**Abstract**

Pain is the cancer-related event that is most disruptive to the cancer patient's quality of life. Although bone cancer pain is one of the most severe and common of the chronic pains that accompany breast, prostate, and lung cancers, relatively little is known about the mechanisms that generate and maintain this pain. Recently, we developed a mouse model of bone cancer pain. Ten days following tumor implantation into the intramedullary space of the femur, significant bone destruction and bone cancer pain-related behaviors were observed and progressed in severity over time. A critical question is how closely this model mirrors human bone cancer pain. In a recent publication, we show that, as in humans, pain-related behaviors are diminished by systemic morphine administration in a dose-dependent fashion that is naloxone-reversible. Humans suffering from bone cancer pain generally require significantly higher doses of morphine as compared to individuals with inflammatory pain and in the mouse model the doses of morphine required to block bone cancer pain-related behaviors were 10 times that required to block peak inflammatory pain behaviors of comparable magnitude induced by hindpaw injection of complete Freund's adjuvant (CFA; 1–3 mg/kg). As these animals were treated acutely, there was not time for morphine tolerance to develop and the rightward shift in analgesic efficacy observed in bone cancer pain versus inflammatory pain suggests a fundamental difference in the underlying mechanisms that generate bone cancer versus inflammatory pain. These results indicate that this model will be useful in defining drug therapies that are targeted for complex bone cancer pain syndromes.

**Key Words**

Pain, metastases, morphine

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**Introduction**

**Clinical Significance of Bone Cancer Pain**

As advances in cancer detection and therapy have extended the life expectancy of cancer patients, more attention must focus on improving patients' quality of life. It is projected that over 1.4 million Americans will be newly diagnosed with cancer each year. Bone pain is a frequent and often disabling symptom associated with cancer pain, particularly in patients with advanced disease. The pain associated with bone metastases can be severe and may be refractory to conventional analgesics. The development of effective therapies for bone cancer pain is critical for improving the quality of life for patients with bone metastases.
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diagnosed with cancer in 2005. Approximately 30–50% of all cancer patients will experience pain, and 75–90% of patients with advanced cancer will experience substantial, life-altering cancer-induced pain. One common type of cancer pain that is difficult to treat is bone cancer pain.

Skeletal malignancies are frequently accompanied by chronic pain and the first symptom of bone cancer is most often pain. Bone cancer pain may arise in humans from either primary bone tumors or more commonly from skeletal metastases from breast, prostate, and lung carcinomas. Cancer patients with skeletal involvement often suffer from fractures, hypercalcemia, spinal cord compression, and severe pain, all of which contribute to an increased morbidity and decreased quality of life.

Pain originating from skeletal metastases usually increases in magnitude over the evolution of the disease and is commonly divided into 2 categories, ongoing pain and breakthrough or incident pain. Ongoing pain, which is usually the first symptom of bone cancer, begins as a dull, constant, throbbing pain that increases in intensity with time and is exacerbated by use of involved portions of the skeleton. With increased bone destruction and time, the pain intensifies; intermittent episodes of extreme pain can occur spontaneously, or more commonly, after weight bearing or movement of the affected limb, and this pain is referred to as breakthrough or incident pain. Of these two types of pain, breakthrough pain is more difficult to control as the dose of opioids needed to control this pain are frequently higher than that needed to control ongoing pain and thus are accompanied by significant unwanted side effects such as sedation, somnolence and constipation.

Treatment of bone cancer pain involves the use of a variety of therapeutic modalities ranging from nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, bisphosphonates, radiation, and surgical intervention. In advanced bone cancer pain, opioids provide the mainstay for analgesic therapy. The escalating doses of opioids required to control the pain may reflect either an evolving opioid tolerance or an increase in the severity of the pain. Although both mechanisms are probably involved in the escalating doses of morphine required for this pain, the ability to notably reduce opioid requirements by the use of palliative therapies such as focal radiation or chemotherapy suggest that the high opioid doses are at least partially a reflection of the intensity associated with the bone cancer pain state. A major problem in dealing with advanced bone cancer pain is that whereas opioids may be able to control ongoing pain, these same doses are frequently insufficient to block movement-evoked breakthrough pain.

