MECHANISMS UNDERLYING CANCER-INDUCED BONE PAIN

By

Devki Sukhtankar

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Devki Sukhtankar
entitled "Mechanisms underlying cancer-induced bone pain"
and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

_____Date: 04 – 12 – 2011
Frank Porreca

_____ Date: 04 – 12 – 2011
Tamara King

_____ Date: 04 – 12 – 2011
Mark Nelson

_____ Date: 04 – 12 – 2011
Todd Vanderah

_____ Date: 04 – 12 – 2011
Anne Cress

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: 04 – 12 – 2011
Dissertation Director: Frank Porreca

_____ Date: 04 – 12 – 2011
Dissertation Director: Tamara King

STATEMENT BY AUTHOR

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SIGNED: Devki Sukhtankar

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DEDICATION

I would like to dedicate this dissertation to those people in my life who have stood by my side through thick and thin and are the pillars of support– my mother, Pallavi Sukhtankar, my father, Dattakumar Sukhtankar, my brother Nikhil Sukhtankar and my husband Sunil Sthalekar.

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ABBREVIATIONS

MCSF: Macrophage Colony Stimulating Factor

RANKL: Receptor Activator of Nuclear factor Kappa B Ligand.

TGF: Transforming Growth Factor

PTHrP: Para Thyroid Hormone related Peptide

PDGF: Platelet Derived Growth Factor

IL-6: Interleukin-6

VLA4: Very Late activating Antigen 4

VCAM: Vascular Cell Adhesion Molecule

SCID: Severe Combined Immuno Deficiency

SDF: Stromal cell Derived Factor

BMP: Bone Morphogenetic Protein

NSAIDs: Non-steroidal Anti-inflammatory Drugs

ROO: Rapid Onset Opioids

PSA: Prostate Specific Antigen

IB4: Isolectin B4

NGF: Nerve Growth Factor

SNL: Spinal Nerve Ligation

CFA: Complete Freund's adjuvant

TRPV1: Transient Receptor Potential Vanilloid 1

CGRP: Calcitonin Gene Related Peptide

ATF3: Activating Transcription Factor 3

AP-1: Activator Protein 1

LIF: Leukemia Inhibitory Factor

PKC: Protein Kinase C

gp130: Glycoprotein 130

s.c.: Subcutaneous

i.t. : Intrathecal

i.v. Intravenous

i.m. Intramuscular

p.o. Per Oral

ABSTRACT

Pain from bone metastases is multifaceted with clinical descriptors including ongoing pain, hypersensitivity to external stimuli and intermittent episodes of breakthrough pain characterized as a sudden and abrupt onset of severe pain on a background of well-controlled pain. Moreover, cancer-induced bone pain remains inadequately managed due to a myriad of side effects associated with the current pain relieving regimens, which primarily rely on administration of opiates. Despite advances made in cancer therapeutics, these patients experience an inferior quality of life with incapacitating pain with limited daily activities. Development of long-term novel, non-opiate mechanism-based therapeutics with limited side effects is considered beneficial in elevating the patients' quality of life. First part of this dissertation encompasses the role of p38 MAPK in a mouse model of cancer-induced bone pain in which breast cancer cells were injected and sealed into the femur. Our data demonstrated that both acute and prolonged inhibition of p38 MAPK blocked cancer-induced spontaneous pain but had no effect on the evoked pain indicating important differences in mechanisms mediating ongoing pain as opposed to evoked pain. Undermanaged control of breakthrough pain is attributed to poor understanding of underlying mechanisms and how they may differ from ongoing pain due, in part, to lack of a pre-clinical model in which these mechanisms can be studied. We have established a rat model of cancer-induced bone pain to examine ongoing pain and pain relief using conditioned place preference paradigm as well as breakthrough pain using palpation-induced conditioned place aversion. We have shown that while peripheral afferent input from the tumor-bearing tibia mediates cancer-induced ongoing

pain and initiation of breakthrough pain, it does not contribute to the maintenance of breakthrough pain. These data suggest that molecular targets mediating these two mechanisms may be different. This hypothesis was confirmed by our findings in this model that acute blockade of interleukin-6 blocked movement-evoked breakthrough pain in tumor-bearing rats, but failed to block tumor-induced ongoing pain. Hence, we provide a platform to manipulate treatments that can be given alone or in combination with opiates in such a way that patients receive adequate control of breakthrough pain.

CHAPTER I

INTRODUCTION

Current understanding of cancer-induced bone pain

Chronic pain is reported by 30-50% of all cancer patients and 70-90% of those with advanced disease (Doyle and Merrilees, 2004; Foley, 2004). Cancer pain is often described as persistent ongoing pain (i.e. pain at rest) along with pain resulting from external stimuli such as touch or temperature (Kuzeyli Yildirim et al., 2005). In addition, 40-80% of these patients may also experience transient episodes of breakthrough pain (BTP)(Davies, 2006; Portenoy et al., 2006). BTP can be defined as an abrupt and rapid onset of excruciating pain despite adequately controlled background cancer pain (Davies et al., 2009; Zeppetella, 2008). BTP is broadly categorized into two subtypes (a) idiopathic pain arising from an unpredictable event reported in 27-38.3 % of BTP and (2) incident pain with a known cause identified in 55-80% of the BTP episodes (Haugen et al., 2010; Mishra et al., 2009). Cancer pain can arise from multiple sources such as the disease itself as a result of direct tumor expansion, invasion and damage to nearby tissues such as bones as well as result of nerve infiltration causing neuropathic pain (Sarantopoulos, 2007). Pain can also be caused due to the treatment modalities used to treat cancer such as chemotherapy, radiation therapy and surgical procedures. Specifically chemotherapeutic agents such as platinum compounds (cisplatin, oxaliplatin or carboplatin) may produce pure painful sensory neuropathies, while others such as

vincristine, or suramin may induce mixed sensorimotor neuropathies with or without autonomic involvement (Quasthoff and Hartung, 2002). Same is the case with paclitaxel or docetaxel, promoters of the polymerization of tubulin that leads to mitotic cell arrest, which also damage neurons and glia and result in sensory, painful peripheral neuropathy (Mielke et al., 2006). The overall incidence of neuropathic pain after chemotherapy is as high as (30-70%) (Mantyh, 2006). Radiation therapy may also induce nerve injury (Polomano and Farrar, 2006) in 25 and 47% patients receiving the treatment (Olsen et al., 1990). Finally, surgery for diagnostic or therapeutic purposes, may lead to chronic pain with 60-90% incidence as seen after surgery for breast cancer (Fassoulaki et al., 2000; Fassoulaki et al., 2001). Post- mastectomy pain syndrome may be particularly troublesome and adversely impact the quality of life of female breast cancer survivors.

Current Treatment of Cancer Pain:

Current analgesic therapies for CIBP have not altered significantly in over a decade. Treatment is multimodal and includes systemic analgesics (opioids, nonsteroidal, anti-inflammatory drugs (NSAIDs), bisphosphonates, anti-tumour chemotherapy, radiotherapy, systemic radioisotopes, local surgery, and anaesthetic techniques (Mercadante, 2002; Mercadante et al., 2002a; Mercadante et al., 2002b; Serafini, 2001).

NSAIDs: According to the guidelines mentioned in World Health Organization's analgesia ladder for treating cancer pain, non-steroidal anti-inflammatory drugs are widely used to treat cancer pain, alone in the initial step or when the pain is mild. It is then used in combination with opioids in the subsequent stages of pain. McNicol et al

looked at 42 pertinent trials involving 3084 patients in order to assess which agent is most clinically efficacious for relieving cancer-related pain, or even what may be the additional benefit of combining an NSAID with an opioid in this setting and found that no generalizations can be made regarding efficacy and safety of NSAIDs for cancer pain (McNicol et al., 2005). Particular subpopulations of patients suffering from cancer pain may particularly benefit from NSAIDs in their analgesic regimen. These may be patients with painful metastatic disease affecting the bones or patients with significant pain, mediated by predominant inflammatory mechanisms.

Opioids: Opioid-based therapy remains the basis for most analgesia in CIBP. Whilst these regimens often attenuate the background (tonic) pain, the doses are insufficient to ameliorate incident pain. Wiffen et al conducted a systematic review in order to assess the efficacy of oral morphine in relieving cancer pain, as well the incidence and severity of side effects (Wiffen et al., 2003). Morphine was shown to be an effective analgesic for cancer pain in 45 published randomized controlled studies on 3061 subjects. Pain relief did not differ between sustained release and immediate release morphine formulations. Sustained release morphine doses were effective for 12 or 24 hour dosing, depending on the formulation. At present the current approach to managing breakthrough pain is using supplemental analgesia employing immediate-release medication at a dose proportional to the total around-the-clock opioid dose. Recently, Mercadante demonstrated the use of fast acting opiates and rapid onset opiates (ROO) in treating breakthrough pain (Mercadante, 2011). Although intravenous morphine (IV-MO) for the rapid onset is

found to be highly effective with minimal side effects even at large doses (Mercadante et al., 2004), the use is limited because of inconvenience of injecting IV-MO at home since the onset of BTP is often unpredictable. ROOs such as Oral trans-mucosal fentanyl citrate (OTFC) dissolve in buccal cavity within 15 min and 25% of the drug is immediately available and side effects are similar to opiate-induced side effects (Aronoff et al., 2005). Fentanyl buccal tablets (FBT) are effective in patients with reasonable amount of salivation. 48% of the drug is immediately available which is the double of OTFC but FBT may not be an option for patients with inadequate salivation. With Intranasal fentanyl spray (INFS), peak plasma concentration can be attained as fast as 12-15 min and control of BTP can be achieved within 10 minutes. INFS has several benefits including fast systemic penetration and the potential for self-administration, as well as being acceptable to patients with reduced salivary flow (Mercadante et al., 2009). Despite being effective in to control cancer-induced BTP, the choice of the dose of prescribed ROO as needed remains controversial (Aronoff et al., 2005). Recent clinical trials have shown statistically significant relationship between the dose of opiates given to control breakthrough pain and the dose given around-the-clock despite the individual difference in doses administered to control BTP (Mercadante, 2011) suggesting importance for titration of ROOs for dose determination. However, patients receiving high doses of opioids as basal analgesic regimen will not be candidates for titration with minimal initial doses of ROOs. Also, no treatment is currently available for patients resistant to opioid therapies. Overall, development of unwanted phenomena associated with the chronic use of opioids, such as side effects associated with high doses, tolerance, and hyperalgesia,

may provide a hindrance to their continuing use on certain patients (Zeppetella and Ribeiro, 2006).

Bisphosphonates: Tumors that compromise bone or nervous structures due to the bone destruction process may be very painful. Pain occurs as a result of bone destruction and as more destruction ensues, more pain can be experienced. Functional limitation and neurological impairment may be additional problems. In the mouse bone cancer pain model it has been clearly demonstrated that biphosphonates inhibit osteoclast-mediated bone resorption and suppress associated pain behaviors (Sevcik et al., 2004). Therapeutically they have a role in treating painful metastatic disease involving bones, as well as tumor related hypercalcemia. Pavlakis et al reviewed the clinical efficacy of biphosphonates in treating bone pain, quality of life and survival in 2189 women with early and advanced breast cancer by looking at 21 randomized studies (Pavlakis et al., 2005). In women with advanced breast cancer and clinically evident bone metastases, the use of bisphosphonates (oral or intravenous) reduced the risk of developing an adverse skeletal event such as pathological fracture, as well as delayed the time to skeletal event. The bisphosphonate most effective in reducing the risk of developing a skeletal event by 41% was intravenous zolendronate (4 mg). Bisphosphonates may also significantly reduce bone pain in women with advanced breast cancer and clinically evident bone metastases, thus improving quality of life. However, treatment with bisphosphonates does not appear to affect survival in women with advanced breast cancer. Yuen et al reviewed the efficacy of bisphosphonates in relieving pain in patients with bone metastases from

prostate cancer, in 10 randomized controlled studies that involved 1955 patients (Yuen et al., 2006). A significant increase in nausea was observed in patients who received bisphosphonates compared to placebo, but no increase in other adverse events was observed. There was no statistically significant difference between the bisphosphonate group and the control group in terms of prostate cancer death, disease progression, radiological response and prostate specific antigen (PSA) levels. Regarding the choice of bisphosphonates or the dose and the route of administration, there are insufficient data so far.

Gabapentin: Confirmation from clinical studies, have shown that gabapentin is effective against pain in cancer patients (Bosnjak et al., 2002; Caraceni et al., 2004; Oneschuk and al-Shahri, 2003; Ross et al., 2005; Ross et al., 2001). Gabapentin can be particularly helpful in patients with neuropathic pain (burning pain, shooting pain, allodynia) due to cancer, particularly when pain does not respond to opioids (Caraceni et al., 2004; Lossignol et al., 2004; Ross et al., 2005; Ross et al., 2001). Both pain and dysesthetic symptoms respond well to this drug, which also has an opioid sparing effect as well (Lossignol et al., 2004). Effective doses range between approximately 100 and 3000 mg daily (Oneschuk and al-Shahri, 2003). Side effects of gabapentin include mainly somnolence, drowsiness and headache (Bosnjak et al., 2002; Lossignol et al., 2004; Oneschuk and al-Shahri, 2003).

The current therapeutic regimens leave up to 45% of patients with inadequate and undermanaged pain control (Sarantopoulos, 2007). The lack of novel therapies is in part due to a lack of understanding of the mechanisms underlying the pathophysiology of CIBP. It was not until 1999, when a novel method of inducing CIBP in an animal model was published, that the unique peripheral and central pathophysiology could be investigated.

Animal models of cancer-induced bone pain:

Until the late twentieth century all animal models of CIBP were based on the systemic injection of carcinoma cells, which resulted in non-specific and/or multiple sited bone metastases. In 1999, Schwei et al. reported a method of local injection of cancer (osteosarcoma) cells directly into the intramedullary space of femur. The cancer cells were sealed in the bone and disease progression remained confined to the bone. Within the 2-3 weeks, these animals displayed progressively severe pain behaviors such as flinching, guarding and tactile allodynia, which correlated with progressive destruction of the femur (Schwei et al., 1999). In presented studies, we use a mouse model (Balb/c females) in which murine mammary adenocarcinoma cells 66.1 are injected and sealed in the femur. Pain behaviors and bone remodeling develop within 6 days post-surgery and are significantly higher than the sham operated rats. Using similar techniques, we have also developed a rat model of CIBP (Fischer 344) in which rat mammary adenocarcinoma cells CRL1666 are injected and sealed in the tibia. Pain behaviors and bone remodeling develop within 10 days post-surgery. This approach involves various

advantages such as (1) the disease progression is confined to the bone (2) the amount of tumor burden introduced in the bone is known (3) this allows for determination of pain behaviors, tumor burden and remodeling in individual animal (4) effects of a drug that modifies tumor, pain and bone remodeling can be studies in individual animals (5) tissue injury from the tibial injection of cancer cells involves minimal tissue injury and animals regain normal behaviors soon after the surgery.

Mechanisms underlying cancer-induced bone pain:

Pain arising from the cancer-bearing bone could be (a) neuropathic i.e. damage or lysis of the nerve ending innervating the bone due to growing tumor burden or acidic environment created by the tumor as well as tumor-induced bone degradation (b) inflammatory pain pro-inflammatory mediators secreted by tumor and tumor associated cells such as macrophages or mast cells (c) mechanical pain arising from bone loss, stress on the brittle bones or spontaneous fractures. Primary afferent nerve fibres transmit non-noxious (Aβ fibres) and noxious stimuli (Aδ and C fibres) to the dorsal horn of the spinal cord. There the signals undergo extensive modulation, both excitatory and inhibitory. Signal is then transmitted to the higher centers in brain where the it is processed and pain is perceived (Perl, 2011). Periosteum and the mineralised bone are richly innervated by peptidergeic as well non-peptidergic primary afferents. Peptidergic sensory neurons can synthesize peptides such as calcitonin gene related peptide (CGRP) and express TrkA, a receptor for nerve growth factor. Non-peptidergic sensory neurons bind to isolectin B4 (IB4) and express P2X receptors (Kaan et al., 2010; Mach et al., 2002).

A variety of transmitters are known to mediate the development of sensitization or excitation of peripheral sensory neurons. These transmitters include protons, adenosine triphosphate (ATP), bradykinin, serotonin, prostaglandins, and leukotrienes. Elevated afferent traffic of nociceptive signaling from peripheral tissues, or aberrant ongoing firing of injured peripheral sensory neurons results in enhanced synaptic transmission via increased release of excitatory amino acids such as glutamate, substance P etc. leading to enhanced post- synaptic responses at dorsal horn neurons. Thus the latter also get sensitized to incoming stimuli. Central sensitization may even result in spontaneous firing of postsynaptic neurons at the dorsal horns even in the absence of incoming stimuli from the periphery. The activation of the NMDA receptor, with aberrant calcium influx and up-regulation of complex intracellular signaling cascades leading to altered gene expression and altered post-translational modification, is pivotal in the mediation of central sensitization (Seybold, 2009). Another mechanism speculated but not yet explored in the model of bone cancer pain is contribution of descending facilitation from the rostral ventromedullary medulla (RVM) that is well documented in headache (Edelmayer et al., 2009), pancreatitis-induced pain (Vera-Portocarrero et al., 2008) and nerve injury (Gardell et al., 2003; Vera-Portocarrero et al., 2006). Central sensitization results in manifestations, such secondary hyperalgesia in tissues beyond the area of the injury, allodynia, and enhanced persistent pain states. Peripheral and central neurons respond with spontaneous and ectopic firing resulting in allodynia and hyperalgesia, constant ongoing pain or excruciating breakthrough pain.

Following mechanisms have been associated with cancer-induced bone pain.

Osteoclasts: Osteoclasts play a key role in the pathogenesis of pain. Osteoclasts are highly differentiated multinucleated cells derived from monocyte that normally play a role in bone remodeling by bone resorption. In metastases to bones, what primarily mediates bone destruction and pain is aberrant activation of osteoclasts. In the bone cancer pain model, within a few days after tumor injection, a marked increase in the number of osteoclasts occurs, associated also with signs of maturation (Sevcik et al., 2004). Proliferation and hypertrophy of osteoclasts develops in both osteolytic and osteoblastic types of metastatic cancer disease. Active osteoclasts in contact with mineralized bone are continually internalizing products of mineralization, thus destroying the bone, and leading to pain (Morrissey et al., 2010). Pain can develop via several mechanisms. As a result of the activity of the osteoclasts leading to osteolysis, normal bone architecture and structural integrity is disrupted, leading to mechanical instability of the bone. Certain parts of the bone, such as the periosteum and bone marrow, contain a rich innervation by peripheral sensory neurons. The disruption of mechanical stability of the bone may produce mechanical deformation of the periosteum, which results in pain by stimulation of mechanoreceptors present on the sensory neurons that densely innervate it, as well as the bone. Deformation of the periosteum may thus generate intense, sharp pain from stimulation of these mechanoreceptors (Mach et al., 2002).

Local extracellular acidosis: Osteoclastic activity leads to release of protons (H+) and the development of an acidic extracellular microenvironment (pH 4.0-5.0) in the vicinity of osteolysis. This stimulates pH-sensitive nociceptors thus producing pain. Additionally, protons may also be released directly by the tumor cells.

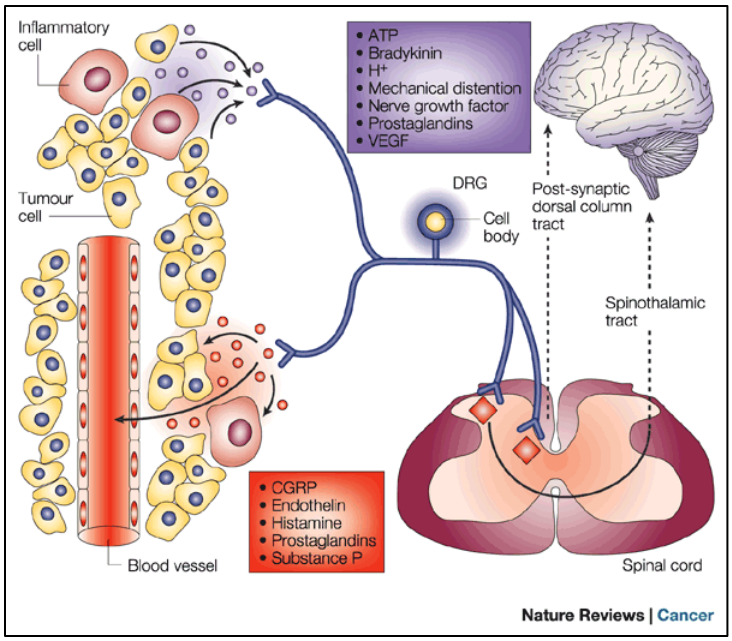


Figure Coutesy: Patrick Mantyh Naturae 2002

Low extracellular pH is a characteristic feature of tumors, and especially those with necrosis. On the other hand, sensory neurons express a variety of channels that can open as a response to sensing protons. These include the transient receptor potential vanilloid 1 (TRPV1) and the acid sensing ion channel 3 (ASIC 3) channels, which are sensitized and activated as a result of pH drop. (Guise, 2000; Luger et al., 2005; Peters et al., 2005; Sabino and Mantyh, 2005; Urch et al., 2005)

Nerve injury: Excessive activity of the osteoclasts may also result in direct injury of peripheral sensory fibers, thus leading to neuropathic pain. It has been shown in the model of cancer-induced bone pain that activating transcription factor 3 (ATF-3), a marker of nerve fiber injury, is upregulated (Sevcik et al., 2004).

Inflammation: Within a tumor mass, in addition to malignant cells, several other cell types may be contained, such as inflammatory cells. Tumor-associated macrophages have been estimated to comprise a considerable portion (2– 60%) of the total tumor mass (McBride, 1986). Inflammatory cells and malignant cells secrete a variety of substances that sensitize or excite primary afferent nociceptors. These mediators include prostaglandins, cytokines such tumor necrosis factor, interleukins and various growth factors such as epidermal growth factor, nerve growth factor, transforming growth factor and platelet-derived growth factor. These mediators have the capacity to sensitize and/or excite primary afferent nociceptors, thus contributing to pain and hypersensitivity (Safieh-Garabedian et al., 1995; Watkins et al., 1994; Woolf et al., 1997).

Nerve growth factor (NGF): The role of NGF in mediating inflammatory and neuropathic pain states is well recognized (Bennett, 2001; Gwak et al., 2003; Lamb et al., 2003). Some reports even indicate secretion of nerve growth factor by cancer cells (Dolle et al., 2003). The results of a study in which anti-NGF therapy produced significant pain relief as well as reversed peripheral and central sensitization in the mouse bone cancer pain model, indicate the potential role of NGF as a mediator of neural sensitization and mediator of pain (Sevcik et al., 2005).

Endothelins: Cancer tumors express high levels of a family of vasoactive peptides, called endothelins. Plasma levels of endothelins have been associated with pain severity in cancer patients (Nelson et al., 1995). Release of endothelin-1 from tumor cells (Wacnik et

al., 2001) has been implicated in the induction of thermal hyperalgesia. Additionally, it has been shown that some primary afferent fibers express endothelin receptors and once activated they may result in sensitization or excitation (Pomonis et al., 2001).

Glial activation: Activation of glial cells, such as astrocytes and microglia contributing to chronic pain is well documented (Watkins et al., 2001a; Watkins et al., 2001b). As a result of tumor growth in the bone and subsequent bone destruction, hypertrophy of astrocytes is observed in ipsilateral spinal cord dorsal horn, as measured using the glial fibrillary acidic protein (GFAP) marker (Honore et al., 2000a; Honore et al., 2000b).

Nerve sprouting and neuroma formation: Recent findings by Mantyh et al in the mouse model of bone cancer pain indicated the role of nerve sprouting in late stage cancer pain (Mantyh et al., 2010). They demonstrated that as tumor burden increases within the bone, TrkA expressing sensory nerve fibers as well as sympathetic nerve fibers undergo sprouting and form neuroma like structures. Since nerve growth factor is implicated in marked sprouting of these nerve fibers, the role of anti-NGF antibody in blocking the pathological nerve sprouting was determined. The studies conclude that only early sequestration i.e. before development of robust pain behaviors or sustained sequestration of nerve growth factor can block the nerve sprouting and structural reorganization thus causing 40-60% reduction in bone cancer-induced pain behaviors.

Data from preclinical models of cancer induced bone pain:

Opiates: High doses systemic of morphine (40 mg/kg) produced slight improvement in pain behaviors in bone cancer mouse model (Luger et al., 2002). In vitro morphine has an inhibitory effect on growth of several human cancer lines by suppression of tumor necrosis factor α (Sueoka et al., 1996). On the other hand, morphine may enhance tumor growth in animals inoculated with tumor cells apparently via an immunosuppressant effect (Ishikawa et al., 1993). Recent finding by King et al shows that sustained morphine accelerates tumor-induced bone loss and spontaneous fractures in a model of bone cancer pain (King et al., 2007).

Biphosphonates: Biphosphonates are predominantly given for management of osteoporosis pain but they are also used to relieve pain from metastases to bones. Bisphosphonate compounds are drugs with high affinity to calcium ions and have the capacity to suppress the activity of the osteoclasts, and consequently to inhibit bone resorption. Alendronate, a bisphosphonate, was shown to decrease pain behaviors as well as cancer-induced bone remodeling and nerve damage in a model of bone cancer pain (Sevcik et al., 2004).

Osteoprotegerin: Osteoprotegerin (OPG), a molecule that belongs to the soluble TNF receptor superfamily (Kong et al., 1999; Lacey et al., 1998; Simonet et al., 1997). OPG acts as a secreted decoy receptor that binds to, and thus sequesters a cognate ligand, the OPG ligand (OPGL). The bound, sequestered ligand is then prevented from activating its cellular target, which is the receptor activator of nuclear factor-K (RANK receptor),

specifically expressed on mature osteoclasts to mediate their activation and activity. The OPG is derived mainly from the osteoblasts and activated T cells (Kong et al., 1999). Considering the capacity of OPG ligand to stimulate osteoclasts, it is an important mediator of bone cancer pain. Honore et al, employing the mouse bone cancer pain model, reported that treatment with OPG completely abolishes cancer-induced bone destruction, reduces the number of osteoclasts at sites of tumor, substantially suppresses spontaneous and evoked pain behaviors and finally prevents the neurochemical reorganization of the spinal cord associated with bone cancer pain (Honore et al., 2000a). Palpation induced substance P release, Neurokinin-1 (substance P receptor) internalization, and palpation-induced c-fos expression seen in the spinal cords of sarcoma-injected mice were also blocked by osteoprotegerin. The action of OPG was rapid. Within 2 days after a single injection there was a substantial inhibition of bone absorption, but in this study it was administered as daily subcutaneous injections (Honore et al., 2000a).

Non-steroidal anti-inflammatory drugs and acetaminophen: Saito et al in the mouse bone cancer pain model investigated the pharmacological efficacy of selective COX-1 or COX-2 inhibitors, non-selective COX inhibitors, acetaminophen and morphine on bone cancer pain. Analgesia was assessed after drugs were administered 2 weeks post-injection of sarcoma cells into mouse femur. Oral administration of acetaminophen, indomethacin, and morphine, but not of SC560 (a COX-1 selective inhibitor) or celecoxib, produced an analgesic effect, while co-administration of subanalgesic doses of morphine with acetaminophen enhanced the analgesic effect of the latter (Saito et al., 2005).

Anti-NGF antibody: Because NGF release by tumor cells or tumor associated cells in the bone microenvironment has been implicated as a possible mediator of cancer pain, Sevcik et al investigated the therapeutic potential of anti-NGF therapy in the mouse model of bone cancer pain (Sevcik et al., 2005). Both spontaneous and movement-induced pain behaviors were attenuated by a monoclonal anti-NGF antibody. The effect was greater than that of high dose morphine. Nerve injury and neurochemical changes indicative of peripheral and central sensitization were also reduced by this therapy.

