blockade of TRPV1 expressing fibers has been found to block both ongoing pain and heat hypersensitivity in preclinical models of inflammation and nerve injury-associated pain.[21, 33] Therefore, KOR agonists may be acting on the same nociceptive fiber population. However, the pattern of expression of KOR in sensory neurons within the dorsal root ganglion (DRG) in relation to other markers indicative of individual fiber populations is not established. Such observations highlight the complexity of tumor-induced bone pain. As CIBP is multifactorial (ongoing pain, breakthrough pain, referred pain), the observations that compounds have differential actions on measures of ongoing and evoked pain states suggest that future studies should examine multiple aspects of cancer pain. These observations also highlight the importance of examining the effects of new drugs on multiple pain states within clinical trials. Drugs that are effective against ongoing pain may fail to block evoked pain such as referred cold allodynia or breakthrough pain, whereas drugs that are not effective against ongoing pain may still diminish or block episodes of breakthrough pain or effectively reduce tactile or cold allodynia commonly experienced by patients with painful bone metastases.

Our finding *in vivo* that KOR agonists block ongoing cancer-induced pain without altering tumor growth is supported by our *in vitro* studies, which strongly suggest that KOR agonists only block proliferation and/or kill cancer cells at extremely high concentrations utilizing a non-KOR mechanism. However, the literature on KOR effects on cancer proliferation is limited. KOR agonists have been shown to induce proliferation of C6 glioma cells,[4] and block proliferation in NSCLC,[25] CNE2,[43] and MCF7[29] cells. The concentrations of U50,488 required in the NSCLC study were similar to the ones required in our study with a concentration range of 15.6-250 μM; however they were able to block this effect with an equal concentration of norBNI. The study investigating MCF7 cells was able to see a strong effect with 10 nM of cyclazocine; however, this drug is not highly selective for the KOR, with MOR partial agonist and delta opioid receptor (DOR) activity that complicates the analysis.[14-16] A recent study demonstrated that KOR agonists can decrease tumor growth *in vivo* by blocking VEGF signaling and tumor angiogenesis.[44] Yamamizu and colleagues demonstrated that Lewis Lung carcinoma and B16 melanoma transplanted subcutaneously showed increased tumor volume and weight in KOR knockout mice

compared to WT mice 19 days following transplantation, along with increased angiogenesis. Furthermore, twice-daily administration of the KOR agonist TRK820 starting 2 days after tumor implantation diminished tumor size measured 7 and 11-14 days after implantation. Their data indicate that this is through an indirect effect on cancer cells as they demonstrate that 1) the treatment with the agonists dramatically reduced angiogenesis and 2) this effect was not observed in KOR knockout mice indicating that the effect was through non-tumor cells in the mouse. Their data indicates that these effects are most likely mediated through reducing tumor angiogenesis.

One potential explanation for the differences of tumor growth *in vivo* between our study and the study by Yamamizu and colleagues is differences in the approaches. Yamamizu and colleagues performed their *in vitro* studies with HUVEC cells. Our *in vitro* studies examined multiple human and rodent cell lines. The HUVEC cells may be more sensitive to KOR agonists, although they did use 10 and 30 μM of U50,488 and TRK820 in their studies. High drug concentrations do suggest non-KOR mechanisms as above. In addition, the tumor grafts were all subcutaneous, whereas our tumors were implanted in intramedullary bone. The well-vascularized bone location could have minimized the need for *de novo* angiogenesis compared to a subcutaneous solid tumor, and should be kept in mind when evaluating the breadth of use of KOR agonists as tumor angiogenesis blockers. One last difference is that whereas Yamamizu and colleagues began drug administration 2 days post-tumor implantation, we began treatment 7 days post tumor implantation into the bone. It is possible that tumor angiogenesis within the bone has been established at that point, and therefore cannot be blocked or reversed by the KOR agonist. This deserves further study to examine whether prevention of angiogenesis by early treatment with a KOR agonist may diminish tumor growth by blocking angiogenesis whereas later treatment with a KOR agonist may fail to alter tumor growth or angiogenesis. This may be particularly relevant in the bone marrow in which the tumor may rapidly fill the marrow space.

Our data indicates that repeated administration of KOR agonist across 7 days fails to worsen disease progression as measured by tumor growth or bone remodeling. However, the KOR agonist blocked tumor-induced spontaneous flinching, a measure of ongoing pain, as well as impaired limb use without altering tactile hypersensitivity. Our data also suggests that chronic dosing of U50,488 has a long-lasting anti-nociceptive benefit even 12 hours after the last drug injection. Such observations suggest that KOR agonists may be beneficial in pain management, particularly for ongoing cancer bone pain, without worsening or accelerating

disease progression. Further studies are required to test the utility of KOR agonists in treating CIBP without adverse effects of MOR agonists including somnolence, constipation and nausea, development of analgesic tolerance, and the potential for enhanced tumor-induced bone loss. Further studies with peripherally restricted or functionally selective KOR agonists will also be required to determine if the dysphoric effects of centrally acting KOR agonists can be avoided in cancer patients, and to determine any potential interactions with typical cancer treatments such as chemotherapy.

Acknowledgments

This work was funded by a generous Pilot Grant from the Maine Cancer Foundation, as well as a Pilot Project grant funded by an institutional COBRE grant (P20GM103643: PI: I. Meng). This work was also supported by institutional funds from the University of Arizona.

References

1. . In: Opioids in Palliative Care: Safe and Effective Prescribing of Strong Opioids for Pain in Palliative Care of Adults. National Institute for Health and Clinical Excellence: Guidance, Cardiff (UK), 2012.

2. Baamonde A, Lastra A, Juarez L, Garcia V, Hidalgo A, Menendez L. Effects of the local administration of selective mu-, delta-and kappa-opioid receptor agonists on osteosarcoma-induced hyperalgesia. *Naunyn Schmiedebergs Arch Pharmacol.* 372:213-219, 2005

3. Biki B, Mascha E, Moriarty DC, Fitzpatrick JM, Sessler DI, Buggy DJ. Anesthetic technique for radical prostatectomy surgery affects cancer recurrence: a retrospective analysis. *Anesthesiology.* 109:180-187, 2008

4. Bohn LM, Belcheva MM, Coscia CJ. Mitogenic signaling via endogenous kappa-opioid receptors in C6 glioma cells: evidence for the involvement of protein kinase C and the mitogen-activated protein kinase signaling cascade. *J Neurochem.* 74:564-573, 2000

5. Breivik H, Cherny N, Collett B, de Conno F, Filbet M, Foubert AJ, Cohen R, Dow L. Cancer-related pain: a pan-European survey of prevalence, treatment, and patient attitudes. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO.* 20:1420-1433, 2009

6. Brigatte P, Konno K, Gutierrez VP, Sampaio SC, Zambelli VO, Picolo G, Curi R, Cury Y. Peripheral kappa and delta opioid receptors are involved in the antinociceptive effect of crotalphine in a rat model of cancer pain. *Pharmacol Biochem Behav.* 109:1-7, 2013

7. Bruchas MR, Yang T, Schreiber S, Defino M, Kwan SC, Li S, Chavkin C. Long-acting kappa opioid antagonists disrupt receptor signaling and produce noncompetitive effects by activating c-Jun N-terminal kinase. *J Biol Chem.* 282:29803-29811, 2007

8. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods.* 53:55-63, 1994

9. Dixon WJ. Efficient analysis of experimental observations. *Annual review of pharmacology and toxicology.* 20:441-462, 1980

10. Eisenach JC, Carpenter R, Curry R. Analgesia from a peripherally active kappa-opioid receptor agonist in patients with chronic pancreatitis. *Pain.* 101:89-95, 2003