The challenges encountered in managing the multiple components of the bone cancer pain raise the question of the biological origin of this pain state. To examine the mechanisms involved in the generation and maintenance of bone cancer pain, we have developed an animal model of bone cancer pain that closely mimics the human condition (see Appendix).

Discussion

Development of a Clinically Relevant Animal Model of Bone Cancer

In assessing any experimental animal model, it is important to determine how well the model approximates the human disease. The murine model we have developed appears to share many features of human bone cancer-induced pain. The osteolytic sarcoma cell line used in this study aggressively destroys bone (Figures 1 and 2) and provides localized pathological findings found in human osteolytic bone cancer. Osteolytic sarcoma cells are injected into the intramedullary space of the mouse femur and the needle hole is then filled with dental amalgam to confine the tumor to bone. As the tumor grows within the mouse femur, the number of osteoclasts increase, bone destruction becomes radiologically evident, and ongoing as well as touch-evoked (breakthrough) pain-related behaviors develop. These pain-related behaviors first appear 10 days following injection and continue to escalate until severe impairment at 17–21 days post-injection when fracture of the affected femur occurs. An essential step in developing this model was to ensure that the osteolytic cancer cells are confined within the intramedullary space of the femur and do not invade soft tissues, which was accomplished with the placement of an amalgam plug. Mice injected with bone cancer exhibit painful behavior in the
Fig. 1. Faxitron images (high-resolution radiographs) of the mouse femur showing the progressive loss of mineralized bone with time after the injection of the tumor in the femur. Bone destruction was quantified on a 0 to 3 scale based on the loss of bone. Images 0 to 5 are examples of each state of destruction: (0) normal bone; (1) small pits of bone destruction (1–3 in number); (2) increased pitted appearance (4–6 in number) and loss of medullary bone; (3) loss of medullary bone and erosion of cortical bone; (4) full thickness unicortical bone loss; (5) full thickness bicortical bone loss and displaced skeletal fracture. Scale bar = 2 mm.

form of guarding of the affected limb, and this behavior correlates with the extent of bone destruction. Severe acute pain is also observed in mice once significant bone destruction has occurred, because normally non-noxious palpation to the affected bone results in behaviors indicative of severe pain, and this severe pain is again correlated with the extent of bone destruction. The pattern of bone destruction in the model can be described as having a moth-eaten appearance on the endosteal surface of both the distal and proximal regions of the femur, similar to that commonly observed in humans with osteosarcoma.27,28 Histologically,

Fig. 2. Bone destruction following injection of osteolytic sarcoma cells into the intramedullary space of the femur. H&E staining of naïve (normal) and 17 day sarcoma-bearing (sarcoma) femora illustrating that in normal bone (a) there is a clear separation of mineralized bone (pink) and marrow cells (purple). In contrast, in animals with osteolytic sarcoma cells in the intramedullary space (b), the smaller, more densely packed sarcoma cells (purple) have largely replaced the marrow cells and induced destruction of the mineralized bone (pink).
tumor presence in the intramedullary space and an increase in the number of activated osteoclasts are also evident in the murine model and in humans.\textsuperscript{22,29} Thus, the quality and severity of the pain and the radiological and histological features of the murine model of advanced bone cancer are similar to what is observed clinically in humans with advanced bone cancer. These features suggest that this murine model shares key features with human bone cancer-induced pain. Importantly, utilizing this model to assess the development of bone cancer pain behaviors and bone alterations while simultaneously assessing neurochemical changes that occur within the spinal cord (Figures 3 and 4) and dorsal root ganglia provides a platform to use an integrated systems approach to understanding the mechanisms that generate bone cancer pain, as well as provides a model in which novel therapeutic strategies can be tested.\textsuperscript{22–25,30,31}