Gabapentin: Gabapentin, a novel anticonvulsant with antihyperalgesic effectts, acting as an inhibitor of voltage-gated calcium channels (Sarantopoulos et al., 2002) and is used widely in the management of neuropathic pain originating from from diabetes mellitus (Backonja, 1999). Chronic treatment with gabapentin did not affect bone destruction or tumor growth but attenuated both spontaneous and movement-evoked bone cancer pain behaviors (Peters et al., 2005). In another study which contained inoculation of cancer cells into the tibia resulting in neuronal hyperexcitability at the superficial dorsal horns of the spinal cord accompanying behavioral manifestations of pain, gabapentin administered acutely and chronically normalized the hyperexcitable superficial dorsal horn neuronal response, significantly reducing electrical-evoked and mechanical-evoked responses. Chronic administration of gabapentin also significantly attenuated pain behavior in injected rats restoring responses to preoperative baseline degrees (Donovan-Rodriguez et al., 2005).

Mechanisms underlying bone metastases

In 1889, Stephen Paget reported the organ specificity of tumors from a study carried out from autopsies of 735 women who died of breast cancer. The most frequent site of metastasis was ovaries followed by the bone. On the basis of these observations, he proposed to the "seed and soil" hypothesis. According to this hypothesis, the microenvironment of the metastatic organ provides soil for the tumor to grow in it. This hypothesis is now widely accepted and believed to be true in the case of bone metastases.

The most common malignancies such as those of breast, prostate and lung have the highest propensity to metastasize to the bone (Clohisy and Mantyh, 2003). 70% of patients dying from breast cancer have skeletal metastases (Guise and Mohammad, 2004). Bone is one of the earliest sites to be affected. The sites within the skeleton that receive the most abundant vascular supply preferentially colonize tumor cells (Guise et al., 2005). Skeletal metastases are essentially incurable, but growth of tumor in bone is often indolent, although it may be rapid and accompanied by visceral metastases (Guise et al., 2005). Median survival is 2 years from time of initial diagnosis of bone metastases, and survival is 5 years in almost 40% of patients (Halvorson et al., 2006). Death in patients with bone metastases, could be from cachexia or secondary metastases to other sites, such as brain or liver (Guise et al., 2005). If skeletal metastases do serve as a reservoir for secondary metastases, reduction of tumor burden in bone with bisphosphonate treatment should impact survival, which has not yet been shown in

clinical trials (Brown et al., 2004). Bone pain is the most common complication of bone metastasis and often the presenting symptom. Pathologic fractures occur in patients with advanced disease. Hypercalcemia caused by excessive bone destruction used to occur in up to 30% of patients with breast cancer with bone involvement (Galasko and Burn, 1971). This complication has been decreased by the use of bisphosphonates. Osteoblastic lesions can cause spinal cord compression and vertebral fractures. Because patients with breast cancer limited to the skeletal metastases have a median survival of 2-4 years (Guise et al., 2005), they have a high cumulative risk of skeletal-related events. The bone metastases seen in animal models represent a simplified version of the clinical picture. Animal models are of much shorter duration and involve homogeneous tumor cell lines, whereas bone metastases in patients with breast cancer are heterogeneous, even between metastatic sites in an individual patient. The frequency of these tumors to metastasize to the bone are reflected are by two events that need to be explored (1) propensity of these tumors to home to the bone (2) capacity of the bone marrow to support the tumor growth.

Interactions between tumor and the bone microenvironment are synergistic since stromal derived growth factors and cytokine aid in the growth of the tumor and the tumor further enhances production of these factors form the bone microenvironment. Tumor induced bone degradation occurs when the process of normal bone turnover is disrupted. The two main important bone-derived cells that are involved in this process are (1) osteoclasts i.e. bone resorbing cells and (2) osteoblasts i.e. bone forming cells. Osteoclasts are derived from the precursors in the mononuclear-phagocyte lineage under the influence of

macrophage colony stimulating factor (MCSF) and RANKL (Yasuda et al., 1998). Osteoblasts are derived from stromal cell lineage and they secrete a lot of growth factors into the bone matrix (Wang et al., 2009).

Following mechanisms are known to mediate tumor-induced bone remodeling:

Adhesion molecules

Interactions between specific cell surface molecules on bone cells, bone marrow cells, and tumors are critical to both tumor invasion and the metastatic process. The importance of these interactions has been demonstrated clearly by studies in human breast carcinoma, myeloma, and prostate carcinoma. Van der Pluijm and coworkers (van der Pluijm et al., 2001) found that the urokinase receptor and β1 integrins formed functional adhesion complexes at distinct sites at the cell surface of metastatic human breast carcinoma cells and that the urokinase receptor is capable of regulating the adhesive function of integrins on breast carcinoma cells. This study showed that the addition of a blocking peptide for the urokinase-integrin complex inhibits the attachment of breast carcinoma cells to vitronectin. Using a mouse model of breast carcinoma metastasis, these authors reported that transplantation of nude mice with MDA-231 breast carcinoma cells, which over express this blocking peptide, results in a significant reduction in tumor progression in bone compared to vector-transfected cells. Furthermore, mice that were transplanted with MDA-231 cells and that received continuous administration of the peptide for 28 days had significantly reduced tumor progression in bone compared with animals that were treated with a control peptide. These data show that breast carcinoma progression in bone

requires adhesive interactions between molecules that are expressed in the bone and molecules that are expressed in tumor cells. Similarly, Sung and coworkers (Sung et al., 1998) have shown human breast carcinoma cells adhere, proliferate, and migrate to bone through the interactions between αvβ3 and αvβ5 integrins and bone sialoprotein.

Transforming growth factor β

Several studies have shown that, when breast carcinoma cells metastasize to bone and induce bone resorption, transforming growth factor (TGF- β) is released in active form from the bone. In particular, Chirgwin and Guise (Chirgwin and Guise, 2000) have shown that breast carcinoma cells produce parathyroid hormone-related peptide (PTHrP), which induces osteoclastic bone resorption and releases TGFβ from the bone matrix. Osteoblasts and bone marrow stromal cells produce TGFβ. TGFβ then increases PTHrP production further, creating a vicious cycle in which tumor cells induce bone destruction and, through this process, release growth factors that enhance the growth of the tumor. TGFβ is a potent, multifunctional cytokine that is produced by many cells, including osteoblasts and bone marrow stromal cells that can regulate cell growth and stimulate matrix production. It has been demonstrated that TGFβ is a major factor in bone remodeling, and tumor-derived agents that enhance TGFβ production have been associated with increased bone formation (Festuccia et al., 1999). TGFβ normally functions as a suppressor of tumor growth. Lang and coworkers (Lang et al., 1999) have shown that mice lacking TGFβ due to haploinsufficiency are more susceptible to tumors. Furthermore, TGFβ is immunosuppressive, which can increase tumor survival further by

suppressing the immune system. TGFβ also can stimulate normal stromal cells and osteoblasts to secrete growth factors that enhance tumor growth. Guise and coworkers (Guise et al., 1996) showed that the administration of a neutralizing monoclonal antibody to PTHrP inhibited the development of breast carcinoma bone metastasis by MDA-MB-231 cells in nude mice. It is noteworthy that these studies also showed that inhibiting TGFβ responsiveness of the tumor by using a dominant negative TGFβ receptor reduced incidences of bone metastasis (Yin et al., 1999).

Platelet derived growth factor

Platelet-derived growth factor (PDGF) is a polypeptide produced by osteoblasts in the bone microenvironment that shows extensive sequence homology with the oncogene c-Cis. PDGF increases cell replication, bone resorption, collagen degradation, and collagenase expression as well as inhibiting osteoblast function. The mitogenic activity of PDGF results in the growth of tumor cells as well as the enhancement of osteoclast activity. Yi and coworkers (Yi et al., 2002) reported that, using MCF-7 cells in a model of breast carcinoma metastasis to bone , breast carcinoma cells that overexpressed HER-2-NEU produced large amounts of PDGF and showed an enhanced propensity to metastasize to bone. Furthermore, they suggested that PDGF played a causative role in the development of osteoblastic bone metastasis in this model. Thus, up-regulation of PDGF may enhance osteoblast formation and activity in bone metastasis and may enhance the growth of tumor cells through its mitogenic effects on tumors.

Interleukin 6

IL-6 is a pleiotropic cytokine that has multiple effects on cell function. IL-6 enhances osteoclast activity in human bone marrow and in murine bone marrow cultures and appears to have direct effects on osteoclast precursors in human systems (Kurihara et al., 1990). IL-6 is also a growth factor for myeloma cells, and its production is up-regulated when myeloma cells bind to bone marrow stromal cells (Chauhan et al., 1996). This up-regulation of IL-6 appears to result from adhesive interactions between very late activation antigen (VLA-4) and VCAM-1. Induction of IL-6 production by bone marrow stromal cells has multiple effects on myeloma growth in bone. It enhances the growth of the myeloma cells and enhances osteoclastic bone resorption, which results in the release of growth factors from the bone matrix. IL-6 can act synergistically or additively with other osteoclast-activating factors produced in the myeloma microenvironment to further increase bone destruction and tumor growth. Recently, Yaccoby and coworkers (Yaccoby et al., 2002) reported that there appeared to be an important link between the growth of myeloma cells and bone disease in a human SCID (SCID-hu) model of myeloma. Those investigators reported that blocking osteoclastic bone resorption decreased the growth of primary myeloma cells from patients in fetal

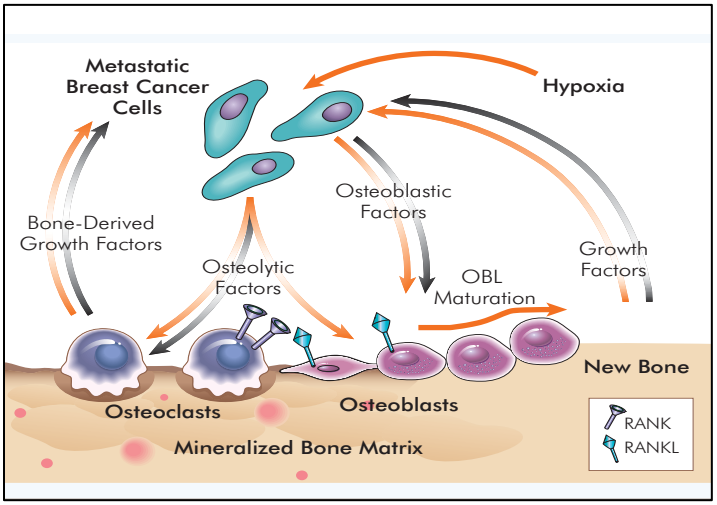


Figure Coutesy: Guise et al Clinical Breast Cancer 2005

bones implanted in SCID mice. Blocking IL-6 production may have important effects on the growth of myeloma cells and bone destruction in patients with myeloma.

Receptor activator of nuclear factor kappa B ligand (RANKL)

RANKL is a recently described factor produced by osteoblasts and stromal cells that is a potent stimulator of osteoclastogenesis. RANKL expression is in- creased when myeloma cells or breast cancer cells bind to marrow stromal cells (Mancino et al., 2001). RANK-L then induces osteoclastogenesis, which results in the release of growth factors that further enhance the growth and

survival of tumor cells. In addition, factors produced by tumor cells directly enhance RANK-L expression. RANKL has no direct effects on the growth of tumor cells, although its increased production enhances bone destruction, which further enhances the bone metastatic process.

Chemoattractants

A number of factors produced by bone marrow stromal cells and osteoblasts can act as chemoattractants to enhance the migration of tumor cells to bone. Monocyte chemoattractant protein 1, which is secreted by bone marrow endothelial cells, produces chemoattraction of the 5T2 multiple myeloma cell line (Vanderkerken et al., 2002). Recently, Muller and coworkers (Muller et al., 2001) reported that breast carcinoma cells from malignant breast tumors and metastases expressed the chemokine receptors CXCR4 and CCR-7, which mediated the chemoattraction and invasive responses in the tumors.

These findings suggest that chemokines and their receptors may play a critical role in determining the metastatic destination of tumor cells. Geminder and coworkers (Geminder et al., 2001) reported a possible role for CXCR4 and its ligand, stromal cell derived factor-1 (SDF-1), in the development of bone marrow metastasis by neuroblastoma. These authors reported that CXCR4 expression may be a general characteristic of neuroblastoma cells and that SDF-1 induces the migration of CXCR4-expressing neuroblastoma cells. These neuroblastoma cells then interact with components of the bone marrow microenvironment to promote neuroblastoma cell adhesion to bone marrow stromal cells and subsequent neuroblastoma cell proliferation. It also has been shown that SDF-1 plays an important role in myeloma cell adhesion to fibronectin and VCAM-1. Bone marrow stromal cells express SDF-1, and SDF-1 expression up-regulates VLA-4-mediated myeloma cell adhesion to fibronectin (Sanz-Rodriguez et al., 2001) (Sanz-Rodriguez 2001).

Bone morphogenetic proteins

BMPs were originally characterized as inducers of bone formation at extraskeletal sites in vivo (Reddi, 1994). BMPs belong to the TGFβ superfamily and stimulate osteoblast differentiation through the activation of transcription factors, in particular Runx-2 (McCarthy et al., 2003). In patients with prostate cancer BMP expression has been shown to correlate with increased recurrence rates and decreased survival (Thomas et al., 2002). BMP-4, -6 and 7 have been shown to be expressed by prostate cancer cells and in addition to their paracrine effects on osteoblasts, also play an important role in the

survival and growth of the cancer cells themselves (Brubaker et al., 2004; Ide et al., 1997). Like TGFβ, BMP appears to differentially affect early and advanced stage cancers (Brubaker et al., 2004). Changes in the responsiveness of prostate cancer cells to BMPs may enable these cells to escape the growth inhibitory autocrine effects of BMPs and at the same time drive bone formation via BMPs paracrine effects on osteoblasts.

Endothelin-1

Endothelin-1 (ET-1) is a potent stimulator of new bone formation that is secreted by tumor cells (Nelson et al., 1995) and can cause osteoblastic metastases in the nude mouse model. Metastases are effectively blocked with a selective antagonist of the endothelin A receptor (Yin et al., 2003) that is in clinical trials in men with advanced metastatic prostate cancer (Rosenbaum and Carducci, 2003). The vicious cycle model predicts that osteoblasts, osteoclasts, and tumor cells cooperate to cause the pathology of bone metastases. The receptor antagonist blocks the activation of osteoblasts by tumor-produced ET-1. It also decreases osteoclastic bone resorption, as indicated by decreases in the serum markers of bone resorption in clinical trials.

Adrenomedullin (AM)

Adrenomedullin (AM) is a vasoactive peptide with potent bone-stimulatory actions (Cornish et al., 2003). It is produced by many tumors (Zudaire et al., 2003) including breast cancer. Using lung and prostate cancer models, AM increases bone metastases.

Mice with AM-overexpressing tumors showed accelerated osteolytic lesions and adjacent areas of osteoblastic new bone formation (Guise et al., 2005).

There is evidence that osteoblastic metastasis also involves considerable osteolysis (Oades et al., 2002; Yonou et al., 2004). In prostate cancer patients with clinical osteoblastic lesions, blood and urinary levels of bone resorption markers are often elevated (Garnero et al., 2000). Clinical trials have suggested that blocking osteoclastic bone resorption reduces related skeletal events in prostate cancer patients (Lipton et al., 2002). In a mouse model of bone metastasis, osteoclasts numbers were shown to markedly increase at sites of early tumor invasion (Yonou et al., 2004). One theory as to why osteoclastogenessis is important for osteoblastic bone metastasis is that bone resorption by osteoclasts releases a variety of growth factors, which are stored as inactive forms in the bone matrix. The activation of growth factors may be required by cancer cells to maintain viability and proliferate and therefore in establishment of bone metastases. Indeed, in our mouse model which includes growth of the tumor within the bone, we see emergence of osteoclastic bone remodeling prior to development of osteoblastic structures suggesting the synergistic interactions between osteoclasts and osteoblasts.

Experimental approach

Detailed study of the metastatic behavior of cancers and of the complex interactions between cancer cells and host cells requires the development of good animal models. The

ideal model would be one in which cancer develops spontaneously in the organ of interest and metastasizes to bone. However, these models are extremely difficult to establish. There are no spontaneous models of breast cancer metastatic to bone. This problem can be partly circumvented in models in which cancer cells are injected directly into the blood stream. The cellular and molecular mechanisms of organ preference of metastatic tumors can be studied in such an experimental model because the steps involved before cancer cells reach their preferential target organs are likely to be non-specific and independent of organ selectivity. Various ways of injecting cancer cells in the circulation can be used such as injection in to the left ventricle of the heart, intramuscular or in tail vein. More recently a model of direct bone injection was developed by Shwei et al (Schwei et al., 1999). Although the cancer cells are directly injected in the bone, the interactions between the tumor and the bone can be studied. Also, there is no involvement of other organs making the interpretation of results less complex.

Role of p38 MAPK signaling pathway

Cells often undergo changes in response to physical and chemical stimuli, disease conditions. As a result of which, the levels of growth factors, cytokines and nutrients are altered. Cells respond to these alterations via signaling molecules called mitogen activated protein kinases or MAPK. Broadly there are three types of MAPKs: ERK, JNK and p38. The focus of this dissertation is mainly on p38 MAPK, which exist in four isoforms: α, β, γ and δ. P38α is the most commonly studied isoform and is expressed almost all cell types. p38γ is mainly expressed by skeletal muscles whereas p38δ is found in testis, pancreas, kidney and small intestines (Cuenda et al., 1997). The 38KDa p38α molecule is shown to undergo phosphorylation is response to endotoxin (J Han 1994, stress and heat shock (Han et al., 1994). It is strongly activated in vivo by environmental stresses and inflammatory cytokines (Kyriakis and Avruch, 2001). p38 undergoes phosphorylation via MKK3 and MKK6 at the Thr-Gly-Tyr motif in the

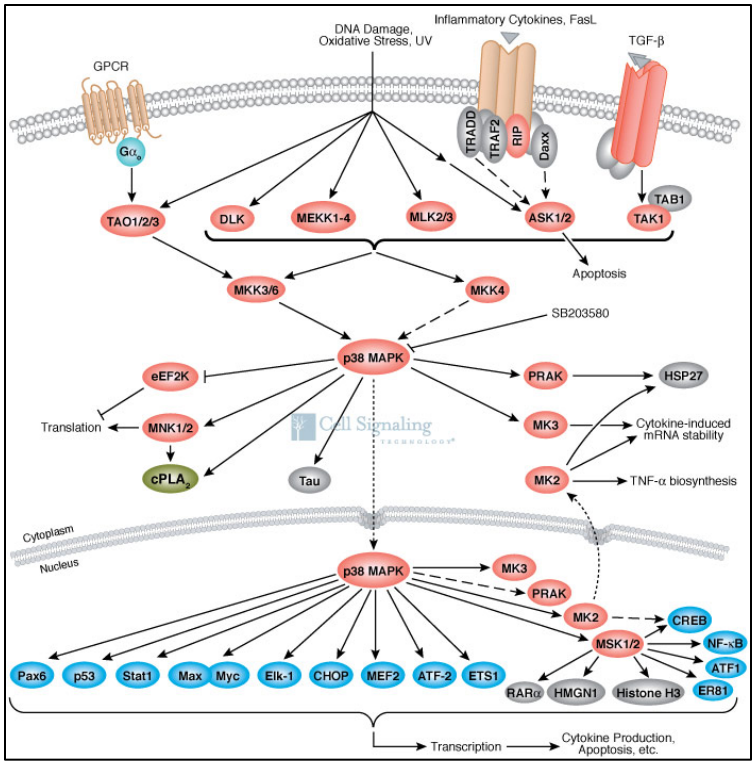


Figure Courtesy: www.cellsignaling.com

activation loop. The signaling then causes adaptive and physiological responses via p38 signaling (Cohen 1997, Kyriakis 2001). In addition to this canonical pathway, p38 also undergoes autophosphorylation independent of MKKs. It has been shown in activated T cells that stimulation of a proximal tyrosine kinase results into phosphorylation of Tyr323 reside of p38α causing changes in the structural conformation leading to phosphorylation other substrates as well as the Thr-Gly-Tyr motif (Salvador et al., 2005a; Salvador et al., 2005b). Multiple studies have demonstrated p38 as a biological switch since magnitude and duration of p38 MAPK signaling is critical in determining the biological effects. Additionally, activation of p38 occurs within minutes and is transient (Takekawa et al., 2000; Takekawa and Saito, 1998; Tanoue et al., 2000).

P38 MAPK is shown to be present in both the nucleus and cytoplasm of the quiescent cells but upon stimulation, some evidence suggests its translocation from cytoplasm to the nucleus (Raingeaud et al., 1995). Other data also indicate presence of activated p38 in the cytoplasm of stimulated cells (Ben-Levy et al., 1998). The most well-known role of the p38 pathway is as a transducer of responses to environment stress (such as hyperosmolarity, ultraviolet irradiation, and heat shock) and as receptors binding by proinflammatory molecules (eg, endotoxin, tumor necrosis factor α [TNF-α], and interleukin [IL 1) (Feng et al., 2010). Once activated, p38 MAPK can have a variety of biological effects such as cellular differentiation, cell migration, inflammation, cancer, cardiovascular dysfunction and Alzheimer's disease as depicted by Cuenda and Rousseau (Cuenda and Rousseau, 2007).

P38 MAPK and Cancer:

p38 MAPK signaling is well established in a number of oncogenic functions that are mediated by its involvement in key processes of cancer progression, such as invasion, inflammation and angiogenesis. The two major groups of proteins that are regulated by p38 MAPK-mediated phosphorylation are (1) transcription factors, such as activating transcription factor 2 (ATF2), ELK1, myocyte-specific enhancer factor 2 (MEF2) and C/EBPβ; and (2) protein kinases, including MAPK-activated kinase 2 (MK2; also known as MAPK2), mitogen- and stress-activated protein kinase 1 (MSK1), MAP kinase-interacting serine/threonine kinase 1 (MNK1) and MNK2 (Cuenda and Rousseau, 2007; Ono and Han, 2000). P38 is also implicated in regulating the cell cycle checkpoints G0, G1/S and G2/M (Ambrosino and Nebreda, 2001). Depending on the cell type and condition, p38 MAPK can either induce or inhibit transition into G1/S by regulating levels of cyclin A or D1 as well as retinoblastoma (pRb) protein and p53 which are important determinants of G1/S progression (Mikhailov et al., 2007). Similarly, G2/M is initiated or halted based on responses to various stimuli such as damage by ultraviolet radiation, disruption of microtubular structure, hyperosmotic stress etc (Bulavin et al., 2001; Lindqvist et al., 2004). p38 can positively regulate proliferation, for example in haematopoietic cells (Platanias, 2003) and several cancer cell lines such as breast cancer (Lee et al., 1999; Neve et al., 2002), prostate cancer (Ricote et al., 2006) and melanoma (Recio and Merlino, 2002). The pro-survival roles for p38 can be mediated by the induction of cell differentiation or by anti-apoptotic inflammatory signals, such as the cytokine interleukin-6 (IL-6), as well as by a quiescent state known as cancer dormancy

that may be important for cancer cells to acquire drug resistance (Silva et al., 2006). Chronic inflammation is a potent cancer promoter (Karin, 2006b; Karin et al., 2006; Mantovani et al., 2008; Mantovani and Pierotti, 2008) and has been linked to increased cancer cell survival and the induction of angiogenesis and invasion. p38 regulates the induction of the pro-inflammatory mediator cyclooxygenase 2 (COX2), which could potentially contribute to cancer progression in non-melanoma skin cancer and breast cancer (Bachelor and Bowden, 2004; Timoshenko et al., 2006). In addition, p38 has a key role in the production of many cytokines, such as TNFα, IL-1 and IL-6, which have pro-inflammatory, pro-survival and angiogenic effects (Kumar et al., 2003). P38 is also linked to carcinogenesis via inflammatory pathways. Production of IL-6 from Tumor Necrosis Factor α and IL-8 from IL1 β has been shown to be mediated via p38 pathway in rheumatoid synovial fibroblasts. Morever, p38 MAPK plays a role in early stages of osteoclastogenesis from bone marrow precursors (Houde et al., 2001; Vachon et al., 2002). p38 can also regulate cytokine expression by modulating transcription factors, such as NF-ḰB (Karin, 2006a) or at the post-transcriptional level, by regulating mRNA stability and protein translation, which is thought to be mostly mediated by the downstream kinase MK2 (Clark et al., 2003).

Morever, p38 MAPK mediates invasion and metastasis by regulating MMP-9 and mediating chemotactic signaling by controlling the ECM remodeling (Simon et al., 1998). p38 MAPK is also shown to be involved in production of as well as downstream signaling of vascular endothelial growth factor or VEGF, which is the key player in

promoting angiogenesis, an important hallmark of tumor progression (Houle and Huot, 2006; Rousseau et al., 2000). P38 induced VEGF synthesis is likely due to activation of p38 by hypoxic conditions often seen in the core of the tumor, which constantly requires formation of new blood vessels (Pages et al., 2000).

P38 MAPK and Pain:

Injury-induced inflammatory mediators such as interleukin-1β, interleukin-6, tumor necrosis factor α, prostaglandin E2 etc. activate neurons in the peripheral and central nervous system causing pain and hypersensitivity (Ji and Suter, 2007a). Not only the non-neuronal cells at the site of injury but also the glial cells in the spinal cord produce inflammatory mediators. Microglia are regarded as the main source of inflammatory mediators in the central nervous system (Tsuda et al., 2005). It is well established that p38 MAPK is the key regulator of synthesis and release of inflammatory mediators and therefore, an important contributor of pain transmission. P38 activation and elevated expression has been shown in the ipsilateral spinal cord dorsal horn three days following the spinal nerve ligation and is maintained for more than three weeks (Jin et al., 2003a). The data further showed that activated or phosphorylated p38 was co-localized with the microglial marker OX-42 indicating activation of p38 in the spinal cord by microglia. In rat models of direct spinal activation i.e. intrathecal substance P administration as well as in the model of inflammation with intradermal formalin injection, increase in phosphorylated p38 was observed in laminae I to IV of spinal cord which was co-localized with the microglial expression (Svensson et al., 2003a; Svensson et al., 2003b).

Similarly, activation of p38 is also observed in dorsal root ganglia following chronic constriction injury (CCI) of the sciatic nerve or peripheral inflammation by injecting formalin into the paw (Kim et al., 2002). P38 can regulate the synthesis of inflammatory mediators by post-translational regulation. One of the speculated mechanisms is via activation of phospholipase A2 (PLA2) via its downstream kinase MAPKAP-2 (Ji and Woolf, 2001). PLA2 plays an important role in inflammatory responses. Activation of PLA2 results in production of arachidonic acid for prostaglandin which can be catalyzed via cyclooxygenases to produce prostaglandin E2 (Ji and Woolf, 2001). Several forms of PLA2 are expressed in the spinal cord and inhibition of PLA2 can have antihyperalgesic effects (Yaksh et al., 2006).

SB203580 is a pyridinyl imidazole compound, which is a highly specific inhibitor of p38 MAPK. In vivo and in vitro assays demonstrate that SB203580 can only α and β isoforms of p38 but γ and δ are completely unaffected. SB203580 inhibits the activity but not activation of p38 MAPK by competitively binding at the ATP-binding site (Badger 1996). A number of studies have indicated the anti-inflammatory nature of SB203580 both in vivo and in vitro (Dong et al., 2009; Han et al., 2006; Simi et al., 2002; Zhou et al., 2010). Sustained administration of SB203580 has been proven to be disease modifying in animal models of collagen-induced arthritis (Badger et al., 1996b) and adjuvant arthritis (Boyle et al., 2006) likely due to inhibition of TNF. Anti-hyperalgesic effects of SB203580 are also well documented. Daily administration of this inhibitor intrathecally prevents nerve-injury induced mechanical allodynia (Jin et al., 2003a; Tsuda

et al., 2004). SB203580 also blocked hyperalgesia arising from intraplantar formalin or carrageenan injection (Svensson et al., 2003b). In addition, pretreatment with SB203580 is shown to block COX2 activity and fos expression of neurons, the events that lead to sensitization of the spinal cord (Svensson et al., 2003b).