11. Exadaktylos AK, Buggy DJ, Moriarty DC, Mascha E, Sessler DI. Can anesthetic technique for primary breast cancer surgery affect recurrence or metastasis? *Anesthesiology.* 105:660-664, 2006

12. Frankowski KJ, Hedrick MP, Gosalia P, Li K, Shi S, Whipple D, Ghosh P, Prisinzano TE, Schoenen FJ, Su Y, Vasile S, Sergienko E, Gray W, Hariharan S, Milan L, Heynen-Genel S, Mangravita-Novo A, Vicchiarelli M, Smith LH, Streicher JM, Caron MG, Barak LS, Bohn LM, Chung TD, Aube J. Discovery of Small Molecule Kappa Opioid Receptor Agonist and Antagonist Chemotypes through a HTS and Hit Refinement Strategy. *ACS Chem Neurosci.* 3:221-236, 2012

13. Gach K, Wyrebska A, Fichna J, Janecka A. The role of morphine in regulation of cancer cell growth. *Naunyn Schmiedebergs Arch Pharmacol.* 384:221-230, 2011

14. Gharagozlou P, Demirci H, Clark JD, Lameh J. Activation profiles of opioid ligands in HEK cells expressing delta opioid receptors. *BMC neuroscience.* 3:19, 2002

15. Gharagozlou P, Demirci H, David Clark J, Lameh J. Activity of opioid ligands in cells expressing cloned mu opioid receptors. *BMC pharmacology.* 3:1, 2003

16. Gharagozlou P, Hashemi E, DeLorey TM, Clark JD, Lameh J. Pharmacological profiles of opioid ligands at kappa opioid receptors. *BMC pharmacology.* 6:3, 2006

17. Glick SD, Maisonneuve IM, Raucci J, Archer S. Kappa opioid inhibition of morphine and cocaine self-administration in rats. *Brain Res.* 681:147-152, 1995

18. Inui S. Nalfurafine hydrochloride to treat pruritus: a review. *Clinical, cosmetic and investigational dermatology.* 8:249-255, 2015

19. Jamshidi RJ, Jacobs BA, Sullivan LC, Chavera TA, Saylor RM, Prisinzano TE, Clarke WP, Berg KA. Functional Selectivity of Kappa Opioid Receptor Agonists in Peripheral Sensory Neurons. *J Pharmacol Exp Ther.* 355:174-182, 2015

20. Kim WM, Jeong CW, Lee SH, Kim YO, Cui JH, Yoon MH. The intrathecally administered kappa-2 opioid agonist GR89696 and interleukin-10 attenuate bone cancer-induced pain through synergistic interaction. *Anesthesia and analgesia.* 113:934-940, 2011

21. King T, Qu C, Okun A, Mercado R, Ren J, Brion T, Lai J, Porreca F. Contribution of afferent pathways to nerve injury-induced spontaneous pain and evoked hypersensitivity. *Pain.* 152:1997-2005, 2011

22. King T, Vardanyan A, Majuta L, Melemedjian O, Nagle R, Cress AE, Vanderah TW, Lai J, Porreca F. Morphine treatment accelerates sarcoma-induced bone pain, bone loss, and spontaneous fracture in a murine model of bone cancer. *Pain.* 132:154-168, 2007

23. Kivell BM, Ewald AW, Prisinzano TE. Salvinorin A analogs and other kappa-opioid receptor compounds as treatments for cocaine abuse. *Advances in pharmacology.* 69:481-511, 2014

24. Kuzmin AV, Semenova S, Gerrits MA, Zvartau EE, Van Ree JM. Kappa-opioid receptor agonist U50,488H modulates cocaine and morphine self-administration in drug-naive rats and mice. *Eur J Pharmacol.* 321:265-271, 1997

25. Kuzumaki N, Suzuki A, Narita M, Hosoya T, Nagasawa A, Imai S, Yamamizu K, Morita H, Nagase H, Okada Y, Okano HJ, Yamashita JK, Okano H, Suzuki T. Effect of kappa-opioid receptor agonist on the growth of non-small cell lung cancer (NSCLC) cells. *Br J Cancer.* 106:1148-1152, 2012

26. Lovell KM, Frankowski KJ, Stahl EL, Slauson SR, Yoo E, Prisinzano TE, Aube J, Bohn LM. Structure-Activity Relationship Studies of Functionally Selective Kappa Opioid Receptor Agonists that Modulate ERK 1/2 Phosphorylation While Preserving G Protein Over betaArrestin2 Signaling Bias. *ACS Chem Neurosci.* 2015

27. Lozano-Ondoua AN, Hanlon KE, Symons-Liguori AM, Largent-Milnes TM, Havelin JJ, Ferland HL, 3rd, Chandramouli A, Owusu-Ankomah M, Nikolich-Zugich T, Bloom AP, Jimenez-Andrade JM, King T, Porreca F, Nelson MA, Mantyh PW, Vanderah TW. Disease modification of breast cancer-induced bone remodeling by cannabinoid 2 receptor agonists. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 28:92-107, 2013

28. Mackiewicz-Wysocka M, Pankowska M, Wysocki PJ. Progress in the treatment of bone metastases in cancer patients. *Expert opinion on investigational drugs.* 21:785-795, 2012

29. Maneckjee R, Biswas R, Vonderhaar BK. Binding of opioids to human MCF-7 breast cancer cells and their effects on growth. *Cancer Res.* 50:2234-2238, 1990

30. McLaughlin JP, Marton-Popovici M, Chavkin C. Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J Neurosci.* 23:5674-5683, 2003

31. Naylor A, Judd DB, Lloyd JE, Scopes DI, Hayes AG, Birch PJ. A potent new class of kappa-receptor agonist: 4-substituted 1-(arylacetyl)-2-[(dialkylamino)methyl]piperazines. *J Med Chem.* 36:2075-2083, 1993

32. Negus SS, Mello NK, Portoghese PS, Lin CE. Effects of kappa opioids on cocaine self-administration by rhesus monkeys. *J Pharmacol Exp Ther.* 282:44-55, 1997

33. Okun A, DeFelice M, Eyde N, Ren J, Mercado R, King T, Porreca F. Transient inflammation-induced ongoing pain is driven by TRPV1 sensitive afferents. *Mol Pain.* 7:4, 2011

34. Paice JA, Ferrell B. The management of cancer pain. *CA: a cancer journal for clinicians.* 61:157-182, 2011

35. Schmid CL, Streicher JM, Groer CE, Munro TA, Zhou L, Bohn LM. Functional Selectivity of 6'-guanidinonaltrindole (6'-GNTI) at Kappa Opioid Receptors in Striatal Neurons. *J Biol Chem.* 2013

36. Su X, Castle NA, Antonio B, Roeloffs R, Thomas JB, Krafte DS, Chapman ML. The effect of kappa-opioid receptor agonists on tetrodotoxin-resistant sodium channels in primary sensory neurons. *Anesthesia and analgesia.* 109:632-640, 2009

37. Sukhtankar D, Okun A, Chandramouli A, Nelson MA, Vanderah TW, Cress AE, Porreca F, King T. Inhibition of p38-MAPK signaling pathway attenuates breast cancer induced bone pain and disease progression in a murine model of cancer-induced bone pain. *Mol Pain.* 7:81, 2011

38. Svensson CI, Medicherla S, Malkmus S, Jiang Y, Ma JY, Kerr I, Brainin-Mattos J, Powell HC, Luo ZD, Chakravarty S, Dugar S, Higgins LS, Protter AA, Yaksh TL. Role of p38 mitogen activated protein kinase in a model of osteosarcoma-induced pain. *Pharmacol Biochem Behav.* 90:664-675, 2008