The ability to test pharmacological agents is an important component to mechanistic investigation of bone cancer pathology. To further validate the animal model by testing a standard drug used to treat bone cancer pain, while simultaneously assessing the opioid requirements for bone cancer pain in comparison to the efficacy and potency of morphine in another well defined pain state, complete Freund’s adjuvant (CFA)-induced inflammation was examined. The following results demonstrate that similar to the clinical situation observed in humans, the delivery of systemic opioids is effective at alleviating bone cancer-induced pain. However, the doses of morphine required to alleviate bone cancer pain were in general 10-fold greater than that required to alleviate pain behaviors of comparable magnitude generated by the CFA inflammatory pain model (Figure 5).\textsuperscript{30} This study suggests that the mouse model of bone cancer mirrors the pain observed in humans with moderate to advanced bone cancer pain and that this model may be useful in defining the mechanisms that generate and maintain bone cancer pain, as well as aid in the development of potential therapies for treating this chronic pain condition.\textsuperscript{30}

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**Fig. 3.** Cancer-induced reorganization of the central nervous system. Chronic cancer pain can cause significant alterations in the central nervous system. This is believed to underlie the phenomena of central sensitization, an increased responsiveness of spinal cord neurons involved in transmission of pain. Confocal imaging of glial fibrillary acidic protein (GFAP) expression in the L4 segment of a tumor-bearing mouse spinal cord shows an increase in the number of astrocytes on the left side of the spinal cord, which receives sensory innervation from the tumor-bearing bone. The right side of the spinal cord, which is not transmitting painful stimuli to the brain, has fewer astrocytes.
Behavioral Analysis of Pain Behaviors in Sarcoma Animals and Sarcoma Animals Treated with Morphine

Eight mice were prepared as sham operated controls and were submitted to the same behavioral assessment procedures. Analysis of the data assessed on day 16 reveals that sarcoma animals showed statistically lower von Frey thresholds, longer guard times, greater incidences of flinching, lower indices of limb use, greater disability on the rotarod, and increased guarding and flinching in response to palpation.
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Fig. 5. Efficacy of systemic morphine in bone cancer and inflammatory pain states represented as a dose response. Sarcoma and CFA injected animals exhibited responses of comparable magnitudes for the guarding and von Frey behavioral tests. However, sarcoma-bearing animals required significantly more morphine sulfate (MS) to reduce the pain, thus suggesting a fundamental difference in the mechanisms that generate bone cancer and inflammatory pain. The gray box indicates significance at $P < 0.05$, one-way ANOVA. Statistical analysis was performed on percent change for sarcoma data and mean values for CFA animals.

Effects of Morphine on Sarcoma-Induced Pain Indices

In these studies, we examined the efficacy of intraperitoneal (i.p.) administered morphine in the sarcoma mice. We first determined that the simple injection of saline had no effect upon the day 17 versus day 16 response on any measure (paired $t$-test, $P > 0.10$, data not shown).

To determine if morphine had a statistically significant effect on several behavioral tests between various doses on the ratio of baseline animals at day 16 to the drug-treated animals on day 17, we examined the effects across dose on each behavioral test using both the non-parametric rank ordered statistics for ordered means (the Jonckhere test) and the one-way ANOVA. As indicated, the results observed with the two analyses were essentially parallel, with the von Frey, limb use, and flinch being significant at the $P < 0.05$ level on both end points and palpation guarding being significant on the rank ordered model and $P = 0.065$ on the ANOVA. These data indicate that in these variables, morphine produced a dose-dependent suppression of pain behavior.

Given the dose-dependent suppression produced by morphine, we sought to define the lowest morphine doses that produced a degree of reversal of the hyperalgesia present in the sarcoma animal. Using a Dunnetts-t comparing each dose to the saline-treated sarcoma group for each endpoint, significant differences were observed at or around 30 mg/kg.
with 10 mg/kg having a significant effect on von Frey thresholds and palpation guarding. In these studies, we routinely examined dose up to 30 mg/kg. Up to 30 mg/kg, there was no change in rota rod performance in the sarcoma mice. Above that dose (100 and 300 mg/kg), however, we observed significant deterioration in rota rod performance. These observations indicate that at doses up to 30 mg/kg, there is no discernable impairment in motor function.