Together, the findings conclude that the p38 MAPK signaling has been implicated in mediating inflammation and neuropathic pain, both of which are thought to play a role in driving cancer-induced bone pain. Moreover the role of p38 MAPK is also demonstrated in maturation and synthesis of osteoclasts, cells responsible for osteolysis. The p38 MAPK cascade is known to be activated by tumor-induced cellular stress such as inflammatory cytokines. The growing tumor burden, acidic tumor microenvironment, an array of inflammatory mediators produced by the tumor and tumor associated cells in the bone microenvironment and tumor-induced bone degradation all contribute to bone pain (Peters et al., 2005). As p38 MAPK plays a key role in each of these variables, we hypothesized that blockade of p38-MAPK signaling will attenuate cancer induced bone pain and bone loss. The present study determined the role of p38 MAPK in tumor growth, tumor-induced bone loss, and tumor-induced bone pain in a mouse model of cancer-induced bone pain in which mammary adenocarcinoma cells were injected and sealed into the intramedullary space of the femur of female mice to allow controlled progression of disease within the bone.

Role of Interleukin-6

Interleukin-6 or IL-6 (184 amino acids) is a pleiotropic cytokine of the interleukin group that is produced by both lymphoid and non-lymphoid cells such as T and B cells, monocytes, fibroblasts, keratinocytes, endothelial cells and several tumor cells. IL-6 is also synthesized by neurons and glia in concentrations ranging from a few picograms to a few nanograms (Sommer and Kress, 2004). Multiple forms of IL-6 are produced, with molecular weights ranging from 21.5 to 28 kDa. IL-6 binds to a heterodimeric receptor with 2 subunits: 80 KDa α subunit (IL-6Rα) and a 130 kDa β subunit (IL-6Rβ) more known as gp130. IL-6 has more affinity for gp130, which is ubiquitous in all receptors for the IL-6 cytokine family including IL-11, ciliary neurotrophic factor, oncostatin M, and leukemia-inhibiting factor. The intracellular signaling initiates when IL-6 binds to IL-6R and gp130 with phosphorylation of signaling molecules called JAK (Janus kinases), which in turn phosphorylates STAT molecules. The STAT molecules are then translocated into the nucleus and transcription of target genes takes place. Interestingly, most cells are devoid of membrane bound IL6R and are unresponsive to IL-6. Such cells, however, can still react to IL-6 complexed with soluble form of IL-6R which can initiate the cascade via gp130 (Brakenhoff et al., 1994). IL-6 can be synthesized at low basal levels in many cell types with local induction in case of injury. Marked overproduction of IL-6 is observed across multiple disease conditions such as diabetes, neuropathic pain, musculoskeletal disorders such as osteoarthritis, osteoporosis, inflammatory or autoimmune diseases such as rheumatoid arthritis, psoriasis, crohn's disease inflammatory bowel disease, pancreatitis and heart failure.

Interleukin-6 and cancer:

High levels of IL-6 have been reported in multiple carcinomas including bladder cancer, prostate cancer, multiple myeloma, colorectal cancer and renal cell carcinoma (Akimoto et al., 1998; Chung and Chang, 2003; Daha et al., 2007; Emile et al., 1994; Nachbaur et al., 1991; Ueda et al., 1994; Walther et al., 1998). It serves as a marker of the progression of pancreatic cancer (Barber et al., 1999; Bharadwaj et al., 2007; Okada et al., 1998).

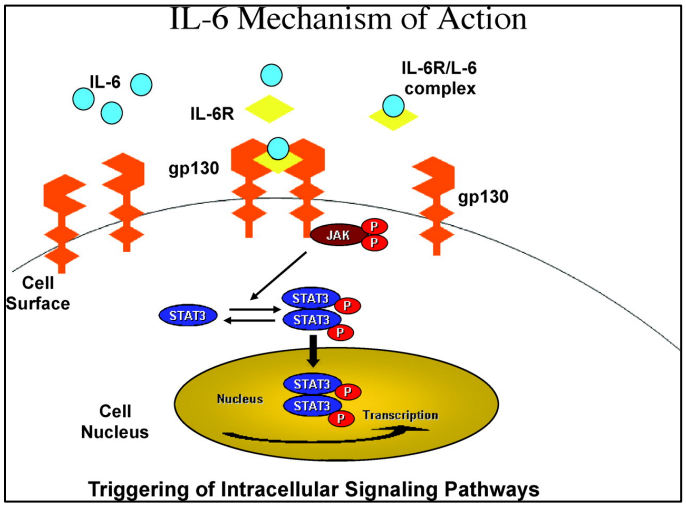


Figure Courtesy: Ahmed et al Molecular Cancer Therapeutics 2007

In addition, elevated IL-6 is also correlated with cachexia (Kuroda et al., 2007; Strassmann et al., 1992). Excessive amounts of IL-6 are found in the sera of patients with bone cancer or bone metastases with osteoclastic bone resorption as compared to patients with benign tumors. IL-6 is also found to promote production of vascular endothelial growth factor (VEGF) and is indirectly responsible for angiogenesis, an important hallmark of cancer (Ara and Declerck, 2010).

IL-6 is found to induce expression of pro-survival or anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL) in esophageal carcinoma and multiple myeloma cells (Jourdan et al., 2000; Leu

et al., 2003; Schwarze and Hawley, 1995). IL-6 was also detected in the supernatant of the multidrug resistant breast cancer cell line MCF-7. When treated with IL-6, MCF-7 cells showed eight to ten fold increase in resistance to doxorubicin (Koren et al., 2000). In some of the breast carcinoma cell lines MCF-7, T47D and MDA-MB-231, addition of recombinant IL-6 to the culture media caused decrease in the expression of E-cadherin, an important factor of cell adhesion indicating the possible role of IL-6 in cancer cell motility and ultimately metastasis (Ara and Declerck, 2010).

Whether IL-6 is significantly associated with breast tumor progression or not is not clearly understood. Green et al identified the expression of IL-6 mRNA in normal as well neoplastic human breast tissue and found that 57% of normal and 58% of neoplastic breast tissue was positive for IL-6 mRNA (Green et al., 1997). However, no significant difference in the expression of IL-6 was noted between the two groups. Basolo et al indicated that IL-6 is in fact found in normal mammary epithelial cells but is absent in ductal infiltrating carcinomas (Basolo et al., 1993). Studied by Fontanini and Karczewska showed that mRNA of IL-6, IL-6R and gp130 was strongly correlated with overall and disease free survival in breast cancer patients (Fontanini et al., 1999). On the contrary, Purohit et al demonstrated significantly higher expression of IL-6 in breast tumor tissue as compared to the normal breast tissue of the same weight (Purohit et al., 1995). Garcia-Tunon et al showed correlation between expression pattern of IL-6 and its receptors with the anti-apoptotic genes bcl-2 and bax (Garcia-Tunon et al., 2005). They also found weak

expression of IL-6 and its receptors in patients with benign lesions. Patients with invasive malignancies showed much higher immunoreactivity for IL-6 and its receptors.

Although the reports of IL-6 expression in the breast tissue being positive or negative prognosticator of disease progression are inconclusive, association of serum levels of IL-6 with breast cancer advancement is clearly and strongly demonstrated. Yokoe et al showed that recurrent breast cancer patients who did not respond to the therapy had much higher IL-6 in their serum as compared to those who responded (Yokoe et al., 2000). Also, patients with 20 pg/ml or more serum IL-6 died within 4 months of the beginning of the treatment. Analyses by Bozcuk and Saldago et al showed association between serum IL-6 and metastases from breast cancer (Bozcuk et al., 2004). Patients with more than one metastatic sites showed higher levels of circulating IL-6 than those with single metastases. They also showed maximum average levels of IL-6 are present in patients with pleural effusions (10.65 pg/ml) followed by those with liver metastases (8.3 pg/ml). Patients with bone dominant metastases show 4.5 pg/ml of IL-6 in the serum.

Studies concerning IL-6 in this dissertation show that IL-6 is released in the media of rat mammary adenocarcinoma cells CRL1666. Bone exudates from cancer treated rats on day 12 post tumor-inoculation also show 5 fold increase in the levels of IL-6 as compared to the bone exudates collected from sham animals at the same time. 16 fold increase in circulating IL-6 was found in cancer bearing rats as compared to the controls. Our studies therefore indicate strong association of IL-6 with breast cancer disease progression.

Interleukin-6 and pain:

Pro-inflammatory mediators such as cytokines are known to enhance pain signaling by sensitizing the primary sensory neurons. Among these mediators, IL-6 is one of the key players responsible for pathophysiology of chronic pain (De Jongh et al., 2003). IL-6 is expressed by both peripheral and central nervous system (Gadient and Otten, 1996; Marz et al., 1996; Schobitz et al., 1992a; Schobitz et al., 1992b). Interleukin-6 is synthesized in more than 75% of human DRG neurons (Nordlind et al., 2000) and only a small amounts of IL-6 are found within the ventral or dorsal horns of the spinal cord (Arruda et al., 1998; DeLeo et al., 1996). Studies have indicated that peripheral nociceptors may not express IL6R but express gp130 (Opree and Kress, 2000). Some findings also suggest that IL-6R mRNA expression is elevated on postnatal day 2 and remains elevated until adulthood i.e. postnatal day 70 (Gadient and Otten, 1996). IL6R mRNA is found in the cell bodies as well as axons of the peripheral afferent neurons (Bolin et al., 1995; Gadient and Otten, 1996). Overall, these findings indicate that IL-6 can act upon neurons in both paracrine and autocrine manner.

Data from IL-6 knockout mice showed decreased nociceptive responses to mechanical and thermal stimulation as compared to wild type mice. These mice also showed reduced hyperalgesia to carrageenin (Xu et al., 1997). IL-6 knockout mice also showed decreased opioid receptors in midbrain and reduced analgesic response to stress or morphine administration suggesting that IL-6 is involved in the responses to nociceptive stimuli and

modulation of the opioid pathway (Bianchi et al., 1999). However, mice with different genetic backgrounds were used to carry out these studies and development compensatory mechanisms in absence of IL-6 cannot be overlooked. Sympathetic nervous system (ganglia and axons) also produces IL-6 (Gadient and Otten, 1996; Marz et al., 1996). It is also shown that IL-6 when administered with soluble IL-6R increased survival of sympathetic neurons and also enhanced release of neuropeptides by these neurons (Marz et al., 1998). Currently all models of IL-6 induced pain are restricted to peripheral injection of IL-6 and not systemic. Cunha et al injected IL-6 in rat hind paw and observed dose dependent mechanical hyperalgesia in both hind paws which was reduced by local pretreatment of indomethacin indicating that at the periphery IL-6 induced hyperalgesia via prostaglandin pathway (Cunha and Tamashiro, 1992). In the rat model of pancreatitis induced pain, acute inhibition of IL-6 blocked abdominal hypersensitivity within 15 minutes when administered subcutaneously or per oral but not intrathecally suggesting that the IL-6 may contribute to pancreatitis induced hypersensitivity via peripheral mechanisms (Vardanyan et al., 2010). Overall, many studies report pro-inflammatory and pro-nociceptive roles for IL-6. Intramuscular, intradermal, intracerebroventricular or intrathecal injection of IL-6 induces allodynia or hyepralgesia in animal models (Dina et al., 2008; Oka et al., 1995). While circulating IL-6 is highly associated with multiple conditions, whether it also results in pain is uncertain.

IL-6 has been identified in a large number of pathological conditions and neuropathic pain. The therapeutic approach to block IL-6 is currently restricted to using a humanized

monoclonal antibody, tocilizumab, which is proved to be therapeutically effective for rheumatoid arthritis (Maini et al., 2006; Smolen and Maini, 2006; Strand and Singh, 2010), systemic juvenile arthritis and Crohn's disease (Nishimoto, 2006). However, the mechanisms through which this monoclonal antibody works cannot be verified in preclinical models because of its humanized origin. Additionally, the use of monoclonal antibodies itself also presents adverse effects and risk of infections irrespective of IL-6 blockade. 44% of rheumatoid arthritis patients that were treated with Tocilizumab showed increased serum lipids whereas liver abnormalities were detected in 12.8% patients. Reduction in white blood cell count was also observed in 15.6% patients in these studies (Nishimoto, 2004). Morever, monoclonal antibodies are difficult to manufacture and are thus extremely expensive (Chatenoud, 2005). This calls for the need of an IL6 pathway inhibitor that can be safely administered and is efficacious for a prolonged period of time. TB-2-081 (3-O-formyl-20R, 21-epoxyresibufogenin, Mw = 428.5) is a high affinity IL-6 receptor antagonist (Enomoto et al., 2004; Kino et al., 2007) that was designed by Scripps Research Institute (La Jolla, CA). This compound has been identified in the extracts of Chinese toad skin (Kamano et al., 2002) and is an orally active small molecule (Vardanyan et al., 2010). TB-2-081 is highly lipophilic and demonstrated minimal observed toxicity in preliminary studies.

Preliminary data from our lab shows that acute oral administration of TB-2-081 reversed bone cancer-induced tactile allodynia, nerve-injury induced mechanical allodynia and thermal hyperalgesia, diabetic neuropathy induced tactile allodynia and pancreatitis

induced tactile allodynia in a dose dependent manner. Based on these preliminary findings, we have tested this drug in the rat model with tibial tumors. Our studies indicate that TB-2-081 was able to reverse cancer-induced tactile allodynia in a dose dependent manner but not ongoing pain suggesting that IL-6 activate sensory nerve fibers driving tonic painful stimuli from the tumor bearing tibia but can sensitize the primary afferent nerve fibers innervating the bone to external stimuli causing evoked pain.

As IL-6 plays a key role in neuropathic and inflammatory pain both of which are components of cancer pain, we hypothesized that blockade of IL6 signaling by a small molecular receptor antagonist will block cancer induced bone pain. The present study determined the role of IL-6 signaling blockade on cancer induced evoked, ongoing and breakthrough pain in a rat model of cancer-induced bone pain in which mammary adenocarcinoma cells were injected and sealed into the intramedullary space of the tibia of female rats to allow controlled progression of disease within the bone.

Preclinical analyses of ongoing and breakthrough cancer pain

Although ongoing pain is a common clinical complaint of patients with neuropathic pain and cancer (Campbell and Meyer, 2006; Kuzeyli Yildirim et al., 2005), it has been difficult to demonstrate in animals. As a consequence, most of the preclinical measures of pain behaviors are based on reflexes to evoked responses (Campbell and Meyer, 2006; Rice, 2008; Vierck et al., 2008) and the subsequent drug development therefore does not correlate with the analgesic efficacy of the drug (Eisenach et al., 2003). The focus of this dissertation is on the ongoing component of cancer-induced bone pain and using similar adaptations, analysis for breakthrough pain is established in the same model.

Pain has both sensory and affective components. The Affective component is characterized by unpleasantness associated with pain. This can reinforce a behavior in an animal by serving as a teaching signal (Johansen et al., 2001). Relief of pain can therefore be a negative reinforcement and hence, rewarding. Manipulations that can alleviate pain can produce conditioned place preference (CPP) in these animals.

Conditioned place preference is the most common means of measuring drug reward in laboratory animals. Although methodological differences exist among laboratories, a typical CPP experiment includes differentially pairing two distinct sets of contextual cue with the stimulus of interest such as drug or food. The contexts typically vary in flooring, color, odors etc. Conditioning involves an animal receiving repeated access to the

appetitive stimulus (unconditioned stimulus or US) in one context (conditioned stimulus or CS). Intermixed with these context-US pairings is similar exposure to the other context without US. Following conditioning is a choice test in which animals receives unrestricted access to both contexts. An increase in time spent in the paired context relative to a control value is considered as evidence that US was rewarding. The learned association between the context CS and US results in animal spending more time in that context.

Our laboratory has demonstrated that single-trial conditioning with a pain alleviating treatment is rewarding and can produce conditioned place preference in animals experiencing pain (King et al., 2009). In rats with spinal nerve ligation, administration of ω-conotoxin reversed tactile allodynia as well as produced preference for the chamber paired with it day 7 post surgery in rats with nerve injury but not in the sham operated rats. Spinal adenosine is known to block secondary hyperalegsia while having no effect on over pain. Similar results were obtained in the rat model of spinal nerve injury with no preference for the adenosine paired chamber. Descending facilitation through rostroventral medullary medulla is known mediate nerve-injury induced hypersensitivity. Its contribution in mediating ongoing pain from nerve injury was demonstrated as the injection of lidocaine into the RVM produced preference for the chamber paired with it both in SNL and SNI (spared nerve injury) operated rats. Of recent, ongoing pain is also validated in models of inflammatory pain and MIA-induced joint pain (Liu et al., 2011; Okun et al., 2011). One possible underlying mechanism is activation of VTA (ventral

tegmental area) dopamine neurons by pain relief. Substantial number of dopamine neurons project to hypothalamus and amygdala, which in turn project directly to PAG (periaquductal gray) providing a strong connection between reward and pain modulating circuitry (Yun et al., 2004). However, some reports indicate that knockout mice lacking either dopamine or serotonin transporter genes show robust cocaine and methylphenidate CPP (Sora et al., 1998), thus calling into question the widely held assumption that these monoamine transporters are critically involved in stimulating reward. Becauses CPP is only produced in animals that experience the pain but not shams, it is confirmed effect of the drug is pain relieving and does not involve intrinsic reward circuit. The importance of studying ongoing pain is heightened by the fact that the preclinical study designs will be more relevant to the human analgesic development as the studies will not only use evoked pain but also use ongoing pain to determine the analgesic efficacy of the drug.

We therefore expanded this approach to study cancer-induced ongoing pain. Female Fischer344 rats are injected with either breast cancer cells CRL1666 or cell free media in the intramedullary space of the tibia. As the tumor grows within the bone, tumor-induced bone loss and pain behaviors increase. Day 12 post-surgery is treated as a conditioning day at which pain alleviating treatment is paired with a chamber. The conditioned chambers are designed with multimodal contextual cues so that distinction can be easily made. One chamber has a striped walls, smooth surface and vanilla odor. The other chamber has grey walls, rough surface and pink lemonade odor. The middle chamber has white walls and is bright. This chamber is mainly used by rats to shuttle between the two

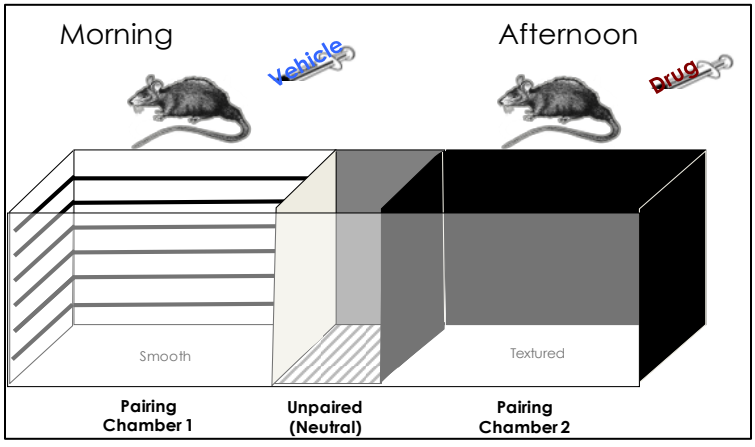
testing chambers. The typical 3 day protocol that is used to perform CPP is as follows. Of note, CPP is carried out from day 11 to day 13 post conditioning.

Day 1 (Pre-conditioning phase): time spent by animal in each chamber is recorded. No biased chamber preference should ideally be seen at this stage.

Day 2 (Conditioning phase): Vehicle is paired with one chamber in the morning and pain alleviating drug is paired with the other chamber in the afternoon

For CPA, animals receive no stimulation in the morning and are paired with a chamber. In the afternoon they receive 2 min of palpation of the cancer bearing limb. Vehicle or drug can be paired either in the morning or the afternoon as per the experimental design.

Day 3 (Testing phase): record the time spent in each chamber - no drug administration



We used this model to determine whether peripheral nerve block will induce conditioned place preference in tumor bearing rats. Previous studies have demonstrated that the rat tibia is primarily innervated by the saphenous nerve (Gajda et al., 2004; Kaan et al., 2010). To determine ongoing bone pain, we examined whether saphenous nerve block induced conditioned place preference in cancer treated rats 12 days following cancer or sham surgeries. Rats underwent baseline testing for time spent in the conditioning chambers 11 days following surgery. No pre-

conditioning differences were observed in the time spent in the chambers across treatment groups, so data were pooled for graphical representation. The following day (D12), rats underwent single trial conditioning in which saline (350 μl) was infused onto the saphenous nerve under isofluorane anesthesia in the morning. Four hours later, rats received lidocaine (4%w/v, 350 μl) onto the saphenous nerve under isofluorane anesthesia. The following day, rats were placed into the conditioning box with access to all chambers. Rats spent significantly more time in the lidocaine paired chamber indicating that cancer growth within the tibia induces ongoing pain and that cancer-induced ongoing pain is mediated by afferent input.

In addition to ongoing pain, patients with metastatic bone cancer may also experience transient episodes of severe pain on a background of otherwise well controlled pain, referred to as breakthrough pain. Breakthrough pain is characterized by severe pain that breaks through the regular pain medication. Breakthrough pain has been categorized into different types, with 2 broad categories (1) unpredictable pain, referred to as idiopathic pain, and (2) incident pain, which has an identifiable cause (Haugen et al., 2010; Mishra et al., 2009). The precipitating event for incident pain can be volitional, triggered when the patient initiates movement such as walking, or non-volitional, such as pain triggered in response to coughing. Some reports indicate that breakthrough pain can be experienced as often as 4-6 episodes within a day (Bennett, 2010; Haugen et al., 2010; Mishra et al., 2009). Precipitating factors, such as movement, can be identified in 55%-80% of the episodes, whereas no identifiable cause is reported in 27-38.3% of breakthrough pain

(Mishra et al., 2009). Although high dose rapidly acting opiates is recommended for treatment of such pain, breakthrough pain remains inadequately treated (Bennett, 2010; Haugen et al., 2010). Increasing efficacy of cancer treatments is extending the lifespan of patients with bone metastases. For this reason, the development of novel, non-opiate mechanism-based therapeutics with limited side effects across prolonged treatment would be beneficial in elevating quality of life in these patients. Limiting the development of novel therapies for breakthrough pain is limited knowledge of the mechanisms underlying breakthrough pain and how they may differ from mechanisms driving cancer-induced ongoing pain. We have developed a model of movement induced breakthrough (incident) pain in which BTP is paired with a distinct context and conditioned place avoidance (CPA) is measured.

Rats are placed into conditioned place pairing boxes and allowed to explore all chamber 11 days following injection of the CLR1666 cells into the tibia. Rats spend equivalent time in the chambers to be paired with no treatment or palpation. The following day (12 days after injection), rats undergo a single trial conditioning. Rats are placed without treatment into one of the conditioning chambers in the morning. Four hours later, rats receive 2 min palpation of the tumor-bearing limb and are immediately placed into the other pairing chamber. The following day (20 hour following the palpation pairing), rats are placed into the conditioning boxes and time spent in each chamber recorded. Cancer treated rats spend significantly less time in the palpation paired chamber whereas control rats that had cell-free media injected into the tibia spent equivalent time in the pairing

chambers. Difference scores calculated as postconditioning time – preconditioning time confirm that tumor bearing rats spent significantly decreased time in the palpation paired chamber compared to the control rats.

It is not known whether all, or some, of these mechanisms underlie movement induce BTP, or whether they play different roles in cancer-induced ongoing and movement-induced breakthrough pain. The experiments proposed in this dissertation explore these possibilities using antagonists of pronociceptive mediators such as Interleukin-6. The proposed model will delineate molecular mechanisms driving breakthrough and ongoing pain, tactile allodynia, and limb use in the model of cancer-induced bone pain. This will increase the understanding of how the neuroadaptive changes associated with cancer-induced bone pain contribute to the different aspects of the pain. These findings may lead to novel therapeutic approaches that may contribute to multiple facets of cancer-induced bone pain with the possibility of opioid sparing effects. For example, a treatment that provides consistent blockade of spontaneous pain may prove adequate for around the clock medication, leaving opioid administration for treatment of breakthrough pain. In addition, combination therapies of these compounds with chronic opioids may decrease instances of tolerance, opioid induced hyperalgesia, and side effects of prolonged opioid administration.

As listed by Brado and Bevins (Bardo and Bevins, 2000), main advantages of CPP are that it (1) tests animals in a drug free state (2) is sensitive to both reward and aversion (3)

allows for simultaneous determination of locomotor activity (4) is adaptable across a number of species (5) yields monophasic dose response curves (6) underlying neural mechanisms can be explored. The main limitations of CPP are (1) novelty seeking behaviors (2) cumbersome if dose response needs to be addressed (3) difficult to interpret if pre-conditioning bias is observed.

Nevertheless, despite these limitations, CPP provides unique information regarding the reward effect produced by the pain alleviating treatment.

The problem at hand

Bone is one of the most common sites of metastasis for frequently diagnosed malignancies such as prostate, breast and lung. Metastatic bone pain is characterized by multiple pain descriptors comprising of ongoing pain i.e. pain at rest, evoked pain such as movement-induced breakthrough pain and hypersensitivity to heat, cold and mechanical stimuli. Pain is the most disruptive symptom of bone metastases that significantly impairs daily activities. The quality of life in these patients is further compromised due to under-managed pain control and treatment related side effects. Inadequate relief of pain is partly due to lack of understanding of molecular mechanisms that initiate and maintain cancer-induced bone pain. A better understanding of neuroadaptive changes that mediate cancer-induced ongoing and evoked pain states such as breakthrough pain, and how they may differ from one another is required for development of more effective therapeutic options for these pain states. This dissertation explores the specific contribution of promising molecular targets, such as p38 MAPK and interleukin-6, that to play a central role in tumorigenesis, bone remodeling and pain, in tumor-induced evoked and ongoing pain. We used preclinical animal models of cancer-induced bone pain in which breast cancer cells are injected and sealed into the bones of immunocompetent mice or rats. In these models, ongoing and evoked pain behaviors develop with disease progression as is described in patients with bone metastases.

Overall hypothesis and specific aims

Hypothesis

We tested the hypothesis that p38 MAPK and IL-6 signaling play differential roles in cancer-induced ongoing as opposed to evoked pain.

Specific aims

1. To determine the role of signaling molecule p38 MAPK in driving cancer-induced bone pain in a mouse model of breast cancer-induced bone pain.
2. To determine the role of the pro-inflammatory cytokine, Interleukin-6, in driving cancer-induced bone pain in a rat model of breast cancer-induced bone pain.

CHAPTER III

MATERIALS AND METHODS

Animal models

Female adult Balb/cfC3H mice, weighing 20–25 g (Jackson Laboratories, Bar Harbor, ME), were chosen for histocompatability with the murine 66.1 cells. Balb/c mice were used for the p38 MAPK studies. Fischer344 rats, weighing 150-175 g (Harlan) were chosen for histocompatibility with the breast cancer cell line CRL1666. Fischer rats were used for conditioned place preference, conditioned place avoidance and Interleukin-6 studies. All animals used in these studies were housed maximum three per cage and used in strict accordance with the NIH Health Guide for the Care and Use of Laboratory Animals and the University of Arizona Animal Care Unit.

Cell lines

Murine cell line 66.1 derived from spontaneously occurring mammary adenocarcinoma was maintained in advanced Minimum Essesntial Medium, Eagle (αMEM) (Cellgro Mediatech) containing 10% Fetal bovine serum (Gemini Bioproducts), 100 units/mL penicillin, and 100 units/mL streptomycin at 37^{o} C and in a 5% CO_2 atmosphere. The cells were passaged every 3 days, and harvested between 12 and 21 passages.

Rat cell line CRL1666 (ATCC) derived form the mammary adenocarcinoma was maintained in McCoy's media (ATCC) containing 10% fetal bovine serum (Gemini Bioproducts),100 units/mL penicillin, and 100 units/mL streptomycin at 37^{o} C and in a

5% CO_2 atmosphere. The cells were passaged every 2 days, and harvested between 2 and 12 passages. Each rat is injected with 1 x 10^5 cells/5µl.

Surgeries

Injection of 66.1 cells in to the intradmedullary space of the femur in Balb/c mice:

Baseline radiograph images (Faxitron X-ray Corporation) of the right femurs were obtained prior to surgery. Animals were anesthetized with ketamine (80 mg/kg)/xylazine (12 mg/kg) i.p. and an arthrotomy was performed exposing the condyles of the distal femur as previously described (King et al., 2007). A hole was drilled into the femur for injecting the needle. Needle placement inside the intramedullary space of the femur was verified using radiograph images and 5 µl of serum-free minimal essential medium (MEM) containing approximately 5x10^5 cells was injected into the intramedullary space of the right femur. For cell-free controls, 5 µl of serum-free MEM alone (no cells) was injected and sealed into the femur. The injection site was sealed with dental cement (Simplex).