39. Tao YM, Li QL, Zhang CF, Xu XJ, Chen J, Ju YW, Chi ZQ, Long YQ, Liu JG. LPK-26, a novel kappa-opioid receptor agonist with potent antinociceptive effects and low dependence potential. *Eur J Pharmacol.* 584:306-311, 2008

40. Vanderah TW, Largent-Milnes T, Lai J, Porreca F, Houghten RA, Menzaghi F, Wisniewski K, Stalewski J, Sueiras-Diaz J, Galyean R, Schteingart C, Junien JL, Trojnar J, Riviere PJ. Novel D-amino acid tetrapeptides produce potent antinociception by selectively acting at peripheral kappa-opioid receptors. *Eur J Pharmacol.* 583:62-72, 2008

41. Vanderah TW, Schteingart CD, Trojnar J, Junien JL, Lai J, Riviere PJ. FE200041 (D-Phe-D-Phe-D-Nle-D-Arg-NH2): A peripheral efficacious kappa opioid agonist with unprecedented selectivity. *J Pharmacol Exp Ther.* 310:326-333, 2004

42. White KL, Robinson JE, Zhu H, DiBerto JF, Polepally PR, Zjawiony JK, Nichols DE, Malanga CJ, Roth BL. The G protein-biased kappa-opioid receptor agonist RB-64 is analgesic with a unique spectrum of activities in vivo. *J Pharmacol Exp Ther.* 352:98-109, 2015

43. Wong N, Diao CT, Wong T. The overexpression of Bcl-2 antagonizes the proapoptotic function of the kappa-opioid receptor. *Ann N Y Acad Sci.* 1010:358-360, 2003

44. Yamamizu K, Furuta S, Hamada Y, Yamashita A, Kuzumaki N, Narita M, Doi K, Katayama S, Nagase H, Yamashita JK, Narita M. small ka, Cyrillic Opioids inhibit tumor angiogenesis by suppressing VEGF signaling. *Scientific reports.* 3:3213, 2013

45. Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ. Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: agonist actions at kappa opioid receptors. *Psychopharmacology (Berl).* 179:551-558, 2005

Figure Legends

Figure 1: Systemic daily administration of a moderate dose KOR agonist partially reverses tumor-induced spontaneous pain but not tactile hypersensitivity. Tumor bearing mice or sham-treated controls were measured for spontaneous and evoked pain behaviors pre-surgery (baseline; BL), pre-injection baselines on days 7, 10, and 14, and in a time course (0.5-8 hrs) after injection on those days. Beginning on day 7, all mice were injected twice daily (every 12 hrs) with vehicle (distilled water) or U50,488 (10 mg/kg, s.c.). The behaviors measured were spontaneous flinching (**A**), limb use scores (**B**), limb guarding (**C**), and evoked tactile hypersensitivity (**D**). Data reported as the mean ± SEM, n = 4-10 mice/group. *, **, ***, **** = p<0.05, 0.01, 0.001, 0.0001 for CIBP-Veh and CIBP-U50 vs. Sham-Veh or Sham-U50 groups; #, ##, ###, #### = p<0.05, 0.01, 0.001, 0.0001 for CIBP-Veh vs. CIBP-U50; both by 2 Way ANOVA with Bonferroni post-hoc test. CIBP groups develop significant spontaneous and evoked pain on all measures by day 10 at latest. U50,488 treatment only had a small acute effect on limb use and hypersensitivity on day 7, but a large effect on all spontaneous pain but not evoked pain on day 10 and 14. On day 14, pre-injection baselines were already improved to drug-treated levels.

Figure 2: Area under the curve and 4 hour time point measurements of KOR agonist treatment in tumor-induced pain. The data from **Figure 1** was analyzed for area under the curve (AUC) of the entire treatment time course, along with highlighting behavioral effects at 4 hours post-injection, a consensus peak effect time for drug administration. This was done for spontaneous flinching (**A**), limb use scores (**B**), limb guarding (**C**), and evoked tactile hypersensitivity (**D**). Data reported as the mean ± SEM. *, **, ***, **** = p<0.05, 0.01, 0.001, 0.0001 for CIBP-Veh and CIBP-U50 vs. Sham-Veh or Sham-U50; #, ##, ###, #### = p<0.05, 0.01, 0.001, 0.0001 for CIBP-Veh vs. CIBP-U50. 4 hour time point data analyzed by 2 Way ANOVA, AUC data by 1 Way ANOVA, both with Bonferroni post-hoc test. The data confirmed that 10 mg/kg U50,488 treatment significantly but partially reversed measures of spontaneous but not evoked pain on days 10 and 14.

Figure 3: Systemic daily administration of a high dose KOR agonist fully reverses tumor-induced spontaneous pain, but not tactile hypersensitivity. Behavior measured 4 hours after the final injection on day 14. **A)** Tumor bearing mice had increased numbers of flinching episodes 14 days post cancer cell implantation. Treatment with U50,488 (40 mg/kg, 2x daily across 7 days) blocked tumor-induced flinching, with values resembling those of baseline controls. norBNI pre-treatment (10 mg/kg, sc, 1x/day) had no effect on its

own, but fully reversed the effects of U50,488 treatment. **** $p < 0.0001$ vs. Day 0 Vehicle group; #### $p < 0.0001$ vs. Day 14 all other groups. **B)** Day 14 tumor-bearing mice showed impaired limb use compared to baseline observations. U50,488 treatment blocked the tumor-induced impaired limb use, with limb use ratings similar to baseline controls. norBNI pre-treatment had no effect on its own, but fully reversed the effects of U50,488 treatment. **** $p < 0.0001$ vs. Day 0 Vehicle; #### $p < 0.0001$ vs. Day 14 all other groups. **C)** Tumor-bearing mice developed tactile hypersensitivity 14 days post cancer cell implantation. Treatment with U50,488 failed to block tumor-induced tactile hypersensitivity. norBNI pre-treatment had no effect on its own, or prior to U50,488 treatment. **** $p < 0.0001$ vs. Day 0 Vehicle. Graphs represent mean ± SEM, n = 7-12/group (2 sets of independent experiments, see Methods).

Figure 4: Systemic daily administration of a KOR agonist failed to alter tumor-induced bone remodeling. **A)** Representative radiograph images showing 3 tumor-bearing mice treated twice daily across 7 days with distilled water (DI H20, left column) or with U50,488 (right column). Arrows indicate tumor-induced bone remodeling. The images indicate mixed osteoclast (dark areas) as well as osteoblast (white areas) bone remodeling. White arrows indicate regions of mixed osteolytic/osteoblastic bone remodeling. **B)** Rating of the tumor-bearing bones demonstrates equivalent levels of tumor-induced bone loss in the distilled water and drug treated mice.Graph represents mean ± SEM, n = 7-12/group (from **Figure 3**), $p > 0.05$.

Figure 5: Systemic daily administration of a KOR agonist failed to alter tumor burden 14 days following tumor implantation. A) Representative H&E stained bone sections demonstrating tumor within the intramedullary space. Brackets indicate areas of tumor within the bone. **B)** Calculation of the percentage of total marrow space that had tumor was calculated. Both vehicle-treated and U50,488-treated mice demonstrated an equivalent amount of tumor growth within the marrow space. For image analysis, 6 sections per femur that contained the marrow space were randomly selected for analysis using Image J (NIH). Femurs from 4 vehicle treated and 4 U50,488 treated mice were analyzed. Graph represents mean ± SEM, $p > 0.05$.

Figure 6: KOR agonist causes cell death at very high concentrations. Cells were grown over 3 days with vehicle or concentration curves of U50,488, and the level of proliferation measured each day by MTS assay. The data was normalized to the value for the Day 1 vehicle, and reported as the mean ± SEM. N=3 independent experiments for each cell line. The values were fit with a nonlinear curve using GraphPad Prism, and the resulting

IC_{50} and Hill Slope values determined (reported in **Table 1**). U50,488 causes cell death at high micromolar concentrations.