Effects of Naloxone

To determine if the effects of morphine were mediated by opioid receptor interactions, we examined the effects of naloxone pre-treatment in mice that subsequently received either morphine or saline (as a control for the possible effects of naloxone alone). No statistically significant effects were noted when comparing the behavior of saline-treated sarcoma mice with mice receiving naloxone. This failure of naloxone alone to alter the pain state in saline-treated animals indicates the absence of any endogenous opioid tone in regulating the observed pain behavior in the sarcoma mice.

As indicated, in von Frey and palpation guarding tests, where morphine 10 mg/kg produced a significant increase in the measured response, the antinociception was reversed by naloxone. For guarding time, limb use, flinching time, and palpation flinch tests, in which morphine 10 mg/kg did not produce a statistically significant increase, naloxone resulted in moving the morphine effect towards values elicited by saline-treated sarcoma animals.

Comparison of Bone Cancer Pain to the CFA-Induced Inflammatory Pain

A principal aim of the present work was to define morphine sensitivity of these observed behavioral pain states induced by sarcoma implantation. We sought to compare the cancer pain state response to morphine to that of the inflammatory pain state induced by CFA. As indicated in Figure 5, the mean response of the animal injected with CFA was a decrease in the von Frey threshold, increase in guarding time, and an increase in flinching response. Comparison of the values demonstrated in Figure 5 suggest that CFA treatment produces a pain state that is comparable to the pain state produced by sarcoma injection at day 16.

On day 3 post CFA-injection, mice were randomly assigned to receive either 1 or 3 mg/kg MS i.p. As indicated in Figure 5, morphine resulted in a powerful reversal of the hyperalgesic state for all three test measures at doses of 1 and 3 mg/kg. In contrast, sarcoma-bearing mice required doses on the order of 10 and even 30 mg/kg to reduce response values to sham levels and, thus, achieve analgesia.

Caliper measurements of the CFA or vehicle injected hindpaw illustrate the development of peripheral edema. The mean value for CFA + morphine (1 and 3 mg/kg) was 3.91 mm and the mean value for vehicle + saline was 2.49, P < 0.001. Additionally, morphine at 1 and 3 mg/kg did not affect the development of peripheral edema in CFA-treated animals.

Factors Contributing to the Generation and Maintenance of Bone Cancer Pain

Although pain is the most frequent and disruptive symptom for patients with bone cancer,9,11,13 the mechanisms responsible for the generation and maintenance of bone cancer pain are not clearly understood. Cancer pain appears to generate a pain state mechanistically unique from inflammatory and neuropathic pains, as each of these specific pains exhibits different profiles of pain-related behavior and neurochemical changes in the spinal cord.32

A variety of factors may play a role in the development and maintenance of the bone cancer pain state (Figure 6). For example, this pain might be caused by pro-hyperalgesic factors, such as prostaglandins and endothelins, which are released by the cancer cells that activate nociceptors in the bone marrow.1,31 Additionally, macrophages, which can represent 260% of the cells in the tumor mass,33,34 produce factors such as tumor necrosis factor and interleukin-1, which have been reported to excite primary afferent neurons.35–38 It has been shown that, as bone resorption occurs, growth factors that are embedded in the mineralized bone are released37,39 and many of the growth factors from bone may directly activate pain fibers which innervate the bone.40 As the tumor continues to grow, sensory neurons that innervate the marrow are compressed and destroyed, potentially causing neuropathic pain.

Pain may also in part be caused by tumor-induced proliferation and hypertrophy of
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Fig. 6. Sensitization of primary afferent fibers may occur via multiple mechanisms. These include release of pronociceptive compounds from tumor cells and/or inflammatory cells or their byproducts, release of factors from resorbing bone, and the interactions of protons released by activated osteoclast with receptors expressed on primary afferent fibers. Cellular and molecular targeting of pain mechanisms may help elucidate the role of cellular elements in the generation of the bone cancer state.