Injection of CRL1666 cells in to the intramedullary space of the tibia in Fischer rats: Baseline radiograph images (Faxitron X-ray Corporation) of the right tibiae were obtained prior to surgery. Animals were anesthetized with ketamine HCL /xylazine (1ml/kg). A 1 cm rostro-caudal incision was made in the skin over the femoral-tibial joint and patellar tendon was exposed. A hole was drilled into the tibia gently under the tendon without causing damage to nearby muscles. The needle was inserted into the

intramedullary canal of the tibia. Needle placement is verified using radiograph images taken on two planes. Each rat was injected with 1 x 10^5 cells/5 μl. Cell free control rats received 5 μl of serum-free McCoy's media alone. The hole was then sealed with dental cement (Simplex) and the site of exposure was sutured with surgery silk (Ethicon). The wound was then flushed with an antibiotic (Gentamicin) and skin incision was sealed with surgery staples. All rats then received an injection of antibiotic gentamicin (4.4 mh/kg s.c.).

Determination of bone destruction

Animals were lightly anesthetized (ketamine 80 mg/kg/xylazine 12 mg/kg i.p. for mice and ketamine 1 mg/ml i.p. for rats) and images of femurs (mice) or tibiae (rats) were captured on the computer using Specimen DR. Mouse radiographs were taken at 26kV with 10 seconds exposure whereas rat radiographs were taken at 35kV with 3 seconds of exposure. Bone destruction was determined on days 7, 10 and 14 post-surgery for mice and days 6, 10 and 13 post-surgery for rats. Bone rating scale: (a) Balb/c mice injected with 66.1 cells: 5=normal bone, 4=epiphyseal bone loss, 3=enhanced bone loss within the shaft, 2=emergence of osteoblastic growth, 1=unicortical fracture, 0=bicortical fracture. (b) Fischer344 rats injected with CRL1666 cells: 4=normal bone, 3=epiphyseal bone loss, 2=enhanced both loss within the shaft, 1=cortical fracture, 0=fibular bone loss.

Drug Administration

SB203580

SB203580-hydrochloride (Tocris # 1402), a selective p38 MAPK inhibitor, was dissolved in saline.. To determine the acute effects of SB203580, the drug was administered (15 mg/kg or 30 mg/kg, i.p.) 13 days following the injection of 66.1 cells into the femur. To determine the chronic effects of the drug, it was administered at two doses (15 mg/kg or 30 mg/kg, i.p. 2x daily across 7 days starting day 7 post surgery). The animals were divided into four groups: Cancer-SB203580, Cancer-saline, Control-SB203580 and control-saline with 10-12 animals in each group.)

Lidocaine

Peripheral nerve blocks were provided by administering 350 μL lidocaine (4% w/v) at day 12 post-surgery. Separate groups of both cancer and control animals received 4% lidocaine or saline (vehicle) over the saphenous nerve or into the popliteal fossa. Each group had 6-12 animals.

TB-2-081

TB-2-081 is an Interleukin-6 receptor antagonist generously provided by Kenner Rice, NIH.. TB-2-081 was prepared by dissolving in 50% ethanol and 500 μL was administered subcutaneously into the scuffs on the rats.10 mg/kg dose of the IL-6 antagonist was used to determine whether blocking IL-6 signaling effectively blocks cancer-induced ongoing bone pain.

Behavioral analyses for evoked and spontaneous pain

Tactile allodynia

Paw withdrawal thresholds in response to probing with calibrated von Frey filaments were determined using the "up-down" method described by Chaplan et al (Chaplan et al., 1994). Animals 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 2 g for mice and 15 g for rats 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 responses were measured as paw withdrawal thresholds using Dixon non-parametric test.

Limb Use

Limb use was assessed as previously described (Luger et al., 2001). The rat or 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.

Flinching and Guarding

Flinching and guarding behaviors were only determined in the mouse model as pilot studies indicate that the cancer treated rats do not display these behaviors. Mice were placed in suspended plexiglass chambers with a wire grid floor and allowed to acclimate to the chamber for 1 h. Guarding and flinching behaviors were measured during a 2 min observation period. The number of flinching episodes and the total time spent guarding the cancer-bearing limb were measured across 2 minutes for each mouse.

Experimental Design for p38 MAPK studies

Each mouse was tested for first movement-evoked pain (limb use), spontaneous pain behaviors (flinching and guarding), and tactile allodynia prior to surgery (pre-sugery baselines). To determine the pain behavior following the injection of breast cancer cells into the femur, mice were tested on days 7, 9, 11 and 13 post-surgery. Acute effects of SB203580 were tested on day 13 post surgery. Pre-drug values were obtained for all groups, followed by drug administration. Behaviors were again assessed at 30, 60, 90, 120 and 180 min following drug administration. All behaviors were assessed within the same animal. Each animal was first tested for flinching and guarding followed by tactile allodynia.

Chronic effects of SB203580 administration was tested following surgery on days 7 (pre-drug), 9, 11 and 13. The behavioral tests were carried out 3 hrs following the morning administration, time points later than observed peak effect of acute drug administration. Mice were first tested for limb use by allowing them to walk in an empty mouse pan and then placed in testing chambers for assessment of spontaneous pain and tactile allodynia.

All pain measures were conducted by the experimenter who was blinded to the treatments.

Experimental design for the studies in rat model

Rats were handled by the experimenter for 4-5 days prior to testing to alleviate stress. Each rat was tested for movement-evoked pain (limb use) and tactile allodynia prior to surgery (pre-sugery baselines). To determine the pain behavior following the injection of breast cancer cells into the tibia, rats were tested for evoked pain and limb use on days 6, 11 and 13 post-surgery. Rats were tested for evoked pain on day 12 post surgery following peripheral nerve block on both ipsilateral and contralateral limbs. Ongoing pain was determined using the conditioned place preference model with conditioning day occurring 12 days after injection of cancer cells into the tibia. Palpation-induced breakthough pain was determined using the conditioned place aversion with conditioning day occurring 12 days after injection of cancer cells into the tibia.

Acute effects of TB-2-081 were tested on day 13 post surgery. Pre-drug values of tactile allodynia were obtained for all groups, followed by drug administration. Tactile allodynia was again assessed at 15, 30, 45, 60 and 90 min following drug administration. This was conducted by the experimenter who was blinded to the treatments.

To determine the effects of acute systemic administration of the IL-6 antagonist (10 mg/kg) on cancer induced ongoing bone pain, effects of IL-6 antagonist in blocking CPP to saphenous nerve block administered 15 min later were examined. This timepoint was

chosen since it corresponded to the peak anti-allodynia effects of systemic administration of the IL-6 receptor antagonist.

Conditioned place preference (CPP)

CPP was performed as a single trial conditioning protocol from day 11 to day 13 post-surgery, modified from the protocol previously described by King et al (King et al., 2009). Both control and cancer rats underwent one-day pre-conditioning period (d1) at which time spent by each rat in striped vs. grey walled chambers was recorded for 15 min as a baseline score using ANY-maze tracking software (Stoelting). On conditioning day (d2), rats were lightly anesthestized under isofluorane, received saline over the saphenous nerve and were paired with a chamber 2 minutes following injection for 30 min in the morning. Four hours later, they were again lightly anesthetized under isofluorane, received 4% lidocaine over the saphenous nerve or into popliteal fossa and were placed into the opposite chamber 2 min following injection for 30 min. Of note, all rats awoke within 2 min of placement into the conditioning chamber. Chamber pairings were counterbalanced. On the test day (d3), rats were once again placed in the conditioning box with open access to all chambers and time spent in striped vs. grey chamber was recorded and determined using the Anymaze software. Preference for the drug-paired chamber was determined as baseline scores subtracted from the test scores (test minus pre-conditioning). Preference is indicated by a positive score.

To determine if acute systemic administration of IL-6 antagonist blocks saphenous nerve block-induced relief from ongoing pain, on the conditioning day all rats received vehicle (50% ethanol) subcutaneously in the morning followed 15 min later by administration of 350μL saline to the saphenous nerve. Four hours later, the first group of rats received TB-2-081 (10mg/kg, s.c.) and second group of rats received the vehicle. Sll rats received saphenous nerve block with 350μL of 4% lidocaine 15 min later.. N=10-12 rats per group.

Conditioned place avoidance (CPA)

Both cancer and control rats underwent one day pre-conditioning period (d1) at which time spent by each rat in striped vs. grey walled chambers was recorded for 15 min as above. On conditioning day (d2), rats were paired with an enclosed chamber (striped or grey) for 30 min without any treatment to avoid accidental palpation of the limb. Four hours later, they underwent 2 min palpation of the treated limb and placed in the opposite chamber within 2 min following the palpations for 30 min. Of note, palpations were done in a room across the hallway from the conditioning chambers to prevent exposure to signs of distress (e.g. vocalizations) during palpation from other rats while in the conditioning chamber. On the test day (d3), rats were once again placed in the CPP box with open access to all chambers and time spent in striped vs. grey chamber was recorded as a test score. Avoidance of the palpation-paired chamber was determined as the preconditioning scores subtracted from test scores (test minus pre-conditioning). A negative score indicates aversion.

To determine the role of afferent drive in maintaining palpation-induced breakthrough pain, separate groups of rats received saphenous nerve block immediately or 10 min following palpation. This nerve block was done in the absence of isofluorane anesthesia as it was determined that isofluorane anesthesia eliminated palpation-induced aversion in the positive control rats (rats that received saline infusion of the saphenous nerve).

In vitro analysis of mouse breast cancer cell growth

The 66.1 cells were maintained in MEM media as mentioned above. To evaluate the effect of SB203580 on tumor cell growth, 66.1 cells were treated with 10 or 20μM of SB203580 dissolved in 4% DMSO or with 4% DMSO alone to serve as controls. Cells were seeded at 15,000 cells/ well in 6-well plates in complete media for 36 h. When cells reached the confluency of 30-50%, they were serum starved overnight with serum free optiMEM media. After 48 h of drug treatment, cell growth was arrested by slowly adding 50% Tri-chloro acetate (TCA) and the cells were kept at 4^{o} C for a minimum of 1 h. All cells were then washed with distilled water 4-5 times and then treated with 1 ml of sulphorhodomine-B (SRB) dye for 10-15 min at room temperature. The cells were again washed with distilled water 4-5 times. They were then precipitated in 0.1% acetic acid on a shaker for 10 min. The absorbance was read at 540 nm. For data analysis, the mean OD_{540} (n = 3) in the SB203580-treated cells is expressed as a percent of that in the control done in parallel. Normalized data from three independent experiments are expressed as means ± SEM.

Determination of Interleukin-6 in sera, bone exudate and cancer cells

Sample collection: (A) Bone exudates*:* On day 12 post-surgery, rats were sacrificed in a CO_2 chamber. Immediately following that, the skin covering the treated limb was opened and the entire tibia was isolated and snipped at both proximal and distal ends. The marrow was then flushed into a microcentrifuge tube with 1 mL of protease inhibitor (1:1000 dilution with 1x PBS). Samples were snap frozen in liquid nitrogen and stored at -80C. (B) Cell media: 25,000 cells were plated in a 10 mL cell culture flask with McCoy's media. 24 hours later, 1 mL media was collected from each flask and snap frozen. Media was stored at -80C. (c) Serum: At day 12 post-surgery, 2 ml of blood was collected from the left ventricles of deeply anesthetized rats into microcentrifuge tubes. Blood samples were allowed to clot for 2 hours at room temperature and centrifuged at 2000 rcf for 30 min. Serum (supernatant) was removed and aliquoted into fresh tubes. Sera were stored at -30C.

Enzyme Linked Immunosorbent Assay (ELISA): The purified interleukin-6 antibody (Invitrogen # CRC0063 anti-rat IL-6) diluted in wash buffer (1 ug/mL) was coated overnight at 4C (100 µL/well) on a 96-well microtiter plate (NUNC # 434797). The following day, plates were washed (3X wash buffer) and then blocked with assay buffer (300 µL/well) at room temperature for one hour. Excess liquid was then aspirated and the standard recombinant rat IL-6 (Invitrogen # CRC0063; serial dilution in assay buffer from 7.8125 to 1000 pg/mL) as well samples (bone exudates, cell media and sera from cancer and control rats) were added to the plate (100 µL//well). Immediately after this step, 50 µL of detection antibody, anti-rat IL-6 Biotin (Invitrogen # CRC0063) diluted in

assay buffer (0.1 μg/mL), was added to each well and the plate was incubated on a shaker (700 rpm) for 3 hours at room temperature. Plates were washed (5X wash buffer) and 100 μL of streptavidin-HRP solution (Invitrogen # CRC0063; diluted 1/1000 in assay buffer) was added to each well and the plate was incubated on a shaker (700 rpm) for 30 min at room temperature. Wells were washed (5X wash buffer) and 100 μL of TMB substrate (Invitrogen) was added to each well. The plate was then incubated on a shaker (700 rpm) at room temperature for 30 min. 100 μL of Stop solution (1N H_2SO_4) was added to each well and as soon as the color turned yellow, the plate was read on a plate reader (MultiSkan Ascent, Thermo laboratories) at absorbance 450 nm.

Statistical analyses

Statistical comparisons between treatment groups were done using ANOVA. Pairwise comparisons were made with Student's *t*-test, multiple comparisons between groups were done using Bonferroni posthoc test. For the rating assays, limb use and bone loss, non-parametric statistical comparisons were made with the Mann–Whitney test. For all analysis, significance was set at $p < 0.05$.

CHAPTER III

ROLE OF p38 MAPK IN CANCER-INDUCED BONE PAIN AND DISEASE PROGRESSION

Implantation of breast cancer cells 66.1 into the intramedullary space of the femur induces pain behaviors in mice.

Injection of the mouse breast cancer cells into the femur produced a time-dependent expression of both spontaneous and evoked pain behaviors (Fig 1). Cancer treated mice demonstrated flinching behavior within 7 days post-surgery, with flinching behavior increasing through the day 14 time-point. (Fig1A). Cancer treated mice showed significant guarding behavior within 7 days post surgery, which increased through day 13. (Fig1B). Cancer treated rats showed robust tactile hypersensitivity by the day 7 time-point, with paw withdrawal thresholds remaining low throughout the testing period. (Fig 1C). Control mice showed slightly lowered withdrawal thresholds at day 7 post-surgery, with withdrawal thresholds returning to baseline by the day 9 time-point. Both cancer-treated and control mice showed impaired limb use on day 7 following the surgery (Fig 1D). Cancer treated mice showed greater impaired limb use than control mice, demonstrating both limping and guarding behaviors whereas control rats only showed limping behaviors, likely due to surgery induced pain. Impaired limb use was not observed at later time points in control mice whereas cancer treated mice continued to guard and/or limp through day 14 post-surgery (Fig1D).

Implantation of breast cancer cells 66.1 into the intramedullary space of femur induces bone remodeling and tumor growth.

Femur radiographs showed a time-dependent change in bone remodeling on days 7, 10, and 13 after the cancer cells were injected (Fig 2A). In comparison with the normal bone (Fig 2A, panel a), cancer-induced bone remodeling was first evident on day 7 post-surgery (Fig 2A, panel b) with bone loss at the proximal end of the bone (knee) at the epiphyseal plate region. Bone loss increased and was extended to the shaft by 10 (Fig 2A, panel c). On day 13 post-surgery, in addition to the bone loss, abnormal bone growth was observed within the shaft (Fig 2A, panel d). Histological analyses of the bones show a time-dependent growth of the tumor within the bone. Bones injected with cell-free media showed normal marrow and continuous cortical bone (Fig 2B, panels a,b). Bones collected on day 3 post-surgery showed the presence of tumor mass within the shaft at the site of injection (Fig 2B, panels c,d). By day 7, tumor burden increased and filled the bone shaft replacing the marrow. In addition, cortical invasion by the tumor was also observed (Fig 2B, panels e,f). On day 13, abnormal osteoblastic bone growth was seen in the shaft as well as growing outside of the cortical bone (Fig 2B, panels g,h).

Acute administration of SB203580 attenuates breast cancer induced spontaneous pain

Pre-drug testing demonstrated that all cancer treated mice showed significant flinching and guarding behaviors 13 days post-surgery whereas control mice did not show any flinching or guarding behavior (Fig 3A-D). Administration of 15 mg/kg or 30 mg/kg of

the drug blocked flinching episodes (Fig 3A,B) as well as time spent guarding (Fig 3C,D), with the peak drug observed 60 min post administration. Flinching and guarding behaviors returned to pre-drug levels by 180 min post administration. Acute systemic administration of p38 MAPK inhibitor decreased flinching in a dose-dependent manner (Fig 3B). Time spent guarding the cancer bearing limb was significantly reduced in mice that received the highest dose (30 mg/kg) of the drug (Fig 3D). Administration of saline vehicle did not alter flinching or guarding in the cancer treated mice.

Acute administration of SB203580 does not block breast cancer induced evoked pain

Pre-drug testing demonstrated that cancer treated mice had decreased response thresholds to von Frey filaments (Fig 3 E,F). Acute administration of either dose (15 or 30 mg/kg, i.p.) of the SB203580 failed to diminish tactile allodynia of cancer bearing limb (Fig 3E,F). Paw withdrawal thresholds of mice that received the vehicle did not alter from the pre-drug threshold. Paw withdrawal thresholds of the control mice remained unchanged after the drug administration.

Chronic administration of SB203580 attenuates breast cancer induced spontaneous pain

Prior to drug treatment (D7), all mice showed spontaneous pain behaviors as compared to the controls (Fig 4 A,C). Administration of SB203580 (15 and 30 mg/kg) diminished cancer-induced flinching episodes by D11 post-surgery as compared to those that

received vehicle (Fig 4A). By D13, flinching was significantly attenuated in cancer bearing mice treated with the drug (15 mg/kg and 30 mg/kg) whereas it was elevated in those that received vehicle (Fig 4A,B). Cancer bearing mice that received the drug showed reduced time spent guarding the cancer bearing limb by D9 post-surgery (Fig 4C). Guarding remained significantly lowered throughout the time course in mice that received the drug as compared to those that received the vehicle (Fig 4C,D). Control mice showed minimal flinching and no guarding irrespective of drug treatment on all the test days. Control or cancer treated mice did not show any adverse drug-related effects on prolonged administration of SB203580.

Chronic administration of SB203580 does not alter breast cancer induced evoked pain

Prior to drug treatment (Day 7), cancer treated mice demonstrated tactile allodynia, as indicated by lowered paw withdrawal thresholds to von Frey filaments. Prolonged treatment with SB203580 (15 and 30 mg/kg) did not alter cancer-induced tactile allodynia across the entire testing period ($p>0.05$, Fig 4E,F). Paw withdrawal latencies remained consistent in control mice throughout the testing period irrespective of drug treatment.

Chronic administration of SB203580 attenuates breast cancer induced impaired limb use.

Cancer bearing mice showed impaired limb use by day 7 post surgery as compared to the control mice which showed slight limping behaviors while walking (Fig 4G). By day 13 post surgery, cancer treated mice that received vehicle or 15 mg/kg of SB203580 continued to show impaired limb use (Fig 4G,H) whereas significant improvement in the limb use rating was observed by day 13 post surgery in mice that received 30 mg/kg of SB203580 (Fig 4d). Control mice regained the normal limb use by day 9 and maintained throughout the testing period irrespective of drug treatment.

Chronic administration of SB203580 prevents and/or delays breast cancer induced bone remodeling

Mice that received the vehicle demonstrated significant bone loss at the epiphyseal region extending to the shaft of the bone. Abnormal osteoblastic bone growth was also observed at the epiphyseal plate and shaft (Fig 5A). In mice that received 15 mg/kg or 30 mg/kg of the drug, bone loss at both the regions was greatly reduced, with no evidence of abnormal bone growth (Fig 5A). Most vehicle treated rats show uni- or bicortical fractures in addition to the abnormal osteoblastic growth on day 12 post-surgery. However, osteoblastic structures are completely eliminated in animals receiving the drug and extent of bone loss is greatly reduced (Fig 5B). In addition, prolonged SB203580 treatment reduced incidence of cortical fractures in a dose dependent manner, with only 30% (15 mg/kg) and 15% (30 mg/kg) of the cancer treated mice showing cortical fractures compared to 50% of the vehicle treated mice (Fig 5C). This indicates that prolonged

inhibition of p38 MAPK diminished bone remodeling. Control mice did not show any abnormal bone remodeling upon drug treatment.

Chronic administration of SB203580 reduces tumor growth within the intramedullary space.

Bones collected from control mice showed completely normal cortical bone and marrow components. Cancer treated mice treated with vehicle showed complete infiltration of the marrow by the growing tumor. Consistency of cortical bone was lost with multiple fractures and abnormal osteoblastic growth throughout the shaft induced by the growing tumor (Fig 6b and c). In cancer treated mice that received 15 mg/kg of the drug, tumor growth was confined to the shaft in the middle of the intramedullary space where the breast cancer cells were initially implanted. No tumor was evident around the ephiphyseal plate indicating the failure of the cancer cells to migrate with SB203580 treatment (Fig 6d and e). In mice that received 30 mg/kg of the drug, only a few tumor pockets were observed within the intramedullary space. Additionally normal marrow components were still present within the bone (Fig 6f and g). This shows that prolonged treatment with SB203580 significantly reduced tumor burden within the bone.

SB203580 reduces breast cancer cell viability in vitro

To determine the effects of p38 MAPK inhibition on 66.1 cell growth, an in vitro sulphorhodomine-B assay was carried out. On treatment with SB203580 for 2 days at 20μM concentration, number of viable cells significantly reduced indicating that p38

MAPK is an important determinant of the growth of 66.1 cells and its blockade results into decreased cell number (Fig 6B).

Figure 1

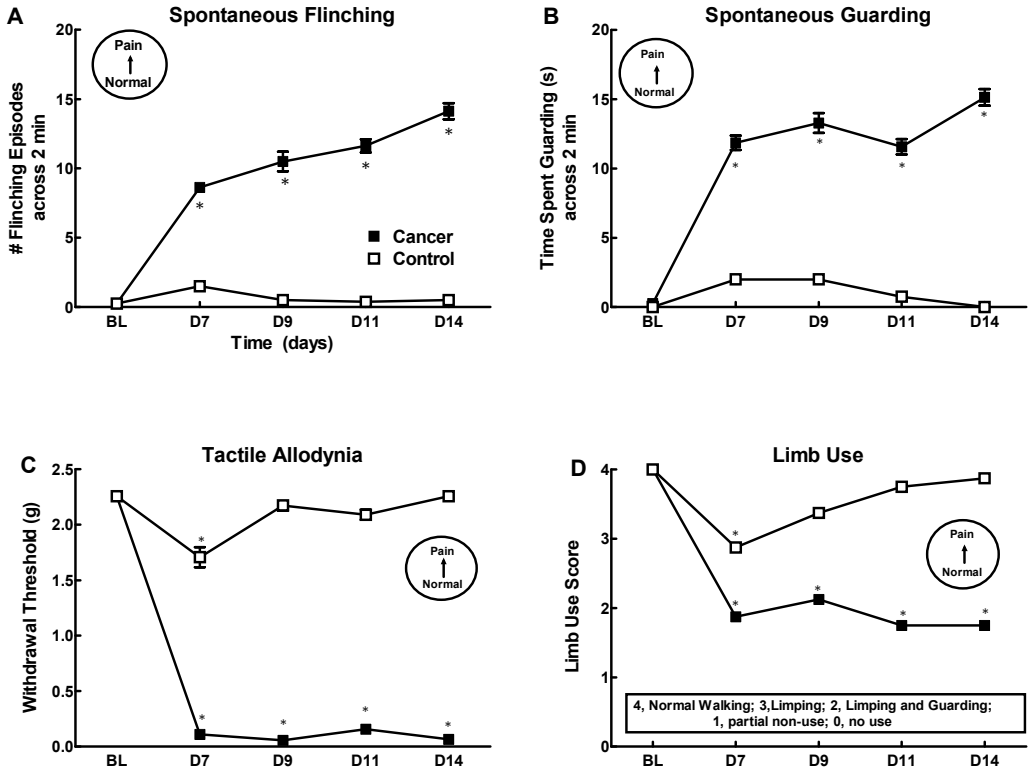


Figure 1. Time course for emergence of pain behaviors showing spontaneous flinching (A), spontaneous guarding (B), tactile allodynia (C) and limb use score (D). n=8 mice/group.

Figure 2

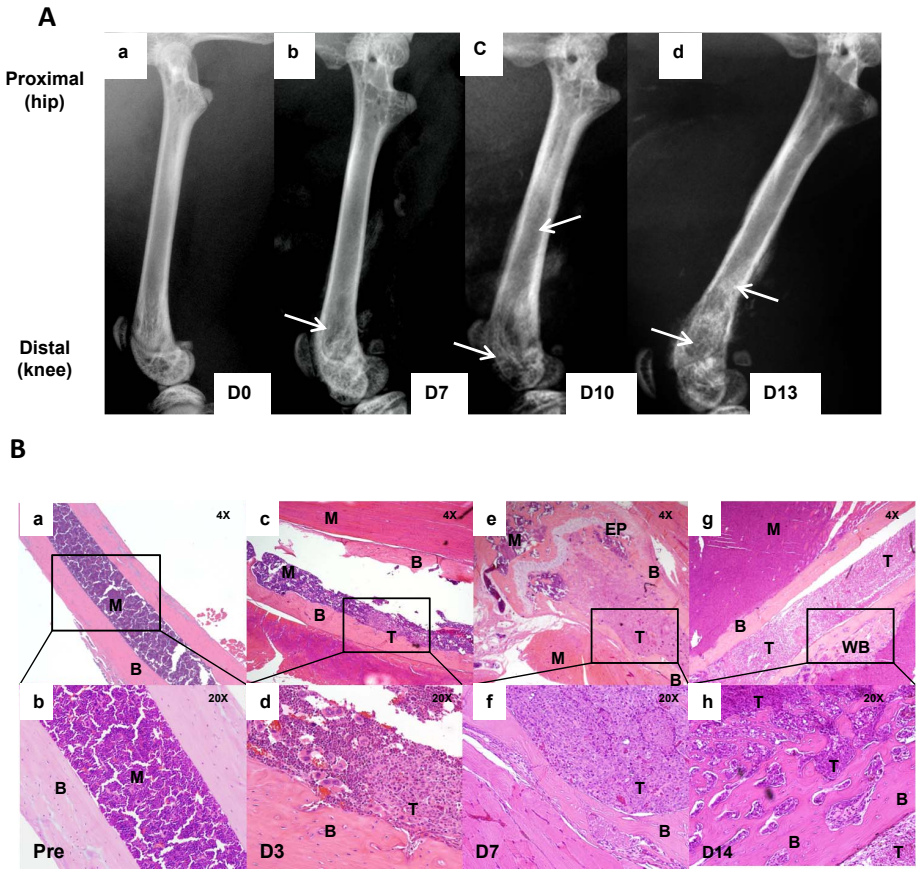


Figure 2. Breast cancer cells when injected in the intramedullary space of the femur result into disease progression and tumor–induced bone remodeling (A). Radiographic images show normal bone with no bone destruction (a), D7 post tumor implantation with little bone loss (osteolytic remodeling; indicated by yellow arrows) observed in the shaft (b), D10 post tumor implantation with increased bone loss in the shaft and knee area (c), D13 post tumor implantation with further increased bone loss in the shaft and the knee region, cortical fractures (indicated by blue arrows) and abnormal bone growth in the shaft (osteoblastic remodeling, indicated by white arrows) (d), Histological analyses of bones (B) show control bone with intact cortical bone and marrow components (a, b), presence of tumor in the intramedullary space D3 post implantation (c, d), tumor invading into cortical bone and growing towards the epiphyseal plate of the knee by D7 post implantation (e,f), tumor infiltrating the entire intramedullary space of the bone with woven bone formation along the cortex by D13(g,h); B, cortical bone; M, bone marrow; T, tumor; EP, epiphyseal plate; WB, woven bone.