Figure 7: **Daily drug replacement does not alter KOR agonist anti-proliferative potency.** MDA-MB-231 cells were grown over 3 days in the presence of vehicle, 10 µM, or 100 µM U50,488, which was replaced daily. Proliferation was measured each day by MTS assay, and normalized to the Day 1 vehicle value. The data is reported as the mean ± SEM, n=3 independent experiments. ***,****p<0.001, 0.0001 vs. same day vehicle group. 100 µM U50,488 causes strong cell death, while 10 µM was not different from vehicle, meaning the potency did not improve with daily replacement.

Figure 8: KOR antagonist co-incubation does not alter KOR agonist induced cell death. All human cancer cell lines were grown for 3 days in the presence of vehicle, 100 µM U50,488, 100 µM nor-BNI, or U50 + nor-BNI. Proliferation was measured each day by MTS assay, and normalized to the Day 1 vehicle value. The data is reported as the mean ± SEM, n=3 independent experiments. *,**,***,****p<0.05, 0.01, 0.001, 0.0001 for U50,488 vs. same day vehicle control group; º, ºº, ººº, ººººp<0.05, 0.01, 0.001, 0.0001 for U50 + norBNI vs. same day vehicle control group. U50,488 alone causes strong cell death, which is not altered by norBNI co-incubation. NorBNI alone also has no significant effect when compared to vehicle treatment.

Figure 9: Alternate high potency KOR agonist causes less cell death than U50,488. All human cancer cells were grown over 3 days in the presence of vehicle, 10 µM, or 100 µM GR89696, a KOR agonist with higher potency and selectivity than U50,488. Proliferation was measured each day by MTS assay, and normalized to the Day 1 vehicle value. The data is reported as the mean ± SEM, n=3 independent experiments. *,**p<0.05, 0.01 vs. same day vehicle control group. GR89696, at best, causes only slight cell death at 100 µM, and only on Day 3, which is less than the effect of U50,488.

Table Legend

IC_{50} and Hill Slope values calculated from the nonlinear fitted curves in **Figure 6**. Values reported as the mean ± SEM. IC_{50} values are in the high micromolar range, and generally decrease over the 3 day treatment period. Hill Slope values are negative since the curves are inhibitory, but are all much steeper than the traditional inhibitory hill slope of -1.0. This suggests that the fitted curves do not match a typical receptor binding model.

Table 1

	U50,488 Proliferation IC_{50} (μM)			Hill Slope		
Cell Line	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day3**
A549	128.6 ± 10.1	77.5 ± 7.8	58.3 ± 7.2	-2.6 ± 0.3	-3.4 ± 0.4	-5.0 ± 2.2
LNCaP	230.7 ± 10.1	180.2 ± 10.7	146.0 ± 12.3	-3.7 ± 0.6	-3.4 ± 0.4	-3.4 ± 1.2
MCF7	238.3 ± 17.7	139.6 ± 19.1	84.2 ± 19.1	-18.2 ± 6.2	-2.1 ± 0.1	-2.9 ± 1.0
MDA-MB-231	264.7 ± 7.5	103.1 ± 6.7	67.6 ± 2.9	-14.8 ± 4.2	-3.7 ± 0.4	-13.0 ± 4.5
SK-BR-3	268.6 ± 54.0	150.9 ± 16.0	148.5 ± 2.2	-2.1 ± 0.6	-2.4 ± 0.3	-2.7 ± 0.5
66.1	79.0 ± 2.4	36.2 ± 0.8	36.1 ± 1.1	-1.3 ± 0.1	-2.6 ± 0.1	-4.4 ± 0.2

Figure 1

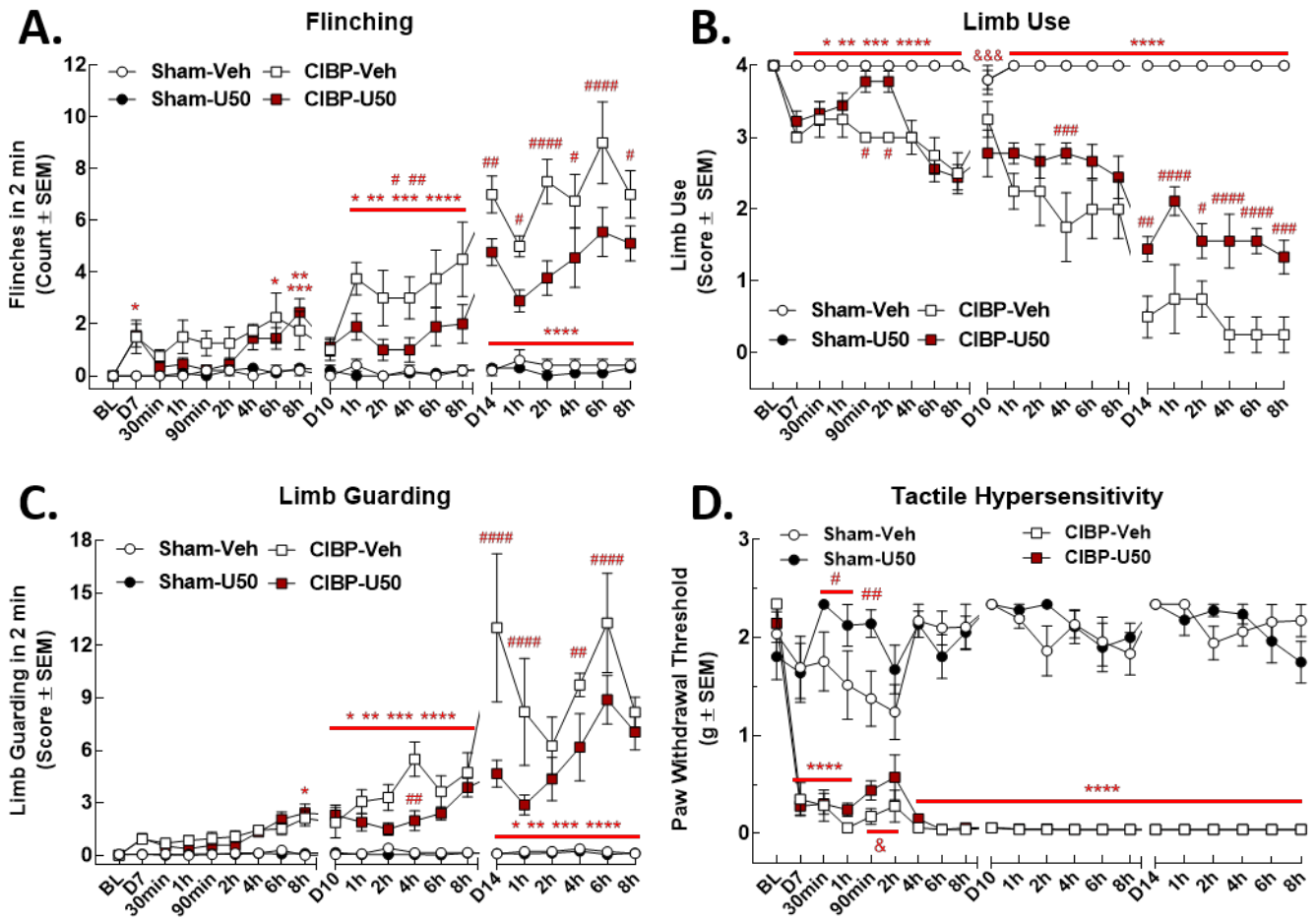

A.
Flinching
Sham-Veh
CIBP-Veh
Sham-U50
CIBP-U50
Flinches in 2 min
(Count ± SEM)
B.
Limb Use
Limb Use
(Score ± SEM)
C.
Limb Guarding
Limb Guarding in 2 min
(Score ± SEM)
D.
Tactile Hypersensitivity
Paw Withdrawal Threshold
(g ± SEM)
BL
D7
30min
1h
90min
2h
4h
6h
8h
D10
D14