Osteoclasts. For osteoclasts to resorb bone, they must maintain an acidic pH (pH 4.0–5.0) microenvironment at the osteoclast-mineralize bone interface,14 and a population of sensory neurons that innervate bone expresses acid-sensing ion channels and vanilloid receptors.41 Additionally, as the cancer cells completely fill the intramedullary space, the high levels of resulting apoptosis may contribute to the generation of an acidic microenvironment. Finally, as bone destruction continues, the mechanical strength of the bone is compromised and ultimately will fracture.1 Mechanical stress on the bone would be expected to place the bone under torsion, which would excite the mechano-sensitive fibers present in the richly innervated periosteum, resulting in significant movement-evoked pain. Although the periosteum is the most densely innervated tissue, when the total volume of each tissue is considered, the bone marrow receives the greatest total number of sensory and sympathetic fibers followed by mineralized bone and then periosteum.12 Patients often perceive bone pain when the pathology is confined principally to the bone marrow or mineralized bone and where there is no obvious periosteal involvement.43,44

Although this pattern of tumor-induced tissue destruction and nociceptor activation might be unique to bone cancer, an evolving set of nociceptive events probably occurs in other cancers. This might, in part, explain why cancer pain is frequently difficult to treat and why it can be so heterogeneous in nature and severity. Because the type of tumor-induced tissue injury, level of nociceptor activation, and the spinal cord and forebrain areas involved in transmitting nociceptive change as the disease progresses, different therapies might be most efficacious at particular stages of the disease.1

Behavioral Correlates of Nociception in Cancer and Inflammatory Pain States

Unilateral injection of sarcoma cells into the intramedullary space of the femur produces a progressive destruction of the murine femur, changes in spinal cord neurochemistry, as well as the development of ongoing, ambulatory and touch-evoked pain related behaviors.22–24,32 At days 16 and 17, when pain-related behaviors are maximal, behavioral assays were assessed. Importantly, the ongoing pain elements measured in sarcoma animals (increased guarding time and increased flinching) and one of the touch-evoked measurements (decreased von Frey thresholds) were also observed in mice treated with CFA.
CFA is a widely used model of inflammatory pain that peaks at 3 days post injection.\textsuperscript{45} Comparing the magnitude of the measured behavioral responses in the CFA model with those in the cancer model strongly suggests that both models develop a comparable level of hyperalgesia. This convergent validation between two separate models suggests that these several indices are robust correlates of the mouse pain state and the model of inflammatory and cancer pain induce a comparable magnitude of the ongoing behavioral pathology.

We used morphine to test both the severity of the pain and the effectiveness of an opioid in blocking behavioral changes in sarcoma and CFA-induced pain states.\textsuperscript{30} Morphine is a \( \mu \) opioid receptor agonist which can exert significant effects at supraspinal, spinal, and, to a lesser degree, peripheral sites.\textsuperscript{46–49} In the present study, systemic morphine was shown to produce a dose-dependent suppression of the pain-related behaviors observed in mice with sarcoma and CFA. An important component of these observations was that this suppression occurred at doses that, while high, did not induce general motor impairment, as assessed by rotarod performance. This suggests that the antinociception observed at this dose was not the result of a significant loss of motor function. As indicated in Figure 5, at higher doses (e.g., 100 and 300 mg/kg), there was a rapid decline in motor function as evidenced by a significant decrease in rotarod performance.

Although these studies demonstrated the effects of systemic morphine in the sarcoma pain state, an important component was the comparison of its potency and efficacy with that observed in the CFA model. As indicated in these studies, and consistent with reports by other laboratories, morphine was extremely effective in blocking the several indices reflecting the inflammatory hyperalgesia.\textsuperscript{50–52} While full dose-effect curves were not established in the CFA-induced inflammatory pain state, it is clear that significant effects were found with doses on the order of 1-3 mg/kg. These values are considerably lower, generally by at a factor of 10, than the doses required to alter the respective behaviors in the sarcoma model.

The differential effect of morphine in the sarcoma pain assays as compared to those associated with the CFA model may arise from several elements. An increase in stimulus intensity will result in a predictable right shift in the dose-effect curve for a given opioid.\textsuperscript{53,54} Because the indices of hyperalgesia are of comparable magnitude in both inflammatory and bone cancer pain, the difference in the dose of morphine does not appear to be due solely to the intensity of inflammatory vs. cancer pain. Rather, it may also be that while the behavioral measurements suggest pain states of comparable magnitudes, the underlying mechanisms that lead to the respective hyperalgesia may be different.