Figure 3

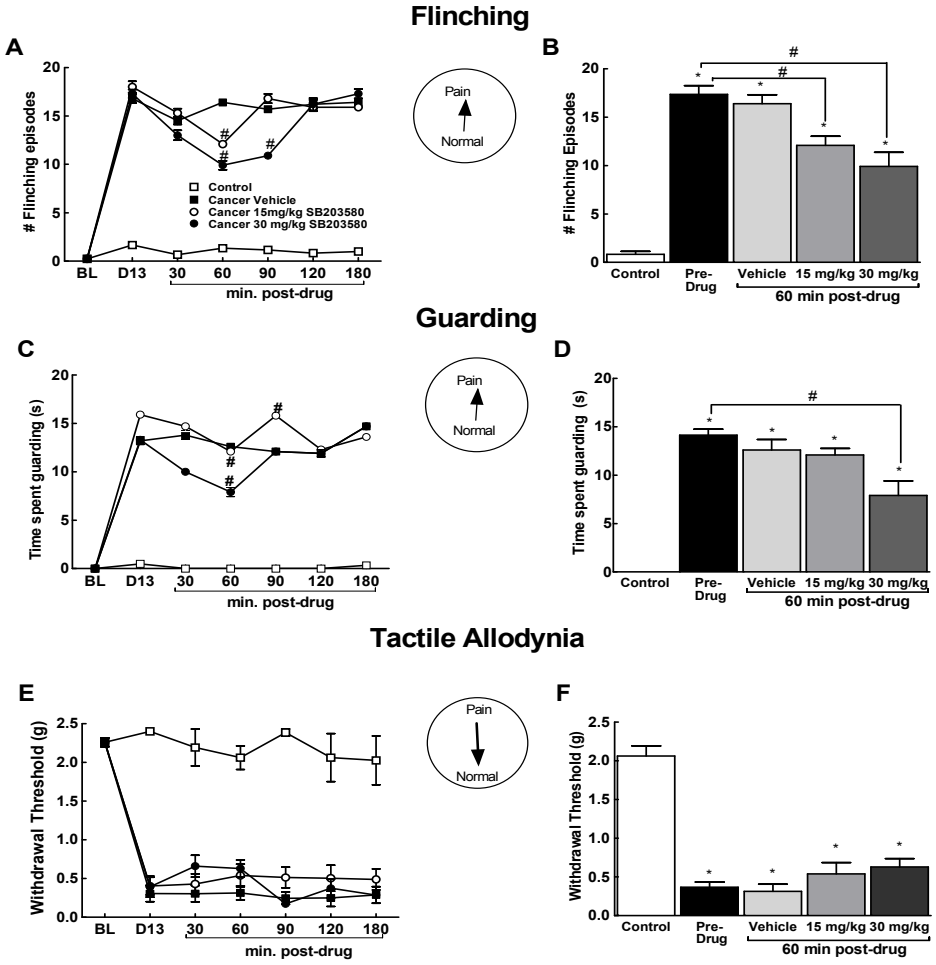


Figure 3. Effects of acute administration of SB203580 (15 and 30 mg/kg, i.p.) at day 13 at different time points on cancer-induced spontaneous flinching (A, B), spontaneous guarding (C,D) and tactile allodynia (E,F) All graphs show means ± SEM. *Indicates significant difference from the control group ($p < 0.05$). # show significant difference from pre-drug levels ($p < 0.05$). n=10

Figure 4

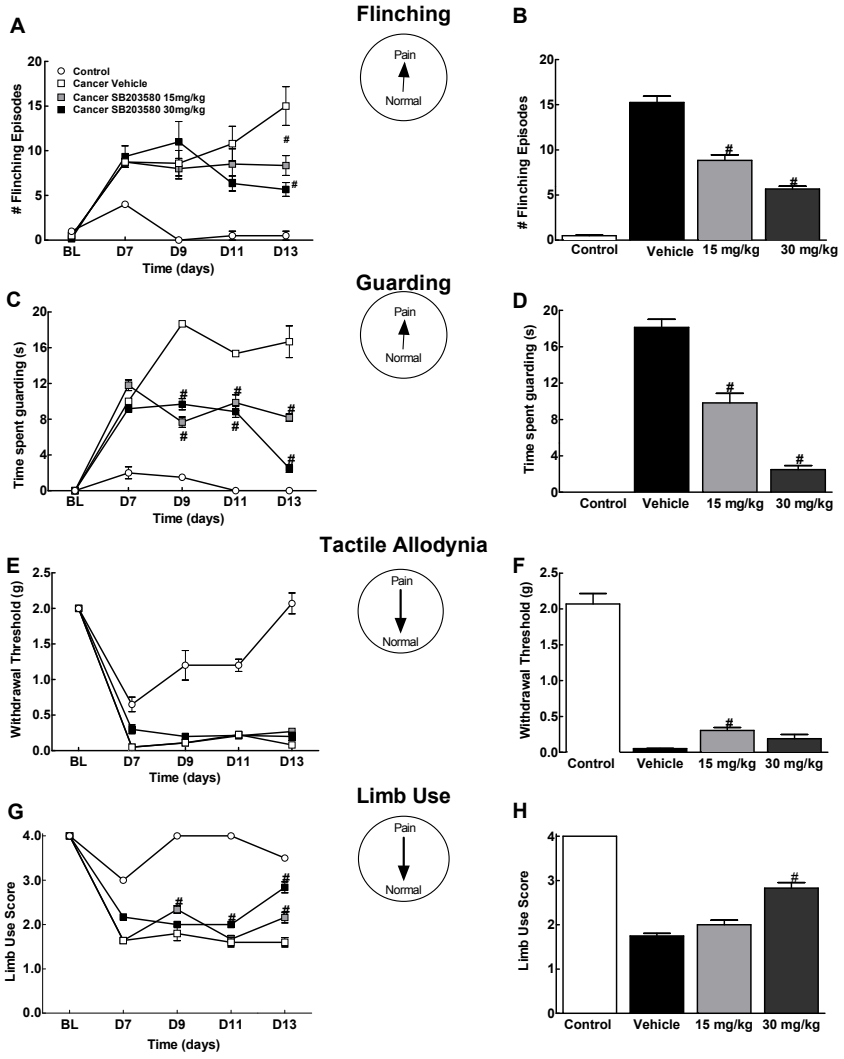


Figure 4. Effects of chronic administration of SB203580 (15 and 30 mg/kg, i.p., 2X daily) on cancer-induced spontaneous flinching (A,B) and spontaneous guarding (C,D). tactile allodynia (E,F) and limb use (G,H). All graphs show means ± SEM. # indicates significant difference from cancer vehicle group ($p < 0.05$). n=6-8

Figure 5

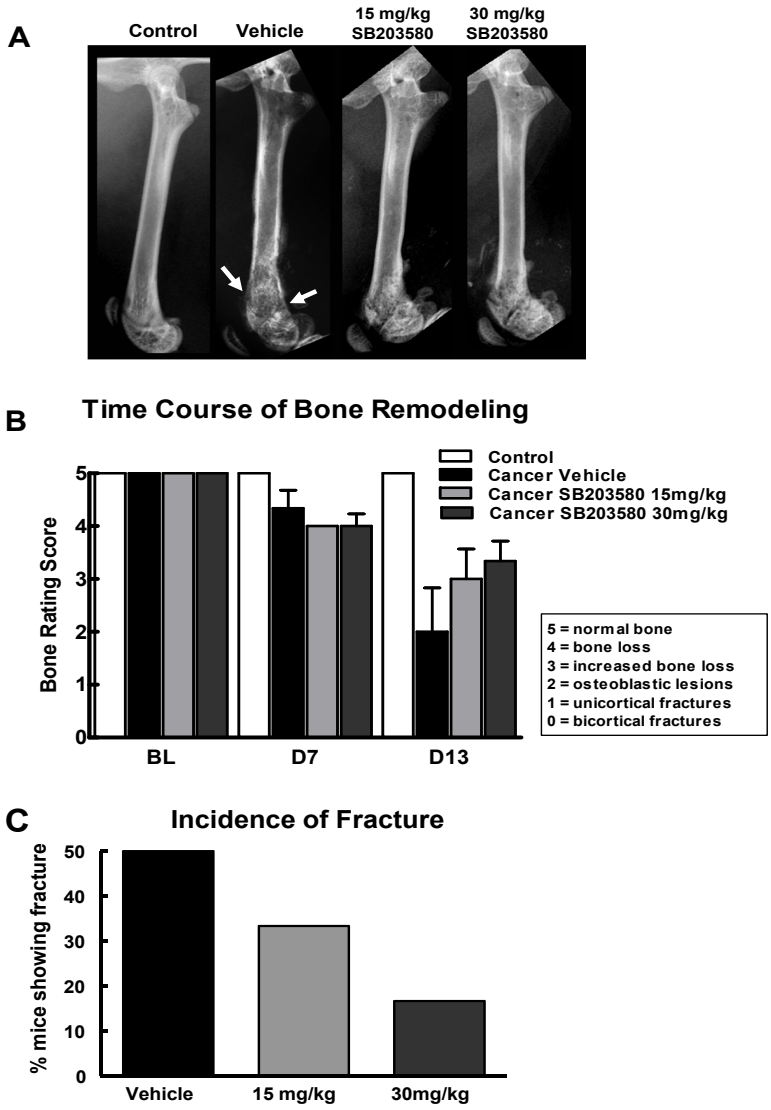
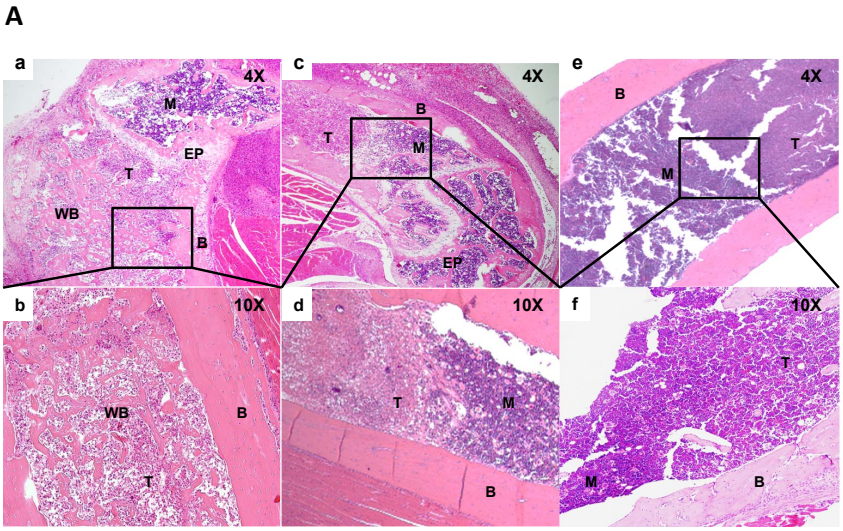


Figure 5. (A) Radiograph images of the injected femur on day 13. Breast cancer-induced bone remodeling is reduced in mice that received systemic administration of SB203580 (15 and 30 mg/kg, i.p., 2x daily, 7 days). Fractures as indicated by arrows were defined as full-thickness cortical loss. (B) Bone remodeling ratings of all treatment groups show that the breast cancer-induced bone remodeling is observed 7 days following the surgery, with no difference between drug- and vehicle-treated mice. Breast cancer-treated mice receiving

SB203580 (15 and 30 mg/kg, i.p. 2x daily, 7 days) demonstrated reduced bone destruction on day 13 compared with breast cancer-treated mice that received saline. (C) Treatment with SB203580 reduced the percentage of mice with breast cancer-induced spontaneous fractures compared to vehicle-treated animals. n=6-8

Figure 6.



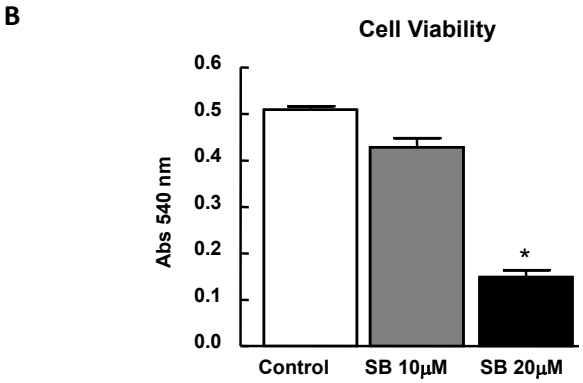


Figure 6. (A) Representative images of H&E staining showing control and cancer treated bones. Breast cancer cells filled the intramedullary space of the femur within 13 days following injection. In addition, areas of new bone growth were evident within the intramedullary space in the breast cancer-treated mice treated with vehicle. The cortical bone displayed an uneven look showing tumor invasion into the bone. Systemic delivery of the p38 MAPK inhibitor (15 and 30 mg/kg, i.p., 2x daily) across 7 days diminished tumor burden, blocked the new bone growth within the intramedullary space and reduced the invasion of breast cancer cells into the cortical bone. (B) Sulforhodomine B (SRB) assay shows dose dependent

reduction in cell viability on treatment of breast cancer cells with SB203580 for 48 h as compared to the breast cancer cells treated with vehicle alone. Graph shows means ± SEM. *Indicates significant difference from DMSO-treated group ($p < 0.05$).

CHAPTER IV

RAT MODEL OF BREAST CANCER-INDUCED BONE PAIN, INCLUDING REFERRED PAIN, ONGOING PAIN AND BREAKTHROUGH PAIN

Implantation of breast cancer cell into the tibia induces pain behaviors in rats

Injection of the rat breast cancer cells into the tibia produced a time-dependent expression of pain behaviors (Fig 7). Cancer treated rats showed tactile hypersensitivity by the day 6 time-point, with paw withdrawal thresholds lowering further throughout the testing period (Fig 7A). Control rats showed slightly lowered withdrawal thresholds at day 6 post-surgery, with withdrawal thresholds returning to baseline by the day 11 time-point. Cancer-treated rats showed impaired limb use on day 6 following the surgery (Fig 7B). Cancer treated rats showed greater impairment of limb use with time progression demonstrating both limping and guarding behaviors whereas control rats did not show impairment at any time after the surgery (Fig 7B).

Implantation of breast cancer cells into the tibia induces bone remodeling

Tibia radiographs showed a time-dependent change in bone remodeling appearing on day 10 after the cancer cells were injected in comparison with the normal bone (Fig 8). On day 10, bone loss at the proximal end of the bone (knee) at the epiphyseal plate region was evident. Bone loss increased and was extended to the shaft by 12. On day 14 post-surgery, in addition to the increasing tibial bone remodeling, some rats showed bone loss in the adjacent fibular bone (Fig 8).

Blockade of peripheral afferent input blocks breast cancer-induced evoked pain

Administration of lidocaine over saphenous nerve on day 12 post-surgery produced a peripheral nerve block as indicated by blockade of tactile allodynia. At 15 min following the lidocaine injection, paw withdrawal thresholds measured by von Frey filaments significantly returned to pre-surgery (Fig 9A). There was no observed motor impairment in the rats that received saphenous nerve block. The withdrawal threshold returned to pre-nerve block levels 45 min after the lidocaine administration.

Blockade of evoked pain following nerve block is due to local but not systemic blockade of afferent input

Administration of lidocaine over saphenous nerve of ipislateral or contralateral limb on day 12 post-surgery indicated blockade of tactile allodynia only on the ipsilateral but not contralateral hind paw (Fig 9B). At 15 min following lidocaine injection, paw withdrawal thresholds were significantly eliminated in rats that received lidocaine on the tumor-bearing limb. Rats that received lidocaine injection on the contralateral limb had no change in their withdrawal thresholds indicating the local effects of the nerve block.

Breast cancer-induced ongoing pain is driven by peripheral afferent input

No pre-conditioning chamber preferences were observed across any groups On the conditioning day (D12 post-surgery), both control and cancer animals underwent light anesthesia with isofluorane and were treated with saline administration over the

saphenous nerve in the morning. Four hours later, they again underwent light anesthesia and received lidocaine administration over the saphenous nerve in the afternoon over saphenous nerve. On test day (D13 post-surgery), animals were tested for chamber preference in absence of saphenous nerve lidocaine. Cancer treated rats that received nerve block demonstrated preference for the chamber paired with saphenous nerve lidocaine whereas control rats that received lidocaine did not show preference for the paired chamber (Fig 9C). Comparison of difference from baseline scores confirmed that cancer-bearing rats spent significantly increased time in the lidocaine paired chamber as compared to the saline paired chamber following conditioning whereas time spent by the control rats in each chamber remained the same as pre-conditioning baseline (Fig 9D)

Palpation-induced conditioned place aversion as a measure of movement-induced breakthrough pain (incident pain)

Movement evoked breakthrough pain was assessed using limb palpation-induced conditioned place aversion (Fig 10). On conditioning day i.e. day 12 post-surgery, rats received no treatment (no palpation) in the morning and 2 min palpation of the cancer bearing limb in the afternoon. On test day, i.e. 20-24 hours following palpation, cancer treated rats spent significantly less time in the palpation-paired chamber whereas control rats spent equivalent time in the pairing chambers (Fig 10a). Difference scores calculated as pre-conditioning time subtracted from post-conditioning time indicated that cancer bearing rats spent significantly decreased time in the palpation paired chamber compared

to the control rats likely due to exacerbation of ongoing pain induced by the limb palpation resulting in breakthrough pain (Fig 10b).

Breast cancer-induced breakthrough pain is driven by peripheral afferent input

The role of peripheral afferent input in driving cancer-induced breakthrough pain was determined by injection of lidocaine or saline over saphenous nerve 15 min prior to or 10 min following 2 min of palpations of the treated limb in the afternoon pairing on conditioning day. Cancer bearing rats that received saline following palpation, showed aversion to the chamber paired with it. Cancer-bearing rats that received lidocaine prior to palpation showed preference for the lidocaine-paired chamber whereas cancer-bearing rats that received lidocaine 10 min after the palpation showed aversion for the chamber paired with it (Fig 11a). Comparison with the pre-conditioning baseline showed that while cancer rats that received lidocaine 10 min later or saline over the saphenous nerve showed aversion to the chamber paired with palpation, rats that received a peripheral nerve block before palpation did not show preference for either chamber, indicating elimination of aversion caused movement-induced pain and blockade of ongoing pain in absence of palpation. Control rats that received lidocaine did not show preference or aversion for the paired chamber (Fig 11b).

Figure 7

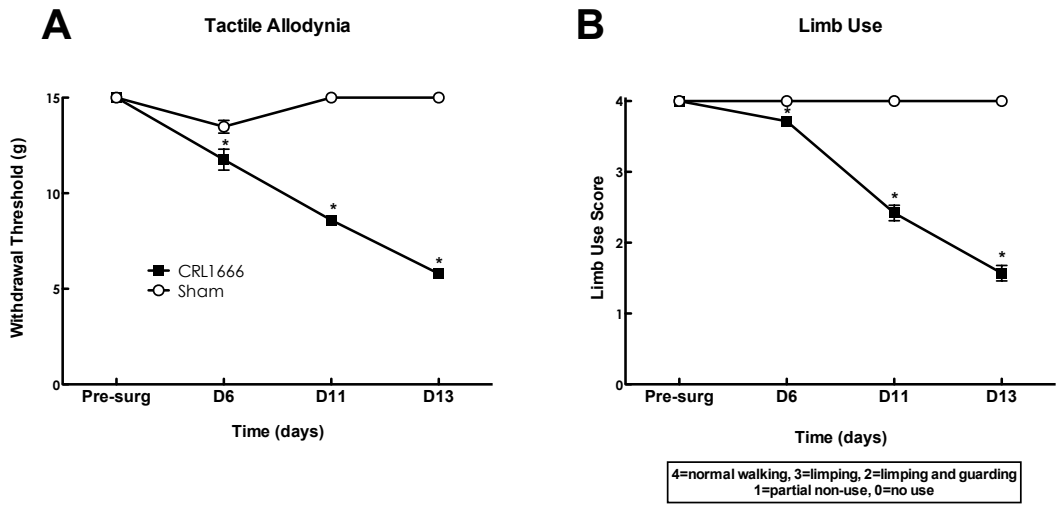


Figure 7. Injection of CRL1666 cells into the tibia produces time-dependent pain behaviors. (a) Tibial tumor-induced hypersensitivity to evoked stimuli measured as tactile allodynia is observed within 6 days post-surgery. Withdrawal threshold decreases with disease progression. *$p<0.05$ compared to shams. (b) Tibial tumor-induced impairment of limb use is observed within 6 days post-surgery. Limb use is decreased with disease progression, *$p<0.05$ compared to shams. Graphs show mean ± SEM, n=10-12.

Figure 8

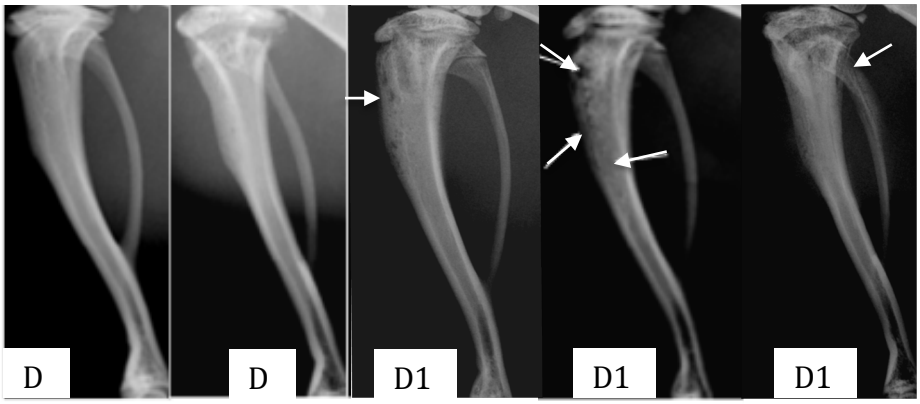


Figure 8. Radiographic analysis of bone remodeling produced by the injection of CRL1666 cells in the tibia. Day 0 indicates normal bone. On day 7 post-surgery, no apparent change is seen in the radiograph. Day 10 shows initiation of bone loss. Bone loss increases and occupies entire shaft by day 12 in addition to cortical fractures. Tumor escapes to fibula on day 14. Arrows indicate bone loss and cortical fractures.

Figure 9

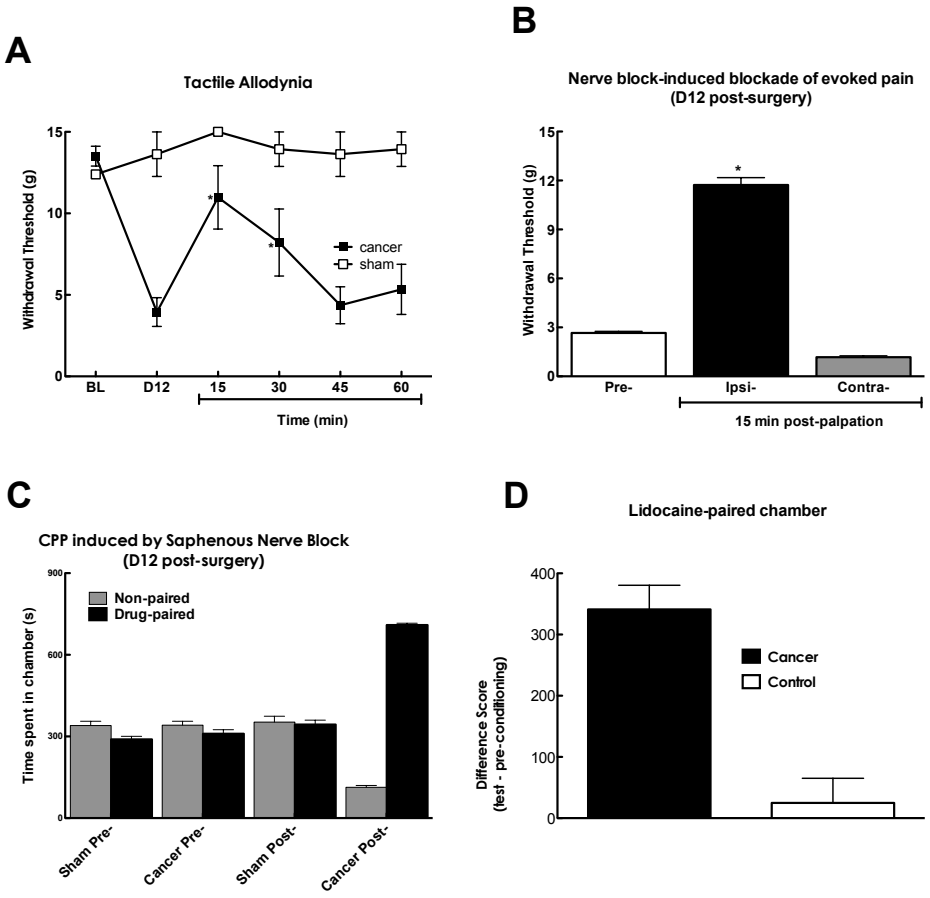


Figure 9. Blockade of peripheral afferent input blocks cancer-induced bone pain. (a) Blockade of tactile allodynia on administration of lidocaine to the saphenous nerve 12 days post-surgery. Maximum blockade of tactile allodynia is seen within 15 min of lidocaine injection. *$p<0.05$ compared to pre-lidocaine withdrawal thresholds. (b) Administration of lidocaine to the saphenous nerve on day 12 post-surgery induced conditioned place preference in tumor-bearing rats. Lidocaine administered to the saphenous nerve does not induce chamber preference in sham-operated rats. *$p<0.05$ compared to pre-conditioning baseline. (c) Difference scores (test time – preconditioning time) confirm that tumor bearing but not sham operated rats spend increased time in the lidocaine paired chamber. *$p<0.05$ compared to time spent in saline paired chamber. Graphs show mean ± SEM, n=10-12.

Figure 10

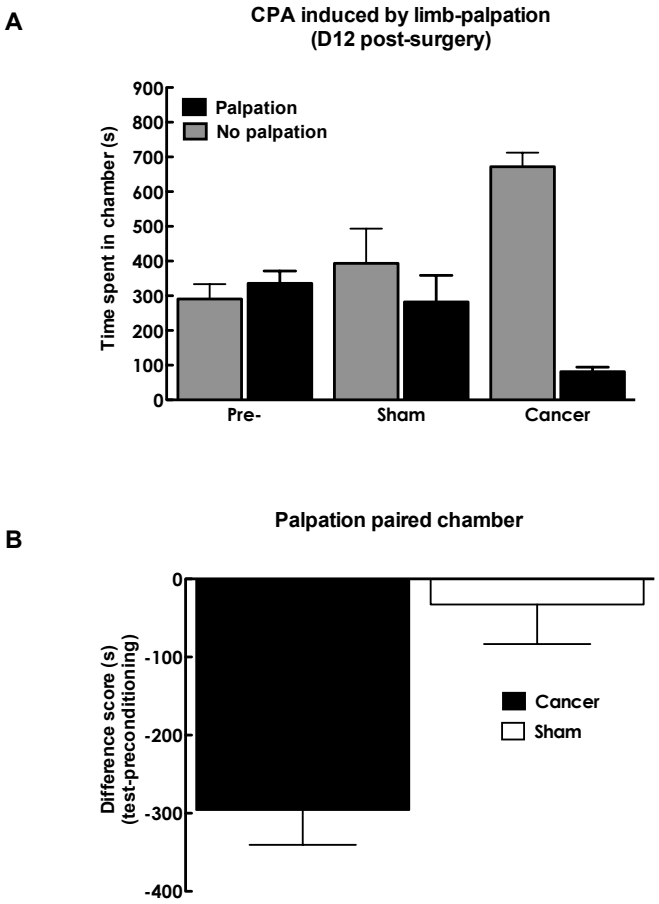


Figure 10. Palpation of tumor-bearing limb produces conditioned place avoidance. (a) A two min. period of palpation of the tumor-bearing limb produces avoidance in cancer treated but not sham operated rats for the chamber paired with it. *$p<0.05$ compared to pre-palpation baseline. (b) difference scores (test-preconditioning time) confirm that tumor-bearing but not sham operated rats spend decreased time in the chamber paired with palpation. *$p<0.05$ compared to time spent in no-stimulation paired chamber. Graphs show mean ± SEM, n=10-12.