Figure 2

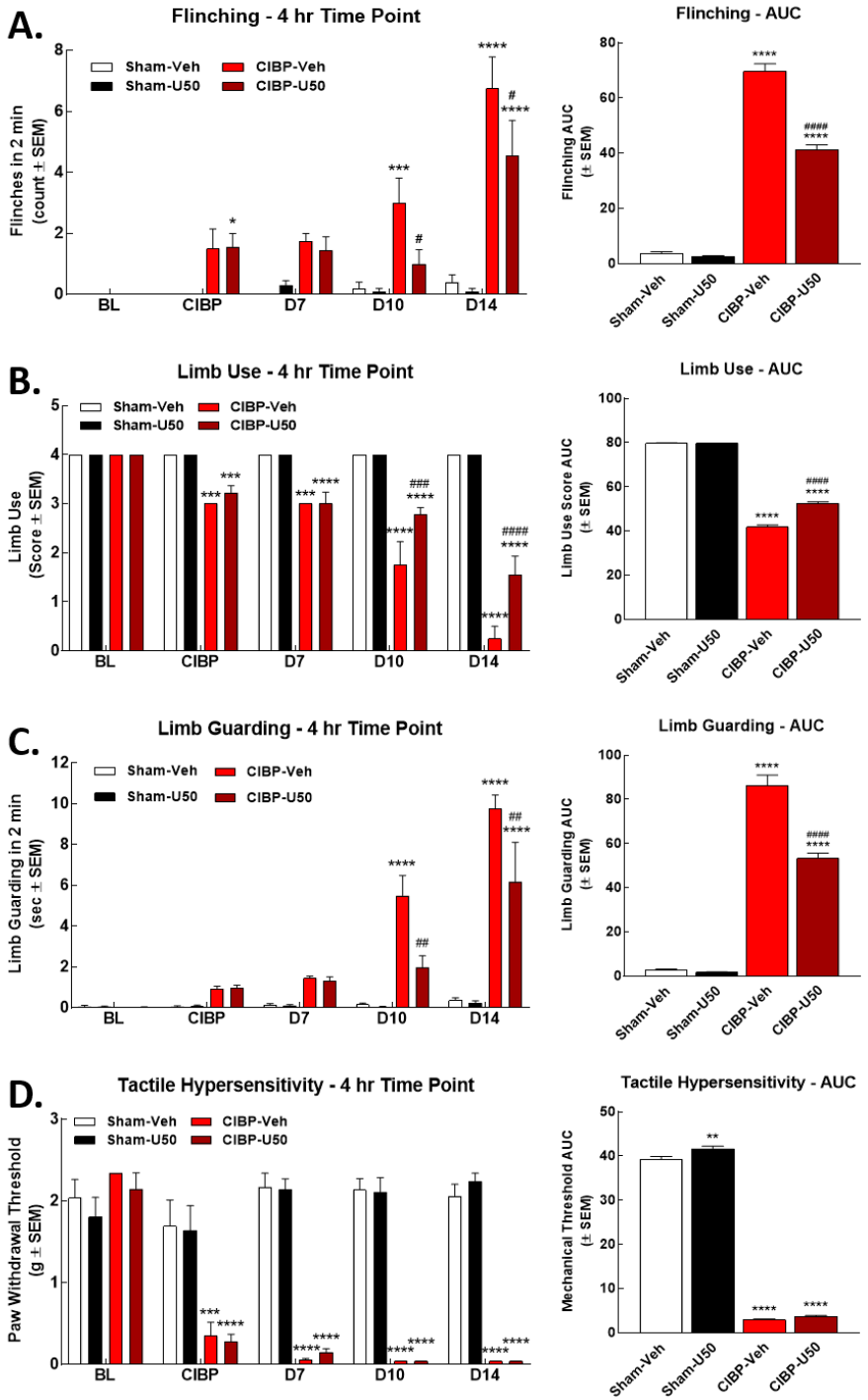

A.
Flinching - 4 hr Time Point
Sham-Veh
Sham-U50
CIBP-Veh
CIBP-U50
Flinches in 2 min (count ± SEM)
BL
CIBP
D7
D10
D14
Flinching - AUC
Flinching AUC (± SEM)
B.
Limb Use - 4 hr Time Point
Limb Use (Score ± SEM)
Limb Use - AUC
Limb Use Score AUC (± SEM)
C.
Limb Guarding - 4 hr Time Point
Limb Guarding in 2 min (sec ± SEM)
Limb Guarding - AUC
Limb Guarding AUC (± SEM)
D.
Tactile Hypersensitivity - 4 hr Time Point
Paw Withdrawal Threshold (g ± SEM)
Tactile Hypersensitivity - AUC
Mechanical Threshold AUC (± SEM)

Figure 3

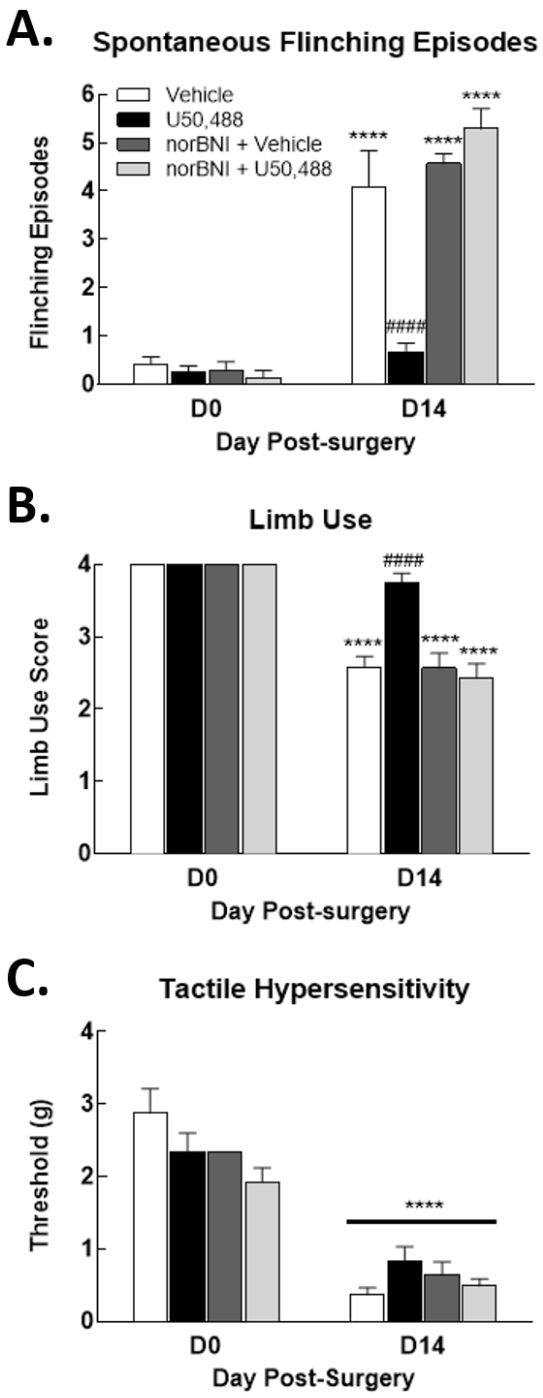

A.
Spontaneous Flinching Episodes
Vehicle
U50,488
norBNI + Vehicle
norBNI + U50,488
Flinching Episodes
0
1
2
3
4
5
6
****
####
****
****
D0
D14
Day Post-surgery
B.
Limb Use
Limb Use Score
0
1
2
3
4
####
****
****
****
****
D0
D14
Day Post-surgery
C.
Tactile Hypersensitivity
Threshold (g)
0
1
2
3
4
****
D0
D14
Day Post-Surgery