Opioids in general are particularly effective in attenuating hyperalgesia in inflammatory pain and less effective in blocking neuropathic pain.\textsuperscript{55–58} CFA is a classic model of inflammatory pain whereas bone cancer pain has changes indicative of both inflammatory and neuropathic pain. Thus, in bone cancer pain,\textsuperscript{22–24,32} there is an upregulation of both c-Fos-immunoreactive (IR) neurons in deep laminae.\textsuperscript{59} An increase in c-Fos-IR neurons have been associated with nociceptive or painful conditions.\textsuperscript{37,60–65} Dynorphin is a pro-hyperalgesic peptide that has been implicated in inflammatory pain states.\textsuperscript{52,66–69} However, in bone cancer pain there is the appearance of reactive astrocytes in the ipsilateral lumbar segments of the spinal cord similar to that observed in neuropathic models of pain involved in peripheral nerve injury.\textsuperscript{63,70–72} These data suggest that bone cancer pain may have both an inflammatory and neuropathic component and both inflammatory and neuropathic components need to be therapeutically addressed to adequately block the overall hyperalgesia that is present.

**Therapeutic Value of Morphine in a Murine Model of Cancer Pain is Similar to Humans**

Previous work has illustrated the similarity between this murine model of bone cancer pain and the human presentation.\textsuperscript{22–24} These similarities are seen at both behavioral, as well as pathological levels. Briefly, tumor-induced bone destruction and tumor-induced pain behaviors are observed in both the mouse and human afflicted with lytic bone cancer pain. The extensive loss of mineralized bone at the metabolically active peripheral and distal ends of the femur as well as the ongoing and movement-evoked pain that accompanies this condition is present in both humans with bone cancer as well as in the murine model employed here.
Importantly, the results obtained in the sarcoma mouse model bear considerable resemblance to the efficacy of morphine in managing advanced stage sarcoma in humans. It is recognized that opioids in this pain state are important but frequently require extreme dosing that leads to significant CNS effects such as respiratory depression, cognitive impairment, sedation, and constipation.  

Although such patients have typically been prescribed opioids for extended periods of time, it has been suggested that the need for such high doses is due to opioid tolerance. In contrast, it has also been reported that the increasing opioid requirements are due to the progressive nature of the disease rather than tolerance. We suggest that additional factors may also be involved as the doses used continue to produce prominent opioid receptor-mediated side effects but fail to produce adequate pain relief. The present studies, wherein prior opioid exposure is not an issue, the right shift in potency, as compared to that observed with peripheral inflammation, suggests that the limited efficacy is due to some intrinsic property of the pain state generated by the sarcoma. We suggest that this may reflect in part a neuropathic element to the pain processing that engages mechanisms that are less effectively modulated by opioid receptor activation.

Over the past decade, a principal thrust in pain research has been to shift the process of discovering new analgesics from one based on empirical observations to one based on a mechanistic understanding of the biology involved in the generation and maintenance of pain states. In addition to the mechanistic insights, the present studies provide support for the contention that the mouse model of bone cancer pain may be an appropriate model for the preclinical assessment of therapies to treat this severe bone cancer pain. Such work will hopefully lead to insights into the biology that leads to the hyperalgesia as well as to the development of new therapies to treat this debilitating condition. For example, a compound known as osteoprotegrin (OPG) has begun Phase I clinical trials.

Appendix

Bone Cancer Model

Experiments were performed on 104 adult male C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine), approximately 4–5 weeks old, weighing 20–25 g at the time of tumor cell injection. The mice were housed in accordance with National Institutes of Health guidelines and kept in a vivarium maintained at 22°C with a 12-hour alternating light-dark cycle and were given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committees at the University of Minnesota. Sarcoma injection protocol was performed as previously described. In brief, an arthrotomy was performed following induction of general anesthesia with sodium pentobarbital (50 mg/kg, i.p.). A needle was inserted into the medullary canal to create a pathway for the sarcoma cells. A depression was then made using a pneumatic dental high speed handpiece. In addition to naïve animals (n = 3), sham animals (n = 8) were generated with an injection of α-minimum essential media (20 µL, SIGMA, St. Louis, MO) into the intramedullary space of the femur (designated sham) whereas sarcoma animals (n = 61) were injected with media containing 10⁵ 2472 osteolytic sarcoma cells (designated sarcoma) (20 µL, ATCC, Rockville, MD). For all animals, the injection site was sealed with a dental amalgam plug to confine the cells within the intramedullary canal and followed by irrigation with sterile water (hypotonic solution). Finally, incision closure was achieved with wound clips. Clips were removed at day 5 so as not to interfere with behavioral testing.