Figure 11

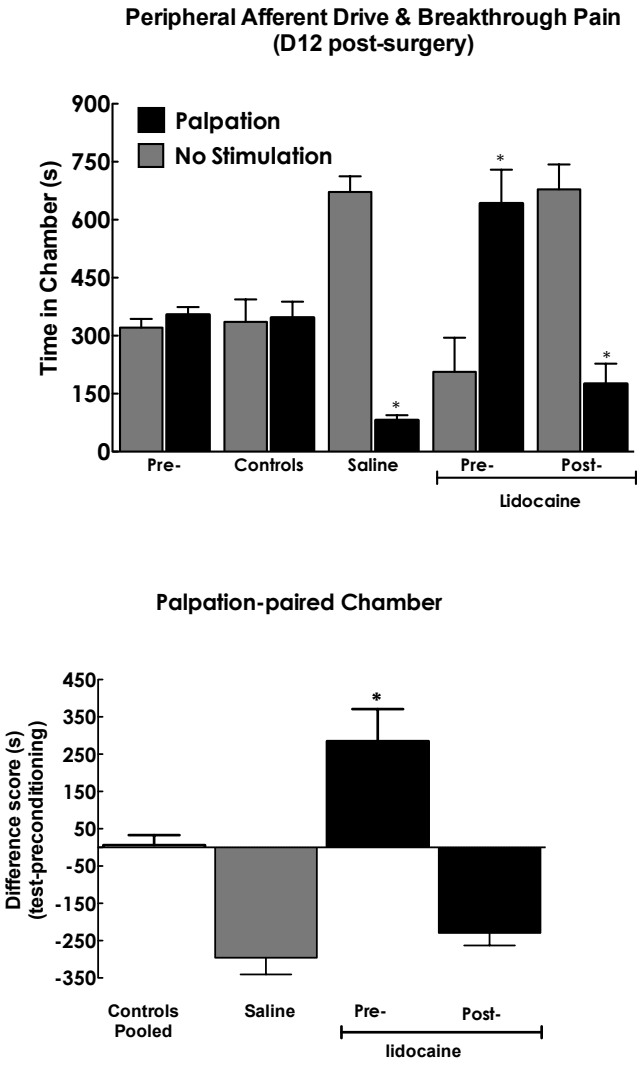


Figure 11. Disruption of peripheral afferent input blocks palpation-induced conditioned place avoidance. (a) Administration of lidocaine immediately after limb-palpation blocks chamber-paired avoidance in tumor bearing but not sham operated rats. In absence of a nerve block, cancer-bearing rats treated with saline show avoidance for the palpation-paired chamber. Cancer-bearing rats that received lidocaine over the saphenous nerve 10 min following the palpation show aversion for the chamber paired with palpation. *$p<0.05$ compared to pre-conditioning baseline. (b) Difference scores (test-preconditioning time) confirm

that saphenous nerve block reverses palpation-induced chamber avoidance in tumor bearing rats when given immediately following palpation whereas in absence of a nerve block or with delayed nerve block, cancer-bearing rats spend significantly reduced time in the chamber paired with limb palpation. Control rats do not show aversion to the palpation paired chamber. Graphs show mean ± SEM, n=10-12.

CHAPTER V

ROLE OF INTERLEUKIN-6 IN CANCER-INDUCED BONE PAIN

Acute inhibition of interleukin-6 blocks breast cancer-induced evoked pain

Subcutaneous administration of IL-6 receptor antagonist TB-2-081 (1 or 10 mg/kg, s.c.) on day 12 post-surgery blocked cancer-induced evoked pain in a dose dependent manner. TB-2-081 (10 mg/kg, s.c.), paw withdrawal threshold significantly increased within 15 min of drug administration demonstrating complete blockade of tactile allodynia (Fig 12). The blockade was maintained through 30 min post drug injection. The withdrawal threshold returned to pre-drug levels 90 min after the drug administration. Paw withdrawal thresholds for control rats did not change.

Acute inhibition of interleukin-6 fails to block breast cancer-induced ongoing pain

To determine the role of IL-6 in cancer-induced ongoing pain, on the conditioning day i.e. day 12 post-surgery, both control and cancer animals received 50% ethanol (s.c.) and saline over saphenous nerve 15 min later. Four hours later, they received either 50% ethanol followed 15 min with saphenous nerve block or TB-2-081 (10 mg/kg s.c.) followed 15 min later with saphenous nerve block. No pre-conditioning chamber preferences were observed across any groups. On test day i.e. day 13 post-surgery, 20-24 hrs following conditioning, animals were tested for chamber preference in absence of any drug treatment. Cancer treated rats demonstrated conditioned place preference for the chamber paired with saphenous nerve block irrespective of drug treatment. Control rats

did not show preference for the saphenous lidocaine paired chamber irrespective of drug treatment, and hence the data were pooled for graphical representation (Fig 13a). Comparison of difference from baseline scores confirmed that cancer-bearing rats treated with the IL-6 antagonist as well as with the vehicle spent significantly increased time in the lidocaine paired chamber following conditioning compared to controls (Fig 13b). Hence our data demonstrate that acute administration of the IL-6 antagonist fails to block saphenous nerve-block induced preference indicating that IL-6 does not mediate cancer-induced ongoing bone pain.

Implantation of breast cancer cells in the bone causes release of interleukin-6 in the bone exudates and serum

Levels of Interleukin-6 in bone exudates and serum were determined on day 12 post-surgery and were compared with those of control rats (Fig 15). A five-fold increase in IL-6 levels was found in the bone exudates of tumor-bearing rats (51.27 ± 10.37 pg/mg protein) as compared to controls (7.68 ± 3.15 pg/mg protein) (Fig 15b). In addition, a sixteen fold increase was observed in the blood plasma levels of IL-6 in tumor-bearing rats (16.989 ± 4.37 pg/mg protein) in comparison with the control rats (1.06 ± 0.18 pg/mg protein) (Fig 15c). Basal release of IL-6 (11.74 ± 2.68 pg/ml) in the rat breast cancer cell line CRL1666 used for bone injection in absence of any treatment was also verified indicating one of the potential sources of IL-6 being the tumor itself (Fig 15a)..

Figure 12

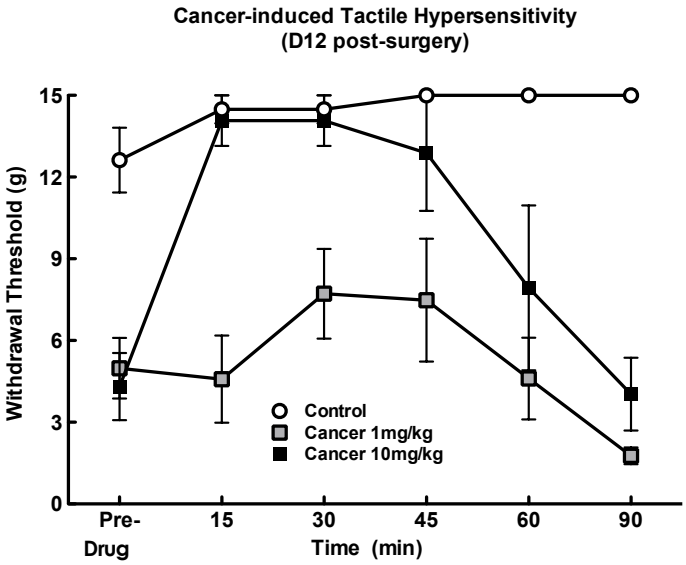


Figure 12. Acute inhibition of Interleukin-6 by TB-2-081 (s.c. 1 or 10 mg/kg) blocks cancer-induced tactile allodynia in a dose dependent manner on day 12 post-surgery. Maximum blockade of tactile allodynia is seen within 15 min of drug injection. *$p<0.05$ compared to pre-drug withdrawal thresholds.

Figure 13

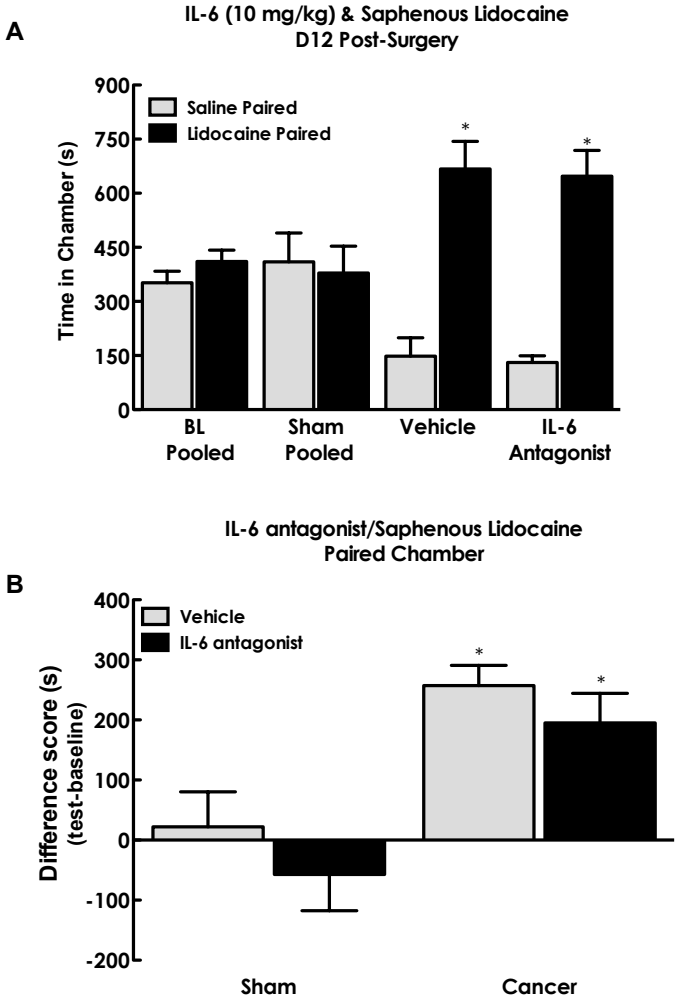


Figure 13. Acute inhibition of Interleukin-6 by TB-2-081 (s.c. 10 mg/kg) fails to block cancer induced ongoing pain (a) Injection of the drug as well as vehicle fails to block saphenous-nerve block induced preference for the chamber paired with it. Lidocaine administered to the saphenous nerve following drug or vehicle administraion does not induce chamber preference in sham-operated rats. *$p<0.05$ compared to pre-conditioning baseline. (b) Difference scores (test time – preconditioning time) confirm that both drug and

vehicle treated rats bearing cancer but not sham operated rats spend increased time in the lidocaine paired chamber. *$p<0.05$ compared to time spent in saline paired chamber. Graphs show mean ± SEM, n=10-12.

Figure 14

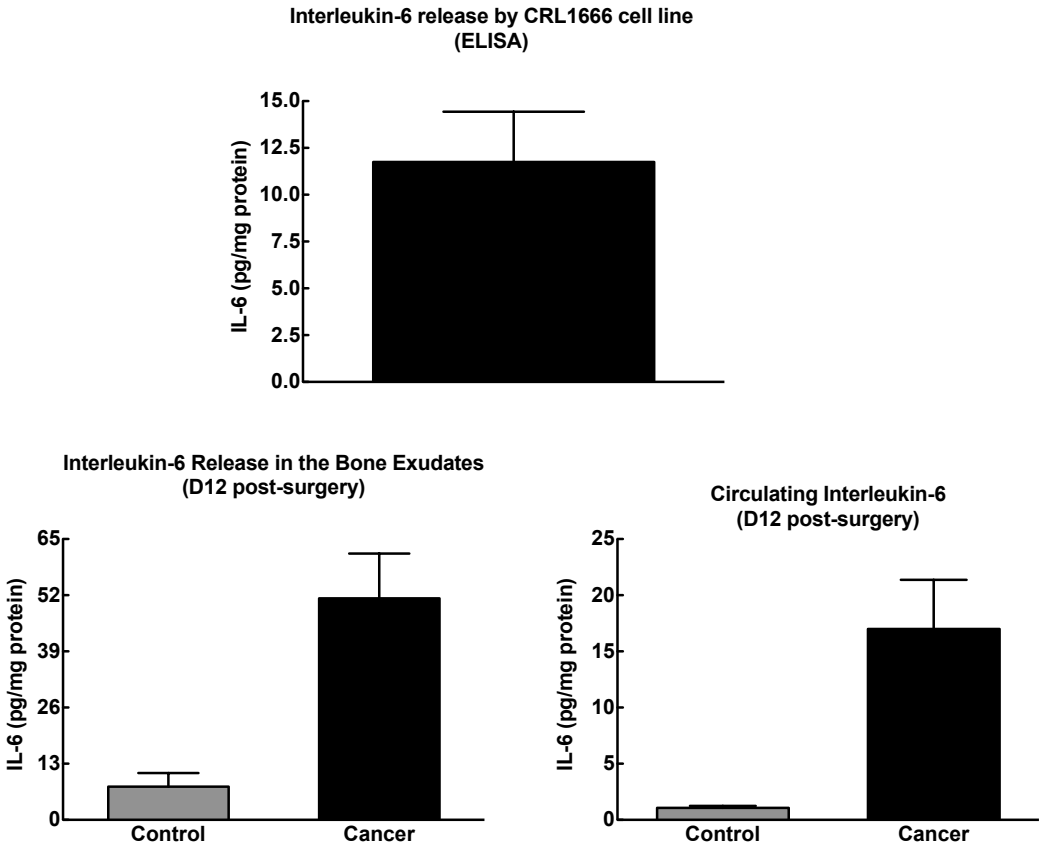


Figure 14. Implantation of breast cancer cells in the tibia results in to release of interleukin-6 (a) media collected from CRL1666 cells cultured overnight without any treatment show basal release of IL-6 (b) IL-6 levels measured in bone exudates of tumor-bearing rats are significantly elevated as compared to control rats (c) circulating levels of IL-6 are significantly high in tumor-bearing rats as compared to control rats. *P<0.05 compared to controls. Graphs show mean ± SEM, n=4.

CHAPTER VI

DISCUSSION

Cancer pain is multifaceted, with clinical descriptors including acute, chronic, nociceptive (somatic), visceral, and neuropathic (Christo and Mazloomdoost, 2008b). Pain limits daily activity in 41% of patients reporting mild to moderate pain and in 94% of patients reporting moderate to severe pain, leading to greatly diminished quality of life in these patients (Coyle et al., 1990). The lifespan of patients diagnosed with metastatic prostate cancer has also increased to from an average of 2.5 years to an average of 55 months i.e. 4.5 years (Halvorson et al., 2006). However, these patients still experience pain from the bone metastases that can be severe and unpredictable and can greatly limit daily activities (Christo and Mazloomdoost, 2008b; Mantyh et al., 2002b). The American Pain Society proposes several important considerations for appropriate pain management for chronic pain and cancer pain such as (1) the source of pain (2) patient's age, general health, and comorbidities (3) potential for medication-related adverse effects (4) potential drug interactions (5) comorbidities that may be relieved by non-analgesic effects of the medications (e.g. sleep disturbances, depression, or anxiety) (6) comorbidities that may be exacerbated by the non-analgesic effects of the medications (e.g. hypertension, ulceration, renal impairment, or cognitive impairment) (7) costs associated with therapy (8) potential risks for medication abuse and (9) risks of overdose (American Pain Society Principles of analgesic use in the treatment of acute pain and cancer pain Glenview: American Pain Society 2008).

Opiates are currently the most recommended front-line treatment for cancer-induced bone pain. Opioids have good efficacy with acute treatment as confirmed by preclinical studies which showed that single bolus injection of morphine can effectively block pain behaviors induced by bone cancer (Luger et al., 2002). However, sustained and long term treatment with opiates that are usually given via controlled release tablets, repeated bolus injections or transdermal patches has always been questioned for a number of factors (1) Irrespective of the type or intensity of pain, the effective opioid dose as well as the relative toxicity ratios may vary greatly across patients (2) Moreover, some patients develop analgesic tolerance to opioids likely from receptor desensitization in which greater doses of opioids are required to produce effective pain management (Christo and Mazloomdoost, 2008a; Silverman, 2009). (3) Especially during rapid dose escalation some patients may experience unexpected development of opioid-induced hyperalgesia and allodynia that is unassociated with original pain (4) Intermittent withdrawals and psychological factors (Silverman, 2009).

Cancer is a chronic condition in which most patients experience advancement of disease and greater pain intensity requires increased doses of opioids explaining the limitation to control pain. Mechanisms responsible for opioid-induced enhancement of pain have been extensively studied in preclinical models (Ossipov et al., 2005). Some of the responsible events are (1) up-regulation of excitatory neurotransmitters, such as substance P and calcitonin gene-related peptide (CGRP) in primary afferent fibers and the spinal cord (2) Increased evoked release of excitatory neurotransmitters within the spinal cord (3) Up-

regulation of spinal dynorphin levels and (4) activation of descending pain facilitation from the rostral ventromedial medulla. Preclinical studies have been exclusively carried out in the mouse model of bone cancer pain which demonstrated enhanced pain when administered prolonged, sustained morphine mini-pumps were determined (King et al., 2007). Some of the proposed mechanisms underlying enhancement of pain in addition to the disease itself are (1) increased the expression of excitatory neurotransmitters (substance P and CGRP) in primary afferent fibers, suggesting the potential for enhanced nociceptive signaling in the morphine-treated animals (2) enhanced sarcoma-induced bone loss and fracture, as well as expression of activating transcription factor 3 (ATF3, a marker of neuronal damage) in cell bodies of primary afferent fibers. Role of mu-opioid receptor in enhanced bone pain and bone loss were confirmed by co-administration of naloxone.

These circumstances highlight the need for a treatment option particularly designed to treat cancer pain that can be given for a prolonged period of time and is associated with minimal side effects. The broad aims of this dissertation are to (1) explore novel molecular targets that effectively control cancer-induced bone pain and are be safe with chronic administration of time (2) to evaluate the effects in various aspects of cancer-induced bone pain such as referred pain, ongoing pain and breakthrough pain.

We first determined the role of a stress-induced signaling molecule p38 MAPK in the mouse model of cancer-induced bone pain. Our data show that (a) acute as well as

prolonged inhibition of p38 MAPK blocks ongoing pain as measured by flinching and guarding behaviors, but not evoked pain; (b) prolonged blockade of p38 MAPK attenuates tumor-induced bone remodeling; and (c) prolonged blockade of p38 MAPK diminishes tumor growth within the bone. Together, these data indicate that the spontaneous and evoked components of cancer-induced bone pain are driven by independent mechanisms. These findings further indicate that tumor-induced ongoing pain is dependent on p38 MAPK signaling. However, inhibition of p38 MAPK signaling is insufficient to block evoked pain, which may be dependent on establishment of central sensitization. Importantly, we demonstrate that chronic inhibition of p38 MAPK failed to attenuate evoked pain despite markedly diminished tumor within the bone and bone remodeling, suggesting that removal of tumor burden after the establishment of tumor-induced bone pain may not be sufficient to block evoked pain.

Both prolonged and acute inhibition of p38 MAPK blocked spontaneous pain behaviors i.e. flinching and guarding. Of note, p38 MAPK has been implicated in translational changes within nociceptive fibers associated with chronic pain states (Cheng et al., 2010; Kim et al., 2002). Previous studies have implicated p38 MAPK in increased transcription of nociceptive peptides, such as SP and CGRP (Ma et al., 2001; Svensson et al., 2005) as well as spinal prostaglandins (Ji et al., 2002; Svensson et al., 2003a). Further, p38 MAPK is implicated in injury-induced increased production and trafficking of the TRPV1 (transient receptor potential vanilloid) channel to the periphery. Together, these changes result in amplification of nociceptive signaling (Ji et al., 2002; Mizushima et al., 2005;

Woolf and Salter, 2000). Thus, prolonged administration of the p38 MAPK inhibitor may diminish cancer-induced ongoing pain behaviors by blocking these pro-nociceptive changes. Notably, p38 MAPK is also implicated in post-translational changes implicated in pain, such as phosphorylation of nociceptive channels such as sodium channel Nav1.8 (Hudmon et al., 2008) and TRPV1 (Ji et al., 2002). Blockade of these post-translational modifications may account for the ability of a single, bolus administration of p38 MAPK to block cancer-induced bone pain.

Importantly, consistent with previous findings in the osteosarcoma pain model (Svensson et al., 2008), neither acute nor prolonged p38 MAPK inhibition blocked tactile allodynia. These observations indicate that ongoing and evoked pain are driven by different mechanisms. One potential explanation for the divergent effects of p38 MAPK inhibition on spontaneous and evoked pain is the site of action. It is possible that peripheral sensitization and activation drives cancer-induced spontaneous pain whereas central mechanisms such as spinal sensitization account for the tactile allodynia. We note that tactile allodynia is a measure of referred pain, which likely reflects expansion of receptive fields that is demonstrated as a sign of central sensitization across multiple models (D'Mello and Dickenson, 2008; Woolf and Salter, 2000). Although p38 MAPK is implicated in initiation of spinal sensitization, it does not appear to be a factor in maintaining central sensitization and the associated enhanced evoked pain. Previous studies have demonstrated phosphorylation of p38 MAPK in glial cells within the spinal cord across several injury states such as inflammation-induced pain (Cui et al., 2008;

Svensson et al., 2003c) neuropathic pain (Garry et al., 2005; Ji and Suter, 2007b; Jin et al., 2003b), spinal injury (Nomura et al., 2005) and incision (Wen et al., 2009). Within these pain states, spinal administration of the p38 MAPK inhibitor starting before or at the time of injury blocks evoked pain (tactile allodynia and thermal hyperalgesia) (Schafers et al., 2003a; Sorkin et al., 2009; Tsuda et al., 2004; Zhang et al., 2005) whereas several studies have demonstrated that spinal administration of the p38 MAPK inhibitors after the pain state is established failed to reverse thermal or tactile hypersensitivity (Raghavendra et al., 2003; Schafers et al., 2003b; Xu et al., 2007). These data indicate that within the spinal cord, p38 MAPK is critical in the induction of nerve injury induced pain, but does not play a role in maintaining pain that is already established. Thus, one possible explanation for the blockade of ongoing pain but not evoked pain could be that p38 MAPK may play a role in generation of cancer-induced bone pain by ameliorating disease progression within the bone, but not in maintenance of pain since the drug was administered after pain behaviors were established.

Inflammation is also an important component of nociception. Cells in the bone microenvironment (macrophages, mast cells etc.) and the tumor-mass itself produce inflammatory mediators such as cytokines, prostaglandins, endothelins (Korf-Klingebiel et al., 2008; Mantyh et al., 2002b; Mercadante, 1997; Mundy, 1991; Temeles et al., 1993). These factors are known to sensitize nociceptive neurons, which are the predominant sensory neurons innervating the bone (Jakobsson, 2010; Mantyh, 2002; Mantyh et al., 2002b). Many studies have revealed the anti-inflammatory activity of

SB203580 both in vivo and in vitro (Badger et al., 1996a; Han et al., 2006; Zhou et al., 2010). Thus, inhibition of p38 MAPK likely reduces the tumor-associated inflammation and up-regulation of pro-inflammatory cytokines that sensitize and activate nociceptive fibers, resulting into blockade of spontaneous pain behaviors.

Histology of bones collected at the end our study (day 13) show significant reduction in tumor burden after the drug treatment. Moreover, administration of the p38 MAPK inhibitor to cultured 66.1 cells diminished viability, indicating a role of p38 MAPK in tumor growth. This is consistent with multiple reports implicating p38 MAPK in proliferation, invasion and migration of a variety of malignancies including breast cancer (Chen et al., 2004; Chen et al., 2009; Kim et al., 2003; Wagner and Nebreda, 2009). Within the bone, treatment with 15 mg/kg of SB203580 diminished tumor growth, with tumor growth restricted to the shaft of the bone, and lack of tumor growth into the epiphyseal plate region. At a higher dose, 30 mg/kg, only small pockets of tumor were seen. These data suggest that removal of tumor burden was one of the key mechanisms that contributed to the blockade of spontaneous pain behaviors. However, the fact that evoked pain still persisted indicates that diminished tumor burden may not be sufficient to block this pain.

Painful bone metastases in humans such as those of prostate and breast cancer often have osteolytic as well as osteoblastic lesions (Keller and Brown, 2004; Winding et al., 2000). Our model shows osteolytic lesions appear by day 10 followed by osteoblastic activity in

the bone. Treatment with SB203580 completely eliminated the abnormal osteoblastic structures that were formed in the vehicle-treated bones by day 13 post-tumor implantation. In addition, the radiograph analyses of bones showed reduced incidences of spontaneous bone fractures in drug treated bones as compared to those treated with the vehicle. Thus, blockade of tumor-induced bone remodeling likely contributed to the blockade of spontaneous pain behaviors in cancer bearing mice treated with prolonged administration of the p38 MAPK inhibitor. Moreover, p38 positively regulates Nuclear Factor –kappa B (NF-kB) activity by a variety of mechanisms including chromatin remodeling by phosphorylating histone H3 at NF-kB dependent promoters such as IL-8 (Saccani et al., 2002). Inflammatory cytokines TNFα and interleukin 1-beta IL-1β are prominent inducers of NF-kB signaling. Receptor activator of nuclear factor kappa B (RANK), which is a type of Tumor Necrosis Factor Receptor and a key player in osteolysis, is a central activator of NF-κB. Osteoprotegerin (OPG), which is a decoy receptor homolog for RANK ligand (RANKL), inhibits RANK by binding to RANKL, and, thus, osteoprotegerin is tightly involved in regulating NF-κB activation (Mori et al., 2007). NF-kB signaling can also lead to inflammatory responses, cell survival and cell proliferation. Hence one of the possible mechanisms by which inhibition of p38 MAPK attenuates the tumor growth and tumor-induced bone remodeling is by blockade of the NF-kB signaling pathway.

Our data indicate that inhibition of the p38 MAPK signaling pathway may be a target for blocking cancer-induced spontaneous pain but may not efficiently block cancer-induced

evoked pain. These findings indicate important differences in mechanisms mediating ongoing and evoked pain. The observation that the p38 MAPK diminished tumor within the bone indicates that spontaneous pain may be dependent on peripheral input from the tumor whereas central mechanisms, likely established prior to diminished tumor burden, likely maintain evoked pain behaviors. Importantly, this may indicate different mechanisms driving the ongoing pain as opposed to movement evoked breakthrough pain, which may reflect evoked pain. A better understanding of how mechanisms driving spontaneous and evoked pain differ, may lead to a more complete treatment strategy for patients with cancer pain.

Few questions need to be addressed in future to determine the extent of redundancy between p38 and other members of the MAPK family since the cross talk between p38 and other members of the MAPK family is reported which includes inhibition of ERK1/2 pathway by p38 MAPK and antagonistic action of p38 towards JNK signaling by cell-type specific mechanisms in myoblasts and haemopoietic cells (Cuadrado and Nebreda, 2010). Also, SB203580 has also been shown to inhibit other members of the MAPK family when given at high concentrations (Fabian et al., 2005; Godl et al., 2003).

As mentioned above, the link between p38 and inflammation is strongly established via release of pro-inflammatory cytokines. Tissue injury (i.e. tumor), stress etc. activate stress-sensitive protein kinases such as ERK, JNK, p38 in tumor cells as well the immune cells (Sommer et al., 2001; Watkins et al., 1999). In a multistep process, these kinases

phosphorylate transcription factors belonging to C/EBP, NF-kB and AP-1 families. On translocation to nucleus, these transcription factors interact with responsive elements in the gene promoters of cytokines such as IL-6, TNFα or IL-1β. Enhanced transcription of these genes is usually followed by rapid translation and protein processing leading to the release of biologically active cytokines (Koj, 1996). TNFα or IL-1β are the cytokines that initiate a cascade in response to a tissue injury. TNFα initiates production of IL-6 cytokine family members such as IL-6, IL-11, leukemia inhibitor factor (LIF), oncostatin M that are involved in systemic responses following tissue injury (Koj, 1996). These cytokines, along with the inflammatory response, also contribute to the pain signaling. In the peripheral nervous system, IL-1β and its receptor are synthesized in dorsal root ganglia indicating paracrine and/or autocrine mechanism of action (Copray et al., 2001). Peripheral pro-nociceptive action of IL-1β may be mediated by a complex signaling cascade and secondary production of nitric oxide, bradykinin or prostaglandins (Poole, 1999). Tumor necrosis factor α is another pro-inflammatory cytokine that initiates a cascade of activation of other cytokines such as IL-6. After nerve injury or tumor growth, TNFα is synthesized and released by cells in the intramedullary space such as tumor and tumor associated macrophages as well as schwann cells (Sommer et al., 1998). TNFα induced hyperalgesia as a result of inflammation or nerve injury is mediated via p38 MAPK pathway (Schafers et al., 2003b). These findings confirm the role of p38 MAPK in cancer-induced bone pain as well as highlight the importance of exploring the role of cytokines in cancer-induced bone pain and disease progression.