Figure 4

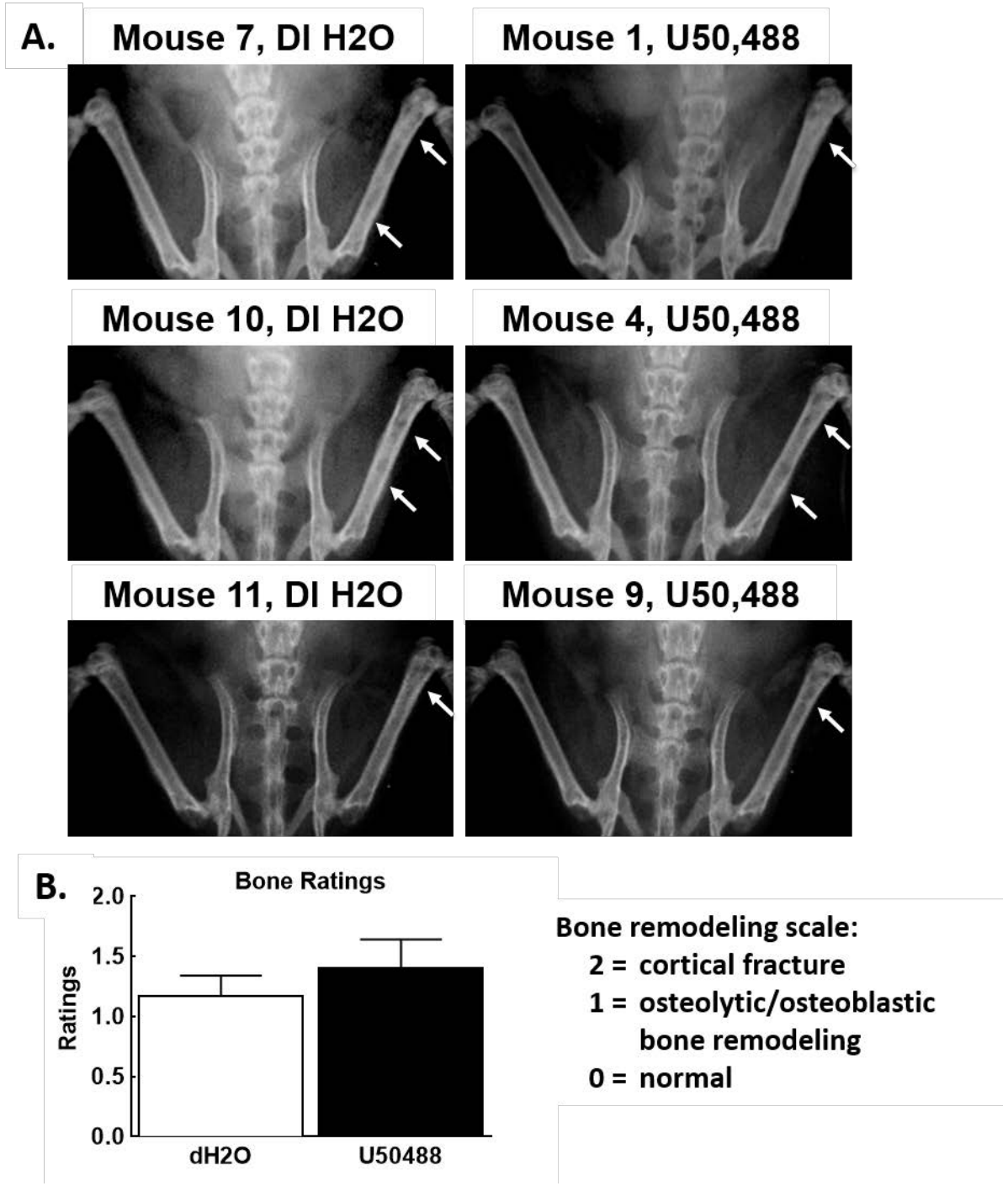

A.
Mouse 7, DI H2O
Mouse 1, U50,488
Mouse 10, DI H2O
Mouse 4, U50,488
Mouse 11, DI H2O
Mouse 9, U50,488
B.
Bone Ratings
Ratings
2.0
1.5
1.0
0.5
0.0
dH2O
U50488
Bone remodeling scale:
2 = cortical fracture
1 = osteolytic/osteoblastic bone remodeling
0 = normal

Figure 5

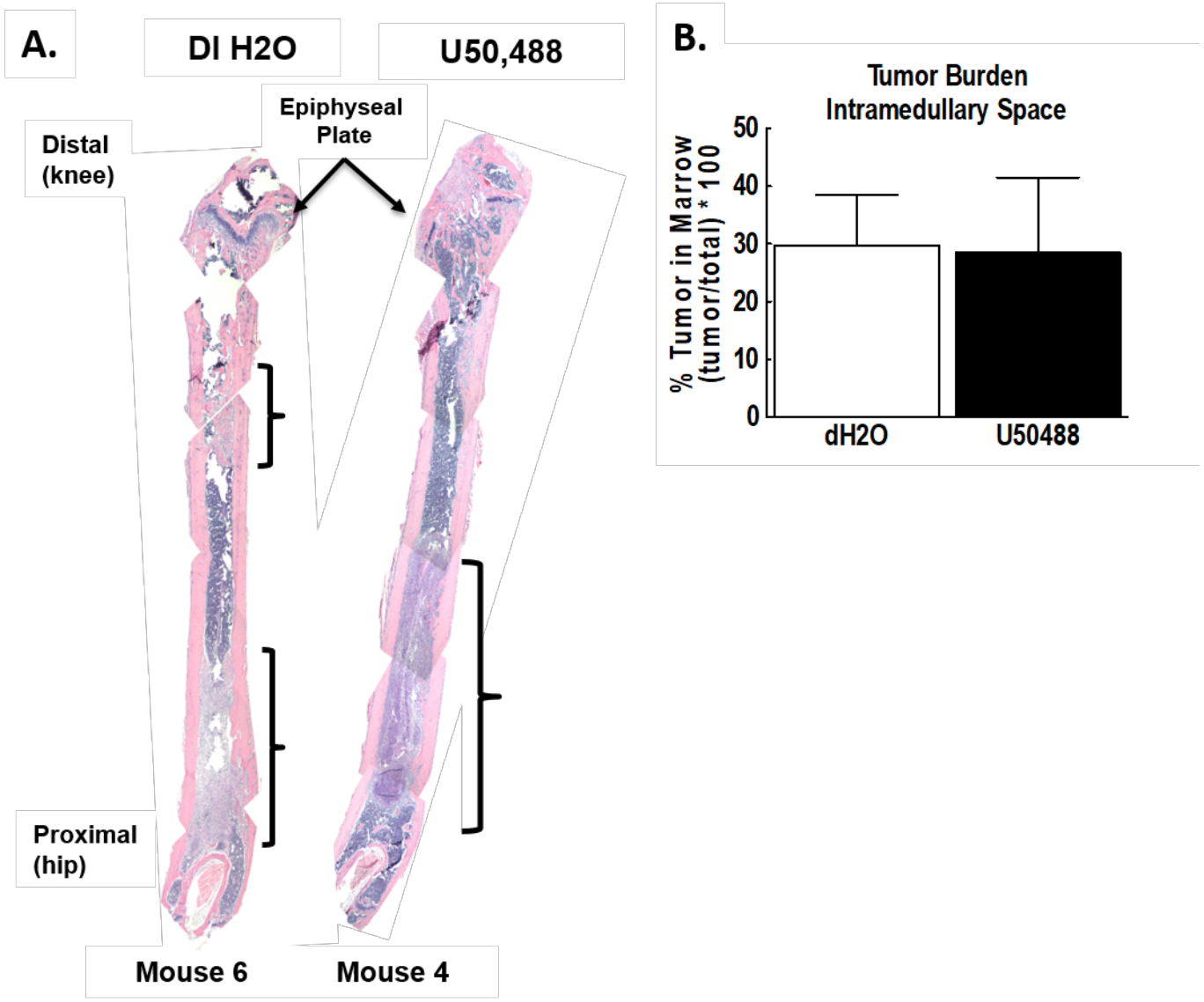

A.
DI H2O
U50,488
Epiphyseal Plate
Distal (knee)
Proximal (hip)
Mouse 6
Mouse 4
B.
Tumor Burden
Intramedullary Space
% Tumor in Marrow (tumor/total) * 100
50
40
30
20
10
0
dH2O
U50488