Complete Freund’s Adjuvant–Induced Inflammation

Mice received a 20 µL subcutaneous injection of CFA (50% dissolved in saline, SIGMA, St. Louis, MO) in the plantar surface of the hindpaw and were behaviorally analyzed and euthanized three days following injection (CFA; n = 16, sham animals received 20 µL saline injections; n = 16). Immediately following behavioral analysis the dorsoventral diameter of the injected hindpaw was measured using a caliper to verify peripheral edema.

Behavioral Analysis

Sarcoma or sham animals were tested for pain related-behaviors before sarcoma or media injections and then tested before and after drug administration at day 17 post-tumor implantation. CFA and sham animals were injected and
behaviorally tested 3 days post-injection. Sarcoma or sham-injected animals were behavioral tested using the following: ongoing pain (spontaneous guarding and spontaneous flinching), ambulatory pain (limb use and rotarod), and movement-evoked pain (von Frey, palpation-evoked guarding, and palpation-evoked flinching). CFA and sham-injected animals were behaviorally examined using the following tests: ongoing pain (spontaneous guarding and spontaneous flinching) and movement-evoked pain (von Frey). In total, seven behavioral indices for sarcoma and three for CFA analysis were systematically assessed and are summarized in Figure 5.

Mice were placed in a clear plastic observation box with a wire mesh floor and allowed to habituate for a period of 30 minutes. After acclimation, mechanical allodynia was assessed followed by spontaneous guarding, spontaneous flinching, limb use during normal ambulation in an open field, and guarding during forced ambulation. Palpation-induced guarding and flinching were measured after the 2-minute period of non-noxious palpation of the distal femur in sarcoma and sham-injected animals.

Mechanical allodynia is measured by von Frey monofilaments on the hindpaw of the injected animal. The Von Frey withdrawal threshold is determined by increasing and decreasing stimulus intensity between 0.2 and 15.1 gram equivalents of force and estimated using a Dixon non-parametric test.80 The number of spontaneous guards and flinches, representative of nociceptive behavior, were recorded simultaneously during a 2-minute observation period. Guarding was defined as the time the hindpaw was held aloft while not ambulatory and flinches were the number of times the animal held the limb aloft.

Normal limb use during spontaneous ambulation was scored on a scale of 4 to 0: (4) normal use, (3) pronounced limp, (2) limp and guarding behavior, (1) partial non-use of the limb in locomotor activity, and (0) complete lack of limb use.

Forced ambulatory guarding was determined using a rotarod (Columbus Instruments, Columbus, OH). The rotarod machine has a revolving rod and is equipped with speed, acceleration, and sensitivity controls. The animals were placed on the rod with ×4 speed, 8.0 acceleration, and 2.5 sensitivity. Forced ambulatory guarding was rated on a scale of 5 to 0: (5) normal use, (4) some limp, but not pronounced, (3) pronounced limp, (2) pronounced limp and prolonged guarding of limb, (1) partial non-use of the limb, and (0) complete lack of use.

Mechanical allodynia at the knee joint was evaluated after a normally non-noxious palpation of the distal femur in sarcoma animals every second for 2 minutes. Following the 2-minute palpation, the mice were placed in the observation box and their palpation-induced guarding and flinching behavior were measured for an additional 2 minutes, as discussed above.

Spontaneous guarding and spontaneous flinches were used to measure ongoing pain. Ambulatory pain was measured from limb use during normal ambulation in an open field and guarding during forced ambulation on a rotarod. Von Frey threshold, palpation-induced guarding and palpation-induced flinching after a 2-minute period of normally non-noxious palpation of the distal femur were used as indicators of touch- or movement-evoked pain.