Our finding that spontaneous and evoked pain each have unique set of underlying mechanisms also provides a platform to closely resolve differences between various aspects of cancer-induced bone pain such as referred pain, ongoing pain and breakthrough pain as well as determine events that drive each of this pain state and how they may differ from each other. In order to address this question more closely we moved to a rat model of cancer-induced bone pain in which breast cancer cells are injected in the tibiae of female rats. Some of the added advantages offered by the rat model are (1) more detailed analyses of behavioral measures of pain (2) determination of the role of higher centers in the brain in driving cancer-induced bone pain (3) no muscle involvement in the surgical procedure and minimal injury (4) feasibility to carry out experiments in the laboratory setting designed for studies in rats.

Neuropathic pain patients commonly experience ongoing i.e. stimulus independent pain (Backonja and Stacey, 2004; Rowbotham, 2005). Although ongoing pain is a common clinical complaint, the current preclinical research is highly based on behavioral measures that rely on hypersensitivity assays based on reflex responses (Campbell and Meyer, 2006; Rice, 2008; Vierck et al., 2008). Besides, modulation of evoked pain responses to an analgesic treatment does not always correlate with the efficacy of the drug to control ongoing pain. Metastatic bone pain is also characterized by the ongoing component (Portenoy et al., 1993). The next part of this dissertation is based on the manipulations that relieve ongoing pain and produce negative reinforcement in such a way that contextual cues associated with pain relief elicit approach following conditioning.

In the rat model of cancer-induced bone pain, we have shown that (a) peripheral afferent input from the tumor-bearing tibia mediates ongoing pain (b) palpation of the tumor-bearing limb causes conditioned place avoidance and (c) disrupting the afferent input immediately after the palpation blocks generation of resulting breakthrough pain but cannot reverse established breakthrough pain.

In the present studies, we tested the role of peripheral afferent input in maintaining cancer-induced ongoing pain as measured by CPP. Peripheral nerve block given on the saphenous nerve produced CPP only in rats with tibial tumors. The local effects of the lidocaine administered over the saphenous nerve were verified using by applying a nerve block on the contralateral limb which did not elevate paw withdrawal thresholds as against the ipsilateral local nerve block which completely eliminated tactile allodynia. This preference indicated reward following pain relief implicating that afferent drive from the tumor-bearing tibia provides an aversive stimulus. Indeed, the tumor progression within the bone causes an array of mediators that directly or indirectly excite or sensitize the primary afferent neurons innervating the bone (Mantyh et al., 2002a). Some of these mechanisms include (a) release of cytokines such as IL-6, TNFα as well as growth factors and prostaglandins released by the tumor itself and the cells associated with it resulting into inflammatory pain (b) local acidosis from tissue injury and bone remodeling causing lysis or damage to the nerve endings innervating the bone leading to

neuropathic pain (c) mechanical pain from bone destruction and tumor-induced spontaneous fractures.

Several mechanisms underlying tumor-induced bone pain have been demonstrated using a mouse model of sarcoma-induced bone pain. However little is known about the cancer breakthrough pain and how it may differ from ongoing pain. BTP is associated with debilitating pain and has a dramatically negative impact on the quality of life (Bennett et al., 2007). As pain relief is required urgently, fast acting parenteral opioids or rapid onset opioids (ROO) are most commonly prescribed (Mercadante, 2011). IV morphine has been found to be highly effective with mild side effects (Mercadante et al., 2004). However, it is often difficult to be administered at 'home settings' given the unpredictable nature of some cancer-induced breakthrough pain (Enting et al., 2005). Although lipophilic opiates such as fentanyl given via transmucosal, buccal, intranasal or sublingual routes are safe and convenient, their administration is limited due to uncertain dosage. The use of ROO remains to be controversial since it largely depends on the anecdotal experience. In addition, patients which receive high doses of opioids as to obtain basal analgesia can not become good candidates for titration of minimal initial ROO doses (Mercadante, 2011). Unfortunately, no alternative medicine is available for patients with neuropathic cancer pain who are resistant to opioid therapy and constitute up to 40-50% of cancer patients (Manfredi et al., 2003).. In the present studies, we have developed a model of movement-induced (incident) BTP in which the BTP is paired with a distinct context and conditioned place avoidance (CPA) is measured. Moreover, CPA is

only produced in rats with tibial tumors indicating that palpations are associated with pain resulting from the movement of tumor-bearing limb. In order to determine whether peripheral afferent input contributes to the generation and maintenance of movement-induced BTP, peripheral nerve block was applied immediately after the palpation or 10 min after the palpation when the pain is believed to be well established. Our data suggest that afferent input from the tumor-bearing tibia is an important determinant of generation of movement-induced BTP however once the painful state is achieved, the peripheral afferent drive may not contribute in maintaining this state indicating the role of central mechanisms. This finding provides a platform to study the role of molecular mediators in the peripheral as well as central nerve system that could be important determinants of cancer-induced breakthrough pain. Central sensitization occurs as a result of persistent firing of peripheral afferent neurons. Delayed blockade of peripheral afferent input fails to block established breakthrough pain likely due to contribution of central mechanisms such as descending facilitation from the rostral ventromedullary medulla (RVM) (Ossipov et al., 2000), enhanced release of pro-nociceptive neuropeptides such as Calcitonin gene related peptide (CGRP) (Neugebauer et al., 1996) and substance P (Schaible et al., 1990) or activation of AMPA and NMDA receptors (Sorkin et al., 1992).

The most commonly diagnosed cancers such as prostate, lung and breast have a high propensity to metastasize to the bone (American Cancer Society). The resultant bone destruction along with disease progression causes pain that is dull and achy in the beginning and increases with advancement of the disease (Halvorson et al., 2006). The

proposed model allows us to further look into the neuroadaptive changes associated with various aspects of cancer-induced bone pain giving us the opportunity to develop therapeutic strategies that target all or individual aspects of cancer-induced bone pain. In addition, this model will also serve as a preclinical means to study the combination of existing therapies for individual pain states arising from cancer leading to prolonged administration with minimal side effects and improved quality of life these patients.

The above findings indicate role of peripheral afferent input from subsequent to the actions of pro-nociceptive mediators from the tumor-bearing tibia in contributing to ongoing pain and initiating of breakthrough pain. This provides basis to identify viable molecular targets that will provide relief from these pain states. One of the most potent peripheral contributors of cancer-induced bone pain are cytokines. Function of cytokines such as TNFα, IL-1 and IL-6 in activation and recruitment of immune cells at the site of injury and subsequent development of neuropathic pain and hyperalgesia is well recognized (Watkins et al., 1995). Two main mechanisms have been proposed to explain the contribution of these pro-inflammatory cytokines to nociception (1) direct action on primary afferent neurons and (2) indirect actions via activation of signaling pathways in immune cells (Thacker et al., 2007).

Both prostate and breast cancer cells produce large amounts of IL-6 and increased IL-6 serum levels of IL-6, sIL-6R and gp130 have been associated with poor clinical outcome in many human cancers, including breast and prostate cancer (Ara and Declerck, 2010).

In addition, IL-6 is produced and released by several cells within the bone microenvironment such as monocytes allowing for paracrine signaling (Schibler et al., 1992). As discussed previously, IL-6 can cause progression of cancer growing in the bone by mechanisms such as stimulating the proliferation and enhancing survival of tumor cells or increasing expression of VEGF from endothelial cells promoting angiogenesis. Although, involvement of IL-6 in neuropathic and inflammatory pain conditions is well documented, no data is available, to the best of our knowledge on its role in blocking cancer-induced bone pain and bone remodeling. Randomized controlled phase III studies of Tocilizumab, a monoclonal antibody for IL-6, in modification of rheumatoid arthritis (RA) provided an insight into the potential of IL-6 blockade in blocking pain (Smolen et al., 2008). Patients who were administered Tocilizumab reported reduced pain intensity as per the visual analog scale in a dose dependent manner as compared to patients who received placebo. Whether the analgesic effect seen from inhibition of IL-6 was via blockade of inflammation or blockade of IL-6 as a target is not concluded since Tocilizumab is a humanized antibody and cannot be tested in preclinical models. This emphasizes the need for the development of a small molecule inhibitor of IL-6 that is orally available. TB-2-081 is one such molecule, which has shown to effectively block pancreatitis-induced pain as shown previously (Vardanyan et al., 2010). In this dissertation, we have focused on the acute blockade of IL-6 by TB-2-081 and analyzed the effects on cancer-induced evoked, ongoing and breakthrough pain using tactile allodynia, conditioned place preference and conditioned place avoidance, respectively.

Our data show that (1) acute systemic blockade of IL-6 blocks tibial tumor-induced evoked pain in a dose dependent manner (2) acute systemic blockade of IL-6 fails to block ongoing pain generated by the tibial tumor (3) rat breast cancer cells release IL-6 (4) Cancer rats show 5 fold increase in the levels of IL-6 in the bone exudates as compared to the controls (5) Cancer rats show 16 fold increase in the levels of circulating IL-6 as compared to the controls.

TB-2-081 has been previously shown to inhibit IL-6 induced phosphorylation of STAT3 and antagonize the IL-6 canonical signaling (Kino et al., 2007). TB-2-081 also displaces binding of IL-6 from sIL-6R with high affinity thus acting as an antagonist for IL-6R (Vardanyan et al., 2010). Previous findings in the pancreatitis model indicate that systemic or oral but not intrathecal administration of TB-2-081 reversed pancreatitis induced referred abdominal hypersensitivity suggesting mainly the peripheral mechanisms of action (Vardanyan et al., 2010). This finding also highlights the importance of IL-6R expressed peripherally and on the sensory nerve fibers. Blockade of evoked pain (tactile allodynia) by TB-2-081 in the time frame consistent with previous findings indicate rapid disruption of signaling cascade such as STAT that modulate the sensory input. This pathway is rapid and is known to further stimulate release of CGRP or substance P via JAK and PKCδ Other mechanism that is proposed to be involved in IL6-IL6R-gp130 is the p38 MAPK (Kang et al., 2007) which we have shown to modulate cancer-induced bone pain in the mouse model. Vardanyan and colleagues determined the

ability of IL-6 to promote excitability of primary afferent neurons, in particular the nociceptors. They found that IL-6 enhances capsaicin evoked but not direct release of CGRP, which can be blocked by TB-2-081. This data indicate that the effect of IL-6 may be mediated by signaling through the IL-6R present on the primary afferent terminals that modulates function of Trpv1, a receptor for capsaicin. It is possible that TB-2-081 can also act on other family members of IL-6 since they share the gp130 receptor and the exact mechanism of action needs to be elucidated in vitro. IL-6 is speculated to be involved at the site of injury thus bringing about changes in the nociceptor sensitization in such a way that nociceptors within the damaged tissue are readily excited by non-noxious stimulus giving rise to a condition called allodynia. Whereas within the cancer bearing bone, IL-6 may sensitize the innervating primary afferent neurons to external tactile stimuli, it may not directly activate the nociceptors to cause continuous neuronal firing that creates a tonic aversive state of ongoing pain. This may partially explain the role of IL-6 blockade in evoked pain but not ongoing on.

In order to determine the source of IL-6 that contributes to cancer-induced bone pain, we first recognized the rat breast cancer cells CRL1666 as a primary source of IL-6. Hence, IL-6 could participate in bone pain and tumor progression by acting in an autocrine manner or in a paracrine manner by acting on other cells in the bone microenvironment such as monocytes, macrophages, osteoclasts, osteoblasts as well as the receptor on the nociceptor terminals. We also found an approximate 5-fold increase in IL-6 in bone exudate and 10-fold in blood serum of cancer treated rats compared to control rats

suggestive of prominent contribution of IL-6 in mediating bone pain and tumor progression within the bone. Although the link between tissue expression of IL-6 and disease progression and whether IL-6 negatively or positively modulates the disease is not well established, increase in serum levels of IL-6 is almost definitely and exclusively concluded as a negative prognosticator of breast cancer and pain conditions such as rheumatoid arthritis.

Overall, a large number of diseases and pathological conditions exist in which IL-6 plays a key role making it a desirable target for drug discovery. The current therapeutics that target IL-6 are limited to Tocilizumab. Our laboratory has shown the efficacy of the small molecule IL-6 blocker TB-2-081 given systemically in models of chronic pain such as cancer pain and pancreatitis. This also provides an alternative strategy to expand the approach of inhibition of chronic pain TB-2-081 across other models of neuropathic and inflammatory pain.

Summary of findings: (1) acute as well as chronic systemic blockade of p38 MAPK blocks cancer-induced spontaneous pain (2) acute as well as chronic systemic blockade of p38 MAPK fails to block cancer-induced evoked pain (3) chronic inhibition of p38 MAPK blocks tumor growth within the bone and in vitro and prevents or delays tumor-induced bone remodeling (4) p38 MAPK may play a role in early development of cancer-induced hypersensitivity but not when the pain is established (5) removal of tumor burden is not sufficient to block sensitivity to evoked stimuli (6) afferent input from the

tumor bearing bone produces ongoing pain (7) afferent input from the tumor bearing bone initiates but does not maintain cancer-induced breakthrough pain (8) blockade of interleukin-6 blocks cancer-induced evoked pain but not ongoing pain (9) elevated levels of IL-6 in bone exudates as well as sera of cancer bearing animals contribute to cancer-induced bone pain.

Overall, our data in mouse model indicate that ongoing and evoked pain are two different entities and may not be driven by common mechanisms. In order to address this further, we established a rat model in which pharmacological analysis of neural mechanisms mediating breakthrough pain and ongoing pain can be carried out.

This dissertation focuses on possible contribution of molecular targets such as p38 MAPK and Interleukin-6 in cancer induced bone pain. It also provides a novel model that serves as a basis to delineate potential important differences between ongoing (persistent) cancer induced bone pain and movement associated breakthrough pain (incident pain). Hence, findings from these studies will identify therapeutic strategies to control persistent relief of ongoing pain and movement evoked breakthrough pain. Importantly, molecular targets such as those blocking p38 MAPK (ARRY-797) and Interleukin-6 (Tocilizumab) are presently approved for human use and are in clinical trials for chronic pain states. These studies may allow discovery of effective therapeutic targets that may have opioid sparing effects. For example, a treatment that provides consistent blockade of spontaneous pain may prove adequate for around the clock medication and opioid

administration can only be used in to control breakthrough pain. In addition, combination therapies of these compounds with chronic opioids may decrease instances of tolerance, opioid induced hyperalgesia, as well as other side effects such as persistent nausea, vomitting and constipation associated with prolonged opioid administration. Such studies would provide significant understanding of the mechanisms that generate and maintain cancer pain.

Future directions

Our data demonstrated that effects of long term p38 MAPK inhibition on blockade of spontaneous pain behaviors in the mouse model of cancer induced bone pain are likely due to removal of tumor burden. We have also shown that blockade of p38 MAPK has inhibitory effects on the growth of mouse breast cancer cells. Since strong evidence exists to link p38 MAPK and inflammation, resultant diminishment in tumor burden on p38 blockade could be due to additional mechanisms such as hindrance to tumor-stroma interaction. It would be therefore interesting to know if (a) SB203580 blocks release of cytokines such as TNFα and IL-6 from mouse breast cancer cells (b) exogenously added cytokines promote proliferation of mouse breast cancer cells. The answers to these questions will provide an insight into direct mechanisms by which p38 MAPK acts to block tumor growth within the bone.

Prolonged p38 MAPK blockade fails to block tumor-induced evoked pain. This raises the possibility of central mechanisms involved in generation of evoked pain. It has been

previously reported that p38 MAPK signaling in the activated glia mediates nerve-injury induced allodynia and hyperalgesia. To verify if central blockade of p38 can block evoked pain, experiments can be carried out to determine (a) extent of p38 activation in the spinal cord (b) if intrathecal administration of SB203580 blocks cancer-induced tactile allodynia.

Our findings in the mouse model of cancer-induced bone pain indicate that mechanisms that target spontaneous pain and evoked pain may be different. Such data may have important implications for potential differences in therapeutic strategies for ongoing pain and evoked pain including movement-evoked breakthrough pain. In order to identify these mechanisms, we have established a rat model of cancer-induced bone pain. The behavioral data and radiographic analyses indicate cancer-induced bone destruction and subsequent pain behaviors. However, it would be noteworthy to determine (a) the progression of tumor burden by the bone histology (b) activation of ATF3, a marker for neuronal damage in the DRG.

Our data showed that peripheral afferent input from the tumor-bearing tibia subsequent to the action of pro-nociceptive mediators within the bone, mediates cancer-induced evoked pain, ongoing pain and only initiation of a breakthrough pain episode. However, blockade of peripheral afferent input does not block maintenance of breakthrough pain. This suggests contribution of central mechanisms such as descending pain facilitation from

rostroventral medullary medulla (RVM). Hence, it would be interesting to know if injection of lidocaine in RVM would completely block established breakthrough pain.

c-Fos is an early transcription factor that indicates presence of activated neurons. Number of Fos-positive neurons in the spinal cord dorsal horn is increased in response to stimulation following injury (Gao and Ji, 2009). It would be of interest to know if palpation of the tumor-bearing limb, which is non-noxious in control rats, would induce increase in the Fos positive-neurons in the spinal cord. Furthermore, we would like to see if prevention or reversal of palpation-induced pain following peripheral or central nerve blocks can reduce the number of Fos positive neurons in the spinal cord laminae.

Pain relief seen in cancer-bearing animals as a result of pain-modulating treatment and conditioned place preference that follows it is a result of pain relief seen as reward. Ventral tegmental area (VTA) is a region in the midbrain widely associated with reward circuitry in the brain as an origin of dopaminergic neurons (Yeomans and Baptista, 1997). It would be interesting to see if pain-relieving manipulations would increase the neuronal activity in the VTA thus increasing the number of Fos positive neurons.

Based on the findings that IL-6 may contribute to evoked but not ongoing pain, it would be interesting to know if IL-6 is involved in generation and maintenance of breakthrough pain. It would also be of interest to know if long term blockade of IL-6 will block either evoked or ongoing pain or both more effectively and if there will be any disease

modifying effects. This can be addressed by (a) treatment of rat breast cancer cells with TB-2-081. If proliferation of breast cancer cells is blocked, presence of IL-6R and gp130 on the breast cancer cells can be confirmed. It could be then determined if prolonged blockade of IL-6 will block (1) cancer-induced ongoing pain (2) cancer-induced referred pain (3) cancer-induced breakthrough pain (4) tumor growth within the bone (5) tumor-induced bone remodeling. The proposed approach could use mini-pump infusion to maintain the constant plasma levels of the drug at 10 mg/kg per rat per day across 7 days (day 7-day 13). Analyses for ongoing and breakthrough pain can be carried out on as previously described. Histological and radiographic analyses of bone will determine the extent of tumor progression and bone remodeling.

APPENDIX A

ABSTRACT PRESENTATIOS OF SUBMITTED WORK

Role of p38 MAPK in a Murine Model of Breast Cancer-Induced Bone Pain*

D. D. Sukhtankar, A. Okun, F. Porreca, T. King, Departments of Cancer Biology and Pharmacology, University of Arizona, Tucson, AZ, USA

Aim of investigation: Bone metastases are associated with adverse events such as tumor-induced bone remodeling and corresponding fractures leading to limited mobility and incapacitating pain. Cancer-induced pain encompasses ongoing pain and evoked pain and additionally, incidences of breakthrough pain that may follow movements. Cancer-induced pain is difficult to control. Increased survival times of cancer patients with bone metastases highlights the need for development of effective therapy that can be administered for extended periods of time without adverse effects associated with many current treatment options. The aim of this investigation was to determine the role of p38 MAPK, implicated in tumorigenesis, bone remodeling and nociception, in multiple aspects of cancer-induced bone pain.

Methods: Mouse breast cancer cells 66.1 or media were injected in the intramedullary space of the femur of the Balb/cfC3H female mice. SB203580, a p38 MAPK inhibitor, was given intraperitoneally (i.p.) twice daily from days 7-14 post tumor injection.

Additionally, potential effects of a single injection of SB203580 on spontaneous and evoked pain were determined 21D post tumor injection. Behavioral analyses of spontaneous pain were performed by measuring flinching and guarding behaviors. Evoked pain was measured by responses to tactile stimulation of the hindpaw. Radiographs of the femurs were taken at different time points and the effects of p38 MAPK inhibition on tumor growth were measured by H&E staining.

Results: Either a single, or repeated, administration of SB203580 blocked cancer-induced spontaneous pain in a dose dependent manner. However, both acute and chronic administration of SB203580 failed to block tumor induced tactile allodynia. Prolonged inhibition of p38 MAPK reduced cancer-induced bone remodeling and fracture indicating ability to modify disease progression in vivo. H&E staining confirmed diminished tumor growth within the bone microenvironment, strengthening the conclusion that prolonged inhibition of p38 MAPK modified disease progression.

Conclusion: We demonstrate that both acute and prolonged p38 MAPK inhibition blocks cancer-induced spontaneous pain. However, prolonged blockade of p38 MAPK did not block tactile allodynia, suggesting a dissociation in mechanisms mediating spontaneous and evoked cancer-induced pain. Prolonged p38 MAPK inhibition further produced a significant reduction in tumor growth and bone remodeling. Thus, reducing tumor burden within the bone microenvironment may diminish cancer-induced ongoing pain, but not evoked hypersensitivity. Such findings implicate that tumor associated factors drive

spontaneous pain whereas sensitization and evoked pain persist following diminishment of these factors. As breakthrough pain may reflect an evoked component of cancer pain, these data may indicate that while inhibition of p38 MAPK may effectively diminish ongoing pain, it may be less effective in treatment of breakthrough pain.

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University of Arizona Biomedical research forum

Preclinical analysis of cancer-induced bone pain: Evaluation of ongoing and breakthrough pain *

Tamara King, Devki Sukhtankar, Frank Porreca.
Department of Pharmacology, Cancer Biology Graduate Interdisciplinary Program
University of Arizona

Metastatic bone pain is characterized by persistent ongoing pain (i.e. pain at rest) as well as intermittent episodes of breakthrough pain on a background of well controlled pain. Increasing efficacy of cancer treatments is extending the lifespan of patients with bone

metastases. For this reason, the development of novel, non-opiate mechanism-based therapeutics with limited side effects across prolonged treatment would be beneficial in elevating quality of life in these patients. Limiting the development of novel therapies for cancer pain, including breakthrough pain, is limited knowledge of the mechanisms driving these components of cancer pain. An added complication is the potential differences that drive these components of cancer induced bone pain, potentially requiring different treatment strategies. We have developed a rat model in which ongoing and breakthrough pain can be examined. Previous studies in rats with neuropathic pain have shown that rats show preference for a chamber previously paired with pain relief, indicating that pain relief is rewarding. We have used this approach in a rat model in which breast cancer cells are injected and allowed to grow in tibia. A peripheral nerve block induced by lidocaine injection onto the saphenous nerve produced a robust conditioned place preference (CPP) in cancer-bearing rats. These findings indicate that afferent drive from the tumor- bearing tibia creates a tonic aversive state (i.e. ongoing pain). Similarly, palpation of the tumor bearing limb selectively produced conditioned place aversion (CPA) in cancer-bearing rats, likely reflecting movement- induced breakthrough pain. In conclusion, we have established a pre-clinical model in which both ongoing and breakthrough components of cancer-induced bone pain can be measured. In addition, we demonstrate that ongoing pain is dependent on afferent input from sensory fibers. Further studies will determine molecular mechanisms driving each of these components of cancer-induced bone pain, serving as the basis for development and testing of novel pain relieving strategies to control multiple components of cancer-

induced bone pain.

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REFERENCES

Akimoto, S., A. Okumura, and H. Fuse. 1998. Relationship between serum levels of interleukin-6, tumor necrosis factor-alpha and bone turnover markers in prostate cancer patients. *Endocr J*. 45:183-189.

Ambrosino, C., and A.R. Nebreda. 2001. Cell cycle regulation by p38 MAP kinases. *Biol Cell*. 93:47-51.

Ara, T., and Y.A. Declerck. 2010. Interleukin-6 in bone metastasis and cancer progression. *Eur J Cancer*. 46:1223-1231.

Aronoff, G.M., M.J. Brennan, D.D. Pritchard, and B. Ginsberg. 2005. Evidence-based oral transmucosal fentanyl citrate (OTFC) dosing guidelines. *Pain Med*. 6:305-314.

Arruda, J.L., R.W. Colburn, A.J. Rickman, M.D. Rutkowski, and J.A. DeLeo. 1998. Increase of interleukin-6 mRNA in the spinal cord following peripheral nerve injury in the rat: potential role of IL-6 in neuropathic pain. *Brain Res Mol Brain Res*. 62:228-235.

Bachelor, M.A., and G.T. Bowden. 2004. UVA-mediated activation of signaling pathways involved in skin tumor promotion and progression. *Semin Cancer Biol*. 14:131-138.

Backonja, M.M. 1999. Gabapentin monotherapy for the symptomatic treatment of painful neuropathy: a multicenter, double-blind, placebo-controlled trial in patients with diabetes mellitus. *Epilepsia*. 40 Suppl 6:S57-59; discussion S73-54.

Backonja, M.M., and B. Stacey. 2004. Neuropathic pain symptoms relative to overall pain rating. *J Pain*. 5:491-497.

Badger, A.M., J.N. Bradbeer, B. Votta, J.C. Lee, J.L. Adams, and D.E. Griswold. 1996a. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. *J Pharmacol Exp Ther*. 279:1453-1461.

Badger, A.M., J.N. Bradbeer, B. Votta, J.C. Lee, J.L. Adams, and D.E. Griswold. 1996b. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone

resorption, endotoxin shock and immune function. *J Pharmacol Exp Ther*. 279:1453-1461.

Barber, M.D., K.C. Fearon, and J.A. Ross. 1999. Relationship of serum levels of interleukin-6, soluble interleukin-6 receptor and tumour necrosis factor receptors to the acute-phase protein response in advanced pancreatic cancer. *Clin Sci (Lond)*. 96:83-87.

Bardo, M.T., and R.A. Bevins. 2000. Conditioned place preference: what does it add to our preclinical understanding of drug reward? *Psychopharmacology (Berl)*. 153:31-43.

Basolo, F., S. Calvo, L. Fiore, P.G. Conaldi, V. Falcone, and A. Toniolo. 1993. Growth-stimulating activity of interleukin 6 on human mammary epithelial cells transfected with the int-2 gene. *Cancer Res*. 53:2957-2960.

Ben-Levy, R., S. Hooper, R. Wilson, H.F. Paterson, and C.J. Marshall. 1998. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr Biol*. 8:1049-1057.

Bennett, D.L. 2001. Neurotrophic factors: important regulators of nociceptive function. *Neuroscientist*. 7:13-17.

Bennett, D.S., S. Simon, M. Brennan, and S.A. Shoemaker. 2007. Prevalence and characteristics of breakthrough pain in patients receiving opioids for chronic back pain in pain specialty clinics. *J Opioid Manag*. 3:101-106.

Bennett, M.I. 2010. Cancer pain terminology: time to develop a taxonomy that promotes good clinical practice and allows research to progress. *Pain*. 149:426-427.

Bharadwaj, U., M. Li, R. Zhang, C. Chen, and Q. Yao. 2007. Elevated interleukin-6 and G-CSF in human pancreatic cancer cell conditioned medium suppress dendritic cell differentiation and activation. *Cancer Res*. 67:5479-5488.

Bianchi, M., G. Rossoni, P. Sacerdote, and A.E. Panerai. 1999. Effects of tramadol on experimental inflammation. *Fundam Clin Pharmacol*. 13:220-225.

Bolin, L.M., A.N. Verity, J.E. Silver, E.M. Shooter, and J.S. Abrams. 1995. Interleukin-6 production by Schwann cells and induction in sciatic nerve injury. *J Neurochem*. 64:850-858.

Bosnjak, S., S. Jelic, S. Susnjar, and V. Luki. 2002. Gabapentin for relief of neuropathic pain related to anticancer treatment: a preliminary study. *J Chemother*. 14:214-219.