Figure 6

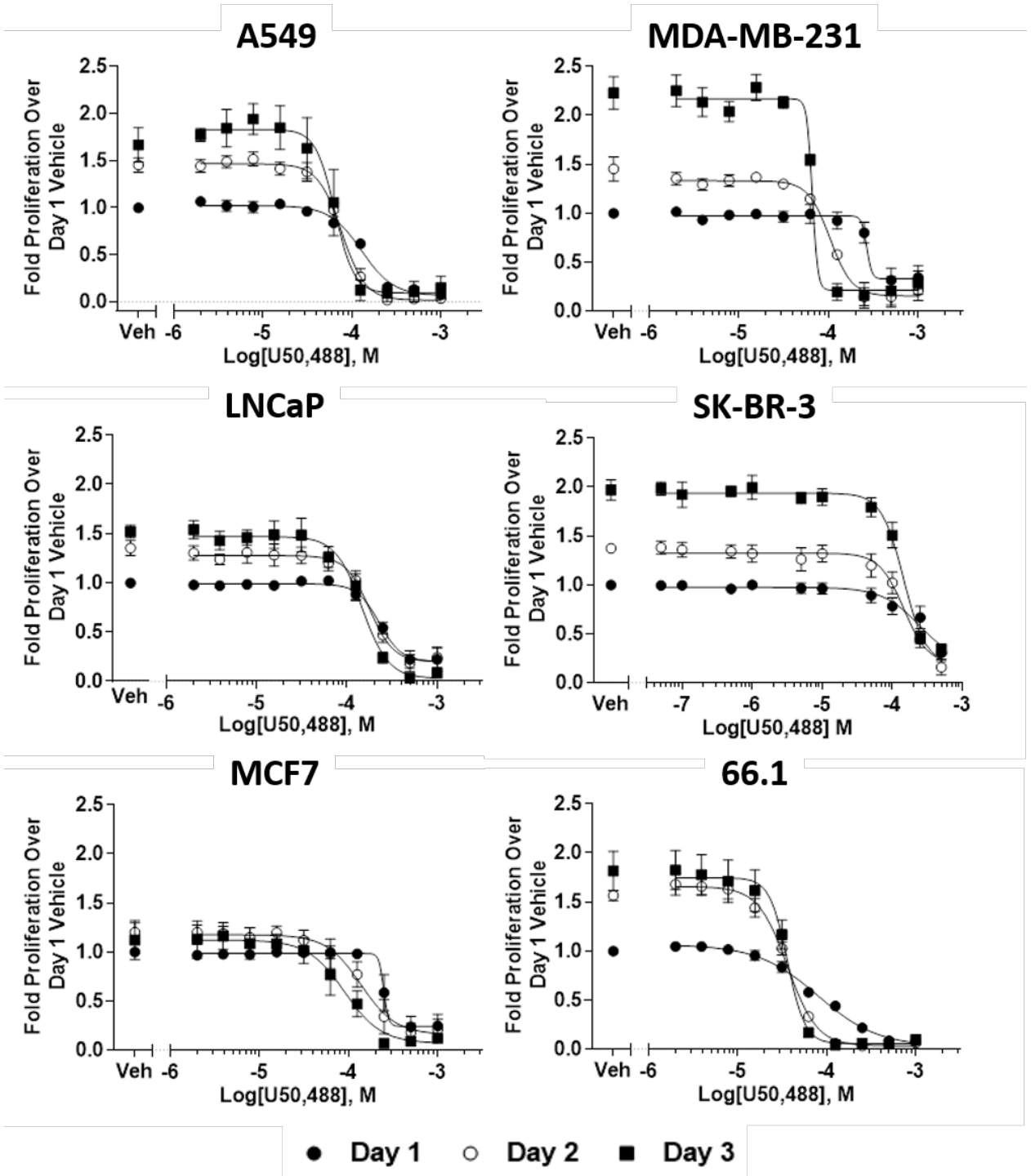

A549
MDA-MB-231
LNCaP
SK-BR-3
MCF7
66.1
Fold Proliferation Over Day 1 Vehicle
Log[U50,488], M
Log[U50,488] M
Veh
Day 1
Day 2
Day 3

Figure 7

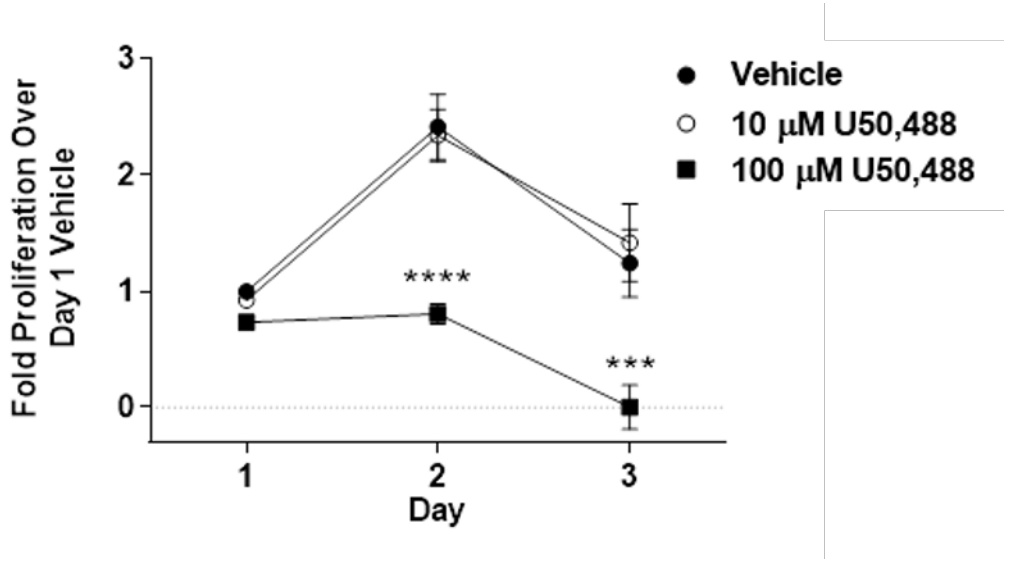

Fold Proliferation Over Day 1 Vehicle
3
2
1
0
1
2
3
Day
****
***
Vehicle
10 μM U50,488
100 μM U50,488