**Acute Treatment of Morphine**

On day 17, sarcoma- and media-injected animals were administered, via i.p. injection, either morphine sulfate (MS, VA Medical Center, Minneapolis, MN), naloxone (SIGMA, St. Louis, MO), MS + naloxone, or saline and were randomly divided into the following groups: sham 17 day + saline: n = 8; sarcoma 17 day + saline: n = 6; sarcoma 17 day + naloxone (3 mg/kg): n = 7, sarcoma + naloxone (3 mg/kg) & MS (10 mg/kg): n = 7, sarcoma + MS (0.3 mg/kg): n = 5, sarcoma + MS (1 mg/kg): n = 5, sarcoma + MS (3 mg/kg): n = 8, sarcoma + MS (10 mg/kg): n = 7, sarcoma + MS (30 mg/kg): n = 4, sarcoma + MS (100 mg/kg): n = 4, sarcoma + MS (300 mg/kg): n = 4. On day 3, CFA- or vehicle-injected animals were divided into the following groups: vehicle + saline: n = 6, vehicle + MS (1 mg/kg): n = 5, vehicle + MS (3 mg/kg): n = 5, CFA + saline: n = 6, CFA + MS (1 mg/kg): n = 5, CFA + MS (3 mg/kg): n = 5. All animals were behaviorally analyzed 15 minutes post-injection using the behavioral paradigms previously described. Analysis was completed by 30 minutes post injection to ensure that the
animals were tested within the therapeutic window of drug action.\textsuperscript{81}

**Assessment of Bone Destruction**

The extent of sarcoma-induced bone destruction (osteolysis) was radiologically assessed at a 4× magnification using a Faxitron machine (Specimen Radiography System Model MX-20, Faxitron X-ray Corporation, Wheeling IL; Kodak film Min-R 2000, Rochester, NY). Faxitron images were taken before sarcoma or sham injections and at day 17 post-injection. Radiographs of tumor-bearing femora were scored on a previously validated 0 to 5 scale.\textsuperscript{22,30} Using this scale, normal bone with no signs of destruction = 0; small pits of bone destruction (1–3 in number) = 1; increased pitted appearance (4–6 in number) and loss of medullary bone = 2; loss of medullary bone and erosion of cortical bone = 3; full thickness unicortical bone loss = 4; full thickness bicortical bone loss and displaced skeletal fracture = 5. Radiological examination of mice surviving through day 17 revealed that femoral bone scores were 3 or greater (e.g., significant bone destruction; Figure 2) in all but 7 mice. These 7 mice were excluded from all subsequent data analyses.

**Euthanasia**

Sarcoma and media-injected animals were euthanized 17 days post-injection, whereas CFA and vehicle injected animals were euthanized 3 days post-injection after all behavioral analyses were complete.

**Statistical Tests**

The pain indices in the bone cancer animals were first examined for distribution by plotting frequency histograms. As will be indicated, the distributions of response end points did not violate assumptions of a normal distribution. Secondly, the baseline responses for each behavioral measure in sarcoma animals was compared with the baseline response in sham animals to determine if the index could distinguish between the behavior of normal and sarcoma animals (paired Student’s $t$-tests / Kruskall Wallace). Endpoints that did not differ were not submitted for further analysis. Third, the several indices consisted of those generating continuous (von Frey thresholds, guarding time, number of flinches, palpation flinching, and palpation guarding) and discontinuous data (ranked categorical data: limb use; rotarod, palpation–agitation). Accordingly, both ANOVA and rank order statistics were carried out in parallel. In the ANOVA, where statistical significance was determined, post hoc analysis was carried out with Dunnetts-$t$ corrected for multiple comparisons. For the rank order statistics, the Jonckhere test was used, which assesses the hypotheses of a designated dose dependency across treatments.\textsuperscript{82} and were typically run in parallel with parametric statistics such as ANOVA and the $t$-test. The CFA data was analyzed by a 1-way ANOVA. Significance level was set at $P < 0.05$. The investigator responsible for scoring bone destruction and assessing behavioral testing was blind to the experimental situation of each animal.

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