Boyle, D.L., T.L. Jones, D. Hammaker, C.I. Svensson, S. Rosengren, S. Albani, L. Sorkin, and G.S. Firestein. 2006. Regulation of peripheral inflammation by spinal p38 MAP kinase in rats. *PLoS Med*. 3:e338.

Bozcuk, H., G. Uslu, M. Samur, M. Yildiz, T. Ozben, M. Ozdogan, M. Artac, H. Altunbas, I. Akan, and B. Savas. 2004. Tumour necrosis factor-alpha, interleukin-6, and fasting serum insulin correlate with clinical outcome in metastatic breast cancer patients treated with chemotherapy. *Cytokine*. 27:58-65.

Brakenhoff, J.P., F.D. de Hon, V. Fontaine, E. ten Boekel, H. Schooltink, S. Rose-John, P.C. Heinrich, J. Content, and L.A. Aarden. 1994. Development of a human interleukin-6 receptor antagonist. *J Biol Chem*. 269:86-93.

Brown, J.E., H. Neville-Webbe, and R.E. Coleman. 2004. The role of bisphosphonates in breast and prostate cancers. *Endocr Relat Cancer*. 11:207-224.

Brubaker, K.D., E. Corey, L.G. Brown, and R.L. Vessella. 2004. Bone morphogenetic protein signaling in prostate cancer cell lines. *J Cell Biochem*. 91:151-160.

Bulavin, D.V., Y. Higashimoto, I.J. Popoff, W.A. Gaarde, V. Basrur, O. Potapova, E. Appella, and A.J. Fornace, Jr. 2001. Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature*. 411:102-107.

Campbell, J.N., and R.A. Meyer. 2006. Mechanisms of neuropathic pain. *Neuron*. 52:77-92.

Caraceni, A., E. Zecca, C. Bonezzi, E. Arcuri, R. Yaya Tur, M. Maltoni, M. Visentin, G. Gorni, C. Martini, W. Tirelli, M. Barbieri, and F. De Conno. 2004. Gabapentin for neuropathic cancer pain: a randomized controlled trial from the Gabapentin Cancer Pain Study Group. *J Clin Oncol*. 22:2909-2917.

Chaplan, S.R., F.W. Bach, J.W. Pogrel, J.M. Chung, and T.L. Yaksh. 1994. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 53:55-63.

Chatenoud, L. 2005. Monoclonal antibody-based strategies in autoimmunity and transplantation. *Methods Mol Med*. 109:297-328.

Chauhan, D., H. Uchiyama, Y. Akbarali, M. Urashima, K. Yamamoto, T.A. Libermann, and K.C. Anderson. 1996. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. *Blood*. 87:1104-1112.

Chen, L., H.Y. He, H.M. Li, J. Zheng, W.J. Heng, J.F. You, and W.G. Fang. 2004. ERK1/2 and p38 pathways are required for P2Y receptor-mediated prostate cancer invasion. *Cancer Lett*. 215:239-247.

Chen, L., J.A. Mayer, T.I. Krisko, C.W. Speers, T. Wang, S.G. Hilsenbeck, and P.H. Brown. 2009. Inhibition of the p38 kinase suppresses the proliferation of human ER-negative breast cancer cells. *Cancer Res*. 69:8853-8861.

Cheng, H.T., J.R. Dauch, S.S. Oh, J.M. Hayes, Y. Hong, and E.L. Feldman. 2010. p38 mediates mechanical allodynia in a mouse model of type 2 diabetes. *Mol Pain*. 6:28.

Chirgwin, J.M., and T.A. Guise. 2000. Molecular mechanisms of tumor-bone interactions in osteolytic metastases. *Crit Rev Eukaryot Gene Expr*. 10:159-178.

Christo, P.J., and D. Mazloomdoost. 2008a. Cancer pain and analgesia. *Ann N Y Acad Sci*. 1138:278-298.

Christo, P.J., and D. Mazloomdoost. 2008b. Interventional pain treatments for cancer pain. *Ann N Y Acad Sci*. 1138:299-328.

Chung, Y.C., and Y.F. Chang. 2003. Serum interleukin-6 levels reflect the disease status of colorectal cancer. *J Surg Oncol*. 83:222-226.

Clark, A.R., J.L. Dean, and J. Saklatvala. 2003. Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. *FEBS Lett*. 546:37-44.

Clohisy, D.R., and P.W. Mantyh. 2003. Bone cancer pain. *Cancer*. 97:866-873.

Copray, J.C., I. Mantingh, N. Brouwer, K. Biber, B.M. Kust, R.S. Liem, I. Huitinga, F.J. Tilders, A.M. Van Dam, and H.W. Boddeke. 2001. Expression of interleukin-1 beta in rat dorsal root ganglia. *J Neuroimmunol*. 118:203-211.

Cornish, J., D. Naot, and I.R. Reid. 2003. Adrenomedullin--a regulator of bone formation. *Regul Pept*. 112:79-86.

Coyle, N., J. Adelhardt, K.M. Foley, and R.K. Portenoy. 1990. Character of terminal illness in the advanced cancer patient: pain and other symptoms during the last four weeks of life. *J Pain Symptom.Manage.* 5:83-93.

Cuadrado, A., and A.R. Nebreda. 2010. Mechanisms and functions of p38 MAPK signalling. *Biochem J*. 429:403-417.

Cuenda, A., P. Cohen, V. Buee-Scherrer, and M. Goedert. 1997. Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *Embo J*. 16:295-305.

Cuenda, A., and S. Rousseau. 2007. p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta*. 1773:1358-1375.

Cui, X.Y., Y. Dai, S.L. Wang, H. Yamanaka, K. Kobayashi, K. Obata, J. Chen, and K. Noguchi. 2008. Differential activation of p38 and extracellular signal-regulated kinase in spinal cord in a model of bee venom-induced inflammation and hyperalgesia. *Mol Pain*. 4:17.

Cunha, F.Q., and W.M. Tamashiro. 1992. Tumour necrosis factor-alpha and interleukin-8 inhibit neutrophil migration in vitro and in vivo. *Mediators Inflamm*. 1:397-401.

D'Mello, R., and A.H. Dickenson. 2008. Spinal cord mechanisms of pain. *British journal of anaesthesia*. 101:8-16.

Daha, L.K., D. Lazar, R. Simak, and H. Pfluger. 2007. Is there a relation between urinary interleukin-6 levels and symptoms before and after intra-vesical glycosaminoglycan substitution therapy in patients with bladder pain syndrome/interstitial cystitis? *Int Urogynecol J Pelvic Floor Dysfunct*. 18:1449-1452.

Davies, A.N. 2006. Cancer-related breakthrough pain. *Br J Hosp Med (Lond)*. 67:414-416.

Davies, A.N., A. Dickman, C. Reid, A.M. Stevens, and G. Zeppetella. 2009. The management of cancer-related breakthrough pain: recommendations of a task group of the Science Committee of the Association for Palliative Medicine of Great Britain and Ireland. *Eur J Pain*. 13:331-338.

De Jongh, R.F., K.C. Vissers, T.F. Meert, L.H. Booij, C.S. De Deyne, and R.J. Heylen. 2003. The role of interleukin-6 in nociception and pain. *Anesth Analg*. 96:1096-1103, table of contents.

DeLeo, J.A., R.W. Colburn, M. Nichols, and A. Malhotra. 1996. Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. *J Interferon Cytokine Res*. 16:695-700.

Dina, O.A., P.G. Green, and J.D. Levine. 2008. Role of interleukin-6 in chronic muscle hyperalgesic priming. *Neuroscience*. 152:521-525.

Dolle, L., I. El Yazidi-Belkoura, E. Adriaenssens, V. Nurcombe, and H. Hondermarck. 2003. Nerve growth factor overexpression and autocrine loop in breast cancer cells. *Oncogene*. 22:5592-5601.

Dong, X., J.J. Xu, and X. He. 2009. [SB203580 reduces proinflammatory cytokine production in rats after cardiopulmonary bypass]. *Nan Fang Yi Ke Da Xue Xue Bao*. 29:2168-2170.

Donovan-Rodriguez, T., A.H. Dickenson, and C.E. Urch. 2005. Gabapentin normalizes spinal neuronal responses that correlate with behavior in a rat model of cancer-induced bone pain. *Anesthesiology*. 102:132-140.

Doyle, A.J., and M. Merrilees. 2004. Synovial cysts of the lumbar facet joints in a symptomatic population: prevalence on magnetic resonance imaging. *Spine (Phila Pa 1976)*. 29:874-878.

Edelmayer, R.M., T.W. Vanderah, L. Majuta, E.T. Zhang, B. Fioravanti, M. De Felice, J.G. Chichorro, M.H. Ossipov, T. King, J. Lai, S.H. Kori, A.C. Nelsen, K.E. Cannon, M.M. Heinricher, and F. Porreca. 2009. Medullary pain facilitating neurons mediate allodynia in headache-related pain. *Ann Neurol*. 65:184-193.

Eisenach, J.C., R.L. Rauck, and R. Curry. 2003. Intrathecal, but not intravenous adenosine reduces allodynia in patients with neuropathic pain. *Pain*. 105:65-70.

Emile, C., J.P. Fermand, and F. Danon. 1994. Interleukin-6 serum levels in patients with multiple myeloma. *Br J Haematol*. 86:439-440.

Enomoto, A., M.C. Rho, A. Fukami, O. Hiraku, K. Komiyama, and M. Hayashi. 2004. Suppression of cancer cachexia by 20S,21-epoxy-resibufogenin-3-acetate-a novel nonpeptide IL-6 receptor antagonist. *Biochem Biophys Res Commun*. 323:1096-1102.

Enting, R.H., C. Mucchiano, W.H. Oldenmenger, M. Fritzon, A. Wallen, S. Goslinga-van der Gaag, P.A. Sillevis Smitt, and E. Delhaas. 2005. The "pain pen" for breakthrough cancer pain: a promising treatment. *J Pain Symptom Manage*. 29:213-217.

Fabian, M.A., W.H. Biggs, 3rd, D.K. Treiber, C.E. Atteridge, M.D. Azimioara, M.G. Benedetti, T.A. Carter, P. Ciceri, P.T. Edeen, M. Floyd, J.M. Ford, M. Galvin, J.L. Gerlach, R.M. Grotzfeld, S. Herrgard, D.E. Insko, M.A. Insko, A.G. Lai, J.M. Lelias, S.A. Mehta, Z.V. Milanov, A.M. Velasco, L.M. Wodicka, H.K. Patel, P.P. Zarrinkar, and D.J. Lockhart. 2005. A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol*. 23:329-336.

Fassoulaki, A., C. Sarantopoulos, A. Melemeni, and Q. Hogan. 2000. EMLA reduces acute and chronic pain after breast surgery for cancer. *Reg Anesth Pain Med*. 25:350-355.

Fassoulaki, A., C. Sarantopoulos, A. Melemeni, and Q. Hogan. 2001. Regional block and mexiletine: the effect on pain after cancer breast surgery. *Reg Anesth Pain Med*. 26:223-228.

Feng, T., N. Yunfeng, Z. Jinbo, Z. Zhipei, Z. Huizhong, L. Li, J. Tao, and W. Yunjie. 2010. Single immunoglobulin IL-1 receptor-related protein attenuates the lipopolysaccharide-induced inflammatory response in A549 cells. *Chem Biol Interact*. 183:442-449.

Festuccia, C., M. Bologna, G.L. Gravina, F. Guerra, A. Angelucci, I. Villanova, D. Millimaggi, and A. Teti. 1999. Osteoblast conditioned media contain TGF-beta1 and modulate the migration of prostate tumor cells and their interactions with extracellular matrix components. *Int J Cancer*. 81:395-403.

Foley, K.M. 2004. Treatment of cancer-related pain. *J Natl Cancer Inst Monogr*:103-104.

Fontanini, G., D. Campani, M. Roncella, D. Cecchetti, S. Calvo, A. Toniolo, and F. Basolo. 1999. Expression of interleukin 6 (IL-6) correlates with oestrogen receptor in human breast carcinoma. *Br J Cancer*. 80:579-584.

Gadient, R.A., and U. Otten. 1996. Postnatal expression of interleukin-6 (IL-6) and IL-6 receptor (IL-6R) mRNAs in rat sympathetic and sensory ganglia. *Brain Res*. 724:41-46.

Gajda, M., J.A. Litwin, D. Adriaensen, J.P. Timmermans, and T. Cichocki. 2004. Segmental distribution and morphometric features of primary sensory neurons projecting to the tibial periosteum in the rat. *Folia Histochem Cytobiol*. 42:95-99.

Galasko, C.S., and J.I. Burn. 1971. Hypercalcaemia in patients with advanced mammary cancer. *Br Med J*. 3:573-577.

Gao, Y.J., and R.R. Ji. 2009. c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? *Open Pain J*. 2:11-17.

Garcia-Tunon, I., M. Ricote, A. Ruiz, B. Fraile, R. Paniagua, and M. Royuela. 2005. IL-6, its receptors and its relationship with bcl-2 and bax proteins in infiltrating and in situ human breast carcinoma. *Histopathology*. 47:82-89.

Gardell, L.R., T.W. Vanderah, S.E. Gardell, R. Wang, M.H. Ossipov, J. Lai, and F. Porreca. 2003. Enhanced evoked excitatory transmitter release in experimental neuropathy requires descending facilitation. *J Neurosci*. 23:8370-8379.

Garnero, P., N. Buchs, J. Zekri, R. Rizzoli, R.E. Coleman, and P.D. Delmas. 2000. Markers of bone turnover for the management of patients with bone metastases from prostate cancer. *Br J Cancer*. 82:858-864.

Garry, E.M., A. Delaney, G. Blackburn-Munro, T. Dickinson, A. Moss, I. Nakalembe, D.C. Robertson, R. Rosie, P. Robberecht, R. Mitchell, and S.M. Fleetwood-Walker. 2005. Activation of p38 and p42/44 MAP kinase in neuropathic pain: involvement of VPAC2 and NK2 receptors and mediation by spinal glia. *Mol Cell Neurosci*. 30:523-537.

Geminder, H., O. Sagi-Assif, L. Goldberg, T. Meshel, G. Rechavi, I.P. Witz, and A. Ben-Baruch. 2001. A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. *J Immunol*. 167:4747-4757.

Godl, K., J. Wissing, A. Kurtenbach, P. Habenberger, S. Blencke, H. Gutbrod, K. Salassidis, M. Stein-Gerlach, A. Missio, M. Cotten, and H. Daub. 2003. An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc Natl Acad Sci U S A*. 100:15434-15439.

Green, A.R., V.L. Green, M.C. White, and V. Speirs. 1997. Expression of cytokine messenger RNA in normal and neoplastic human breast tissue: identification of interleukin-8 as a potential regulatory factor in breast tumours. *Int J Cancer*. 72:937-941.

Guise, T.A. 2000. Molecular mechanisms of osteolytic bone metastases. *Cancer*. 88:2892-2898.

Guise, T.A., W.M. Kozlow, A. Heras-Herzig, S.S. Padalecki, J.J. Yin, and J.M. Chirgwin. 2005. Molecular mechanisms of breast cancer metastases to bone. *Clin Breast Cancer*. 5 Suppl:S46-53.

Guise, T.A., and K.S. Mohammad. 2004. Endothelins in bone cancer metastases. *Cancer Treat Res*. 118:197-212.

Guise, T.A., J.J. Yin, S.D. Taylor, Y. Kumagai, M. Dallas, B.F. Boyce, T. Yoneda, and G.R. Mundy. 1996. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest*. 98:1544-1549.

Gwak, Y.S., T.S. Nam, K.S. Paik, C.E. Hulsebosch, and J.W. Leem. 2003. Attenuation of mechanical hyperalgesia following spinal cord injury by administration of antibodies to nerve growth factor in the rat. *Neurosci Lett*. 336:117-120.

Halvorson, K.G., M.A. Sevcik, J.R. Ghilardi, T.J. Rosol, and P.W. Mantyh. 2006. Similarities and differences in tumor growth, skeletal remodeling and pain in an osteolytic and osteoblastic model of bone cancer. *Clin J Pain*. 22:587-600.

Han, J., J.D. Lee, L. Bibbs, and R.J. Ulevitch. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*. 265:808-811.

Han, S.K., S.J. Jeon, K. Miyazawa, S.Y. Yi, and Y.S. Yoo. 2006. Enhancement of anti-inflammatory tendency by SB203580, p38alpha specific inhibitor, in human fibroblast-like synoviocyte cell line, MH7A. *Rheumatology international*. 26:972-978.

Haugen, D.F., M.J. Hjermstad, N. Hagen, A. Caraceni, and S. Kaasa. 2010. Assessment and classification of cancer breakthrough pain: a systematic literature review. *Pain*. 149:476-482.

Honore, P., N.M. Luger, M.A. Sabino, M.J. Schwei, S.D. Rogers, D.B. Mach, F. O'Keefe P, M.L. Ramnaraine, D.R. Clohisy, and P.W. Mantyh. 2000a. Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. *Nat Med*. 6:521-528.

Honore, P., S.D. Rogers, M.J. Schwei, J.L. Salak-Johnson, N.M. Luger, M.C. Sabino, D.R. Clohisy, and P.W. Mantyh. 2000b. Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. *Neuroscience*. 98:585-598.

Houde, M., P. Laprise, D. Jean, M. Blais, C. Asselin, and N. Rivard. 2001. Intestinal epithelial cell differentiation involves activation of p38 mitogen-activated protein kinase that regulates the homeobox transcription factor CDX2. *J Biol Chem*. 276:21885-21894.

Houle, F., and J. Huot. 2006. Dysregulation of the endothelial cellular response to oxidative stress in cancer. *Mol Carcinog*. 45:362-367.

Hudmon, A., J.S. Choi, L. Tyrrell, J.A. Black, A.M. Rush, S.G. Waxman, and S.D. Dib-Hajj. 2008. Phosphorylation of sodium channel Na(v)1.8 by p38 mitogen-activated protein kinase increases current density in dorsal root ganglion neurons. *J Neurosci*. 28:3190-3201.

Ide, H., T. Yoshida, N. Matsumoto, K. Aoki, Y. Osada, T. Sugimura, and M. Terada. 1997. Growth regulation of human prostate cancer cells by bone morphogenetic protein-2. *Cancer Res*. 57:5022-5027.

Ishikawa, M., K. Tanno, A. Kamo, Y. Takayanagi, and K. Sasaki. 1993. Enhancement of tumor growth by morphine and its possible mechanism in mice. *Biol Pharm Bull*. 16:762-766.

Jakobsson, P.J. 2010. Pain: how macrophages mediate inflammatory pain via ATP signaling. *Nat Rev Rheumatol*. 6:679-681.

Ji, R.R., T.A. Samad, S.X. Jin, R. Schmoll, and C.J. Woolf. 2002. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron*. 36:57-68.

Ji, R.R., and M.R. Suter. 2007a. p38 MAPK, microglial signaling, and neuropathic pain. *Mol Pain*. 3:33.

Ji, R.R., and M.R. Suter. 2007b. p38 MAPK, microglial signaling, and neuropathic pain. *Mol Pain*. 3:33.

Ji, R.R., and C.J. Woolf. 2001. Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. *Neurobiol Dis*. 8:1-10.

Jin, S.X., Z.Y. Zhuang, C.J. Woolf, and R.R. Ji. 2003a. p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci*. 23:4017-4022.

Jin, S.X., Z.Y. Zhuang, C.J. Woolf, and R.R. Ji. 2003b. p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci*. 23:4017-4022.

Johansen, J.P., H.L. Fields, and B.H. Manning. 2001. The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulate cortex. *Proc Natl Acad Sci U S A*. 98:8077-8082.

Jourdan, M., J. De Vos, N. Mechti, and B. Klein. 2000. Regulation of Bcl-2-family proteins in myeloma cells by three myeloma survival factors: interleukin-6, interferon-alpha and insulin-like growth factor 1. *Cell Death Differ*. 7:1244-1252.

Kaan, T.K., P.K. Yip, S. Patel, M. Davies, F. Marchand, D.A. Cockayne, P.A. Nunn, A.H. Dickenson, A.P. Ford, Y. Zhong, M. Malcangio, and S.B. McMahon. 2010. Systemic blockade of P2X3 and P2X2/3 receptors attenuates bone cancer pain behaviour in rats. *Brain*. 133:2549-2564.

Kamano, Y., T. Nogawa, A. Yamashita, M. Hayashi, M. Inoue, P. Drasar, and G.R. Pettit. 2002. Isolation and structure of a 20,21-epoxybufenolide series from "Ch'an Su". *J Nat Prod*. 65:1001-1005.

Kang, K.W., Y. Wagley, H.W. Kim, Y.R. Pokharel, Y.Y. Chung, I.Y. Chang, J.J. Kim, J.S. Moon, Y.K. Kim, S.Y. Nah, H.S. Kang, and J.W. Oh. 2007. Novel role of IL-6/SIL-6R signaling in the expression of inducible nitric oxide synthase (iNOS) in murine B16, metastatic melanoma clone F10.9, cells. *Free Radic Biol Med*. 42:215-227.

Karin, M. 2006a. NF-kappaB and cancer: mechanisms and targets. *Mol Carcinog*. 45:355-361.

Karin, M. 2006b. Nuclear factor-kappaB in cancer development and progression. *Nature*. 441:431-436.

Karin, M., T. Lawrence, and V. Nizet. 2006. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell*. 124:823-835.

Keller, E.T., and J. Brown. 2004. Prostate cancer bone metastases promote both osteolytic and osteoblastic activity. *J Cell Biochem*. 91:718-729.

Kim, M.S., E.J. Lee, H.R. Kim, and A. Moon. 2003. p38 kinase is a key signaling molecule for H-Ras-induced cell motility and invasive phenotype in human breast epithelial cells. *Cancer Res*. 63:5454-5461.

Kim, S.Y., J.C. Bae, J.Y. Kim, H.L. Lee, K.M. Lee, D.S. Kim, and H.J. Cho. 2002. Activation of p38 MAP kinase in the rat dorsal root ganglia and spinal cord following peripheral inflammation and nerve injury. *Neuroreport*. 13:2483-2486.

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

King, T., L. Vera-Portocarrero, T. Gutierrez, T.W. Vanderah, G. Dussor, J. Lai, H.L. Fields, and F. Porreca. 2009. Unmasking the tonic-aversive state in neuropathic pain. *Nat Neurosci*. 12:1364-1366.

Kino, T., T.L. Boos, A. Sulima, E.M. Siegel, P.W. Gold, K.C. Rice, and G.P. Chrousos. 2007. 3-O-Formyl-20R,21-epoxyresibufogenin suppresses IL-6-type cytokine actions by targeting the glycoprotein 130 subunit: potential clinical implications. *J Allergy Clin Immunol*. 120:437-444.

Koj, A. 1996. Initiation of acute phase response and synthesis of cytokines. *Biochim Biophys Acta*. 1317:84-94.

Kong, Y.Y., U. Feige, I. Sarosi, B. Bolon, A. Tafuri, S. Morony, C. Capparelli, J. Li, R. Elliott, S. McCabe, T. Wong, G. Campagnuolo, E. Moran, E.R. Bogoch, G. Van, L.T. Nguyen, P.S. Ohashi, D.L. Lacey, E. Fish, W.J. Boyle, and J.M. Penninger. 1999. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature*. 402:304-309.

Koren, R., D. Rocker, O. Kotestiano, U.A. Liberman, and A. Ravid. 2000. Synergistic anticancer activity of 1,25-dihydroxyvitamin D(3) and immune cytokines: the involvement of reactive oxygen species. *J Steroid Biochem Mol Biol*. 73:105-112.

Korf-Klingebiel, M., T. Kempf, T. Sauer, E. Brinkmann, P. Fischer, G.P. Meyer, A. Ganser, H. Drexler, and K.C. Wollert. 2008. Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. *Eur Heart J*. 29:2851-2858.

Kumar, S., J. Boehm, and J.C. Lee. 2003. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov*. 2:717-726.

Kurihara, N., D. Bertolini, T. Suda, Y. Akiyama, and G.D. Roodman. 1990. IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. *J Immunol*. 144:4226-4230.

Kuroda, K., J. Nakashima, K. Kanao, E. Kikuchi, A. Miyajima, Y. Horiguchi, K. Nakagawa, M. Oya, T. Ohigashi, and M. Murai. 2007. Interleukin 6 is associated with cachexia in patients with prostate cancer. *Urology*. 69:113-117.

Kuzeyli Yildirim, Y., M. Uyar, and C. Fadillioglu. 2005. [Cancer pain and its influence on quality of life]. *Agri*. 17:17-22.

Kyriakis, J.M., and J. Avruch. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev*. 81:807-869.

Lacey, D.L., E. Timms, H.L. Tan, M.J. Kelley, C.R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, H. Hsu, J. Sullivan, N. Hawkins, E. Davy, C. Capparelli, A. Eli, Y.X. Qian, S. Kaufman, I. Sarosi, V. Shalhoub, G. Senaldi, J.

Guo, J. Delaney, and W.J. Boyle. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*. 93:165-176.

Lamb, K., Y.M. Kang, G.F. Gebhart, and K. Bielefeldt. 2003. Nerve growth factor and gastric hyperalgesia in the rat. *Neurogastroenterol Motil*. 15:355-361.

Lang, S.H., N.W. Clarke, N.J. George, and N.G. Testa. 1999. Scatter factor influences the formation of prostate epithelial cell colonies on bone marrow stroma in vitro. *Clin Exp Metastasis*. 17:333-340.

Lee, R.J., C. Albanese, R.J. Stenger, G. Watanabe, G. Inghirami, G.K. Haines, 3rd, M. Webster, W.J. Muller, J.S. Brugge, R.J. Davis, and R.G. Pestell. 1999. pp60(v-src) induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways. A role for cAMP response element-binding protein and activating transcription factor-2 in pp60(v-src) signaling in breast cancer cells. *J Biol Chem*. 274:7341-7350.

Leu, C.M., F.H. Wong, C. Chang, S.F. Huang, and C.P. Hu. 2003. Interleukin-6 acts as an antiapoptotic factor in human esophageal carcinoma cells through the activation of both STAT3 and mitogen-activated protein kinase pathways. *Oncogene*. 22:7809-7818.

Lindqvist, A., H. Kallstrom, and C. Karlsson Rosenthal. 2004. Characterisation of Cdc25B localisation and nuclear export during the cell cycle and in response to stress. *J Cell Sci*. 117:4979-4990.

Lipton, A., E. Small, F. Saad, D. Gleason, D. Gordon, M. Smith, L. Rosen, M.O. Kowalski, D. Reitsma, and J. Seaman. 2002. The new bisphosphonate, Zometa (zoledronic acid), decreases skeletal complications in both osteolytic and osteoblastic lesions: a comparison to pamidronate. *Cancer Invest*. 20 Suppl 2:45-54.

Liu, P., A. Okun, J. Ren, R.C. Guo, M.H. Ossipov, J. Xie, T. King, and F. Porreca. 2011. Ongoing pain in the MIA model of osteoarthritis. *Neurosci Lett*.

Lossignol, D.A., B. Plehiers, and J.J. Body. 2004. [Gabapentin (Neurontin) and cancer pain: a pilot study]. *Rev Med Brux*. 25:429-435.

Luger, N.M., P. Honore, M.A. Sabino, M.J. Schwei, S.D. Rogers, D.B. Mach, D.R. Clohisy, and P.W. Mantyh. 2001. Osteoprotegerin diminishes advanced bone cancer pain. *Cancer Res*. 61:4038-4047.

Luger, N.M., D.B. Mach, M.A. Sevcik, and P.W. Mantyh. 2005. Bone cancer pain: from model to mechanism to therapy. *J Pain Symptom Manage*. 29:S32-46.

Luger, N.M., M.A. Sabino, M.J. Schwei, D.B. Mach, J.D. Pomonis, C.P. Keyser, M. Rathbun, D.R. Clohisy, P. Honore, T.L. Yaksh, and P.W. Mantyh. 2002. Efficacy of systemic morphine suggests a fundamental difference in the mechanisms that generate bone cancer vs inflammatory pain. *Pain*. 99:397-406.

Ma, W., W.H. Zheng, K. Powell, K. Jhamandas, and R. Quirion. 2001. Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: an in vitro and in vivo study. *Eur J Neurosci