Figure 8

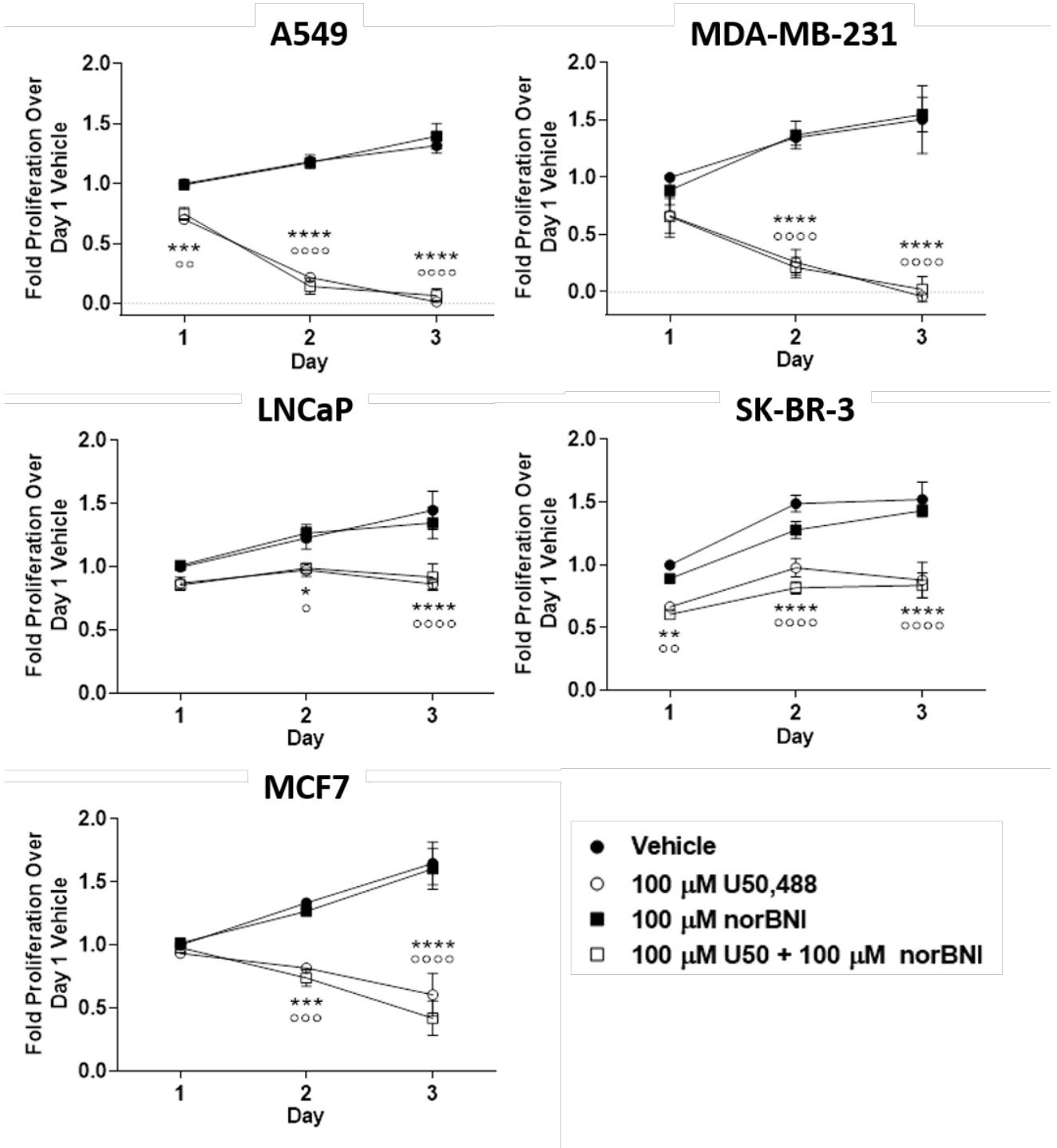
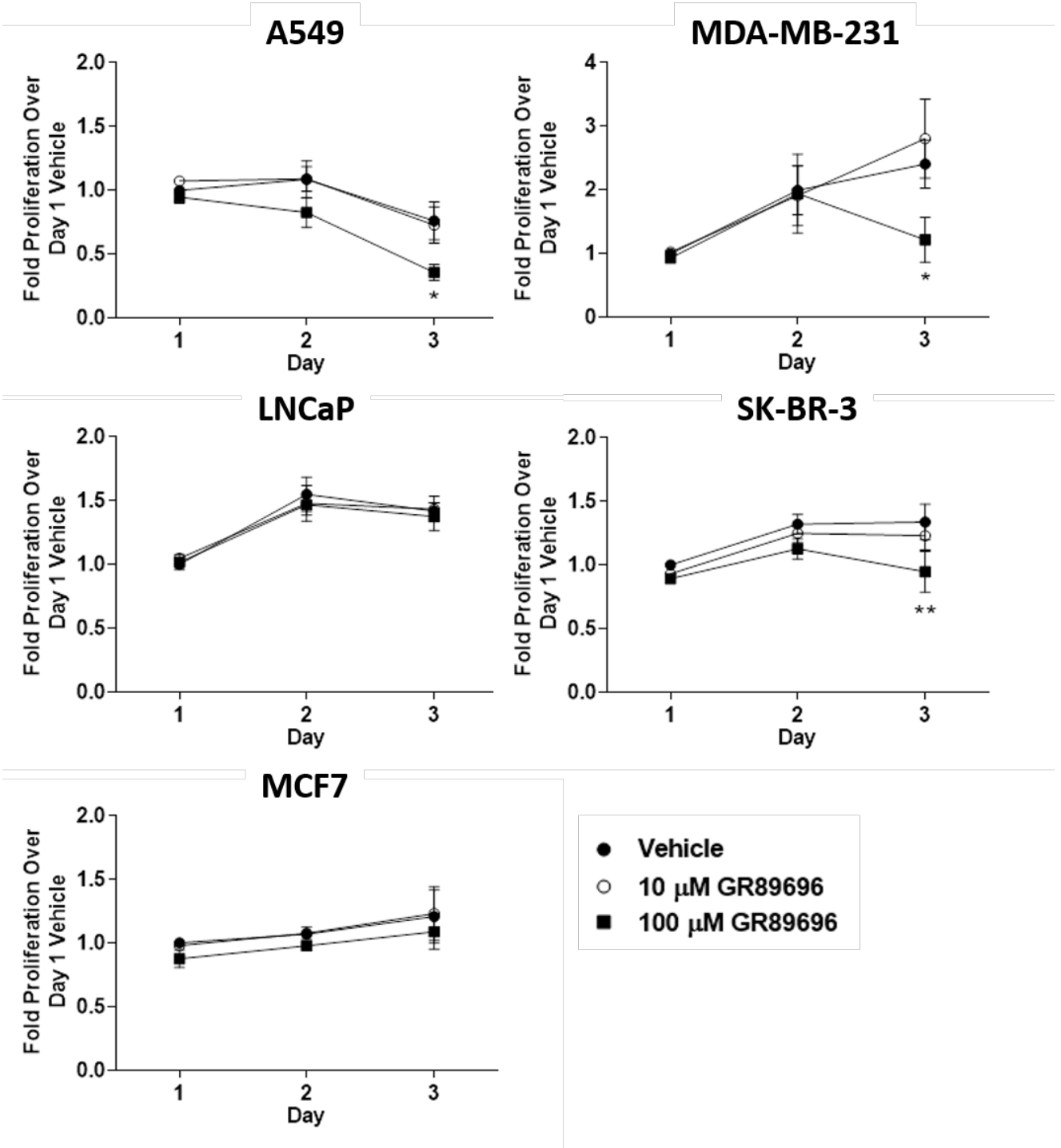

A549
MDA-MB-231
LNCaP
SK-BR-3
MCF7
Fold Proliferation Over Day 1 Vehicle
Day
2.0
1.5
1.0
0.5
0.0
1
2
3
Vehicle
100 μM U50,488
100 μM norBNI
100 μM U50 + 100 μM norBNI

Figure 9


A549
MDA-MB-231
LNCaP
SK-BR-3
MCF7
Fold Proliferation Over Day 1 Vehicle
Day
*
**
Vehicle
10 μM GR89696
100 μM GR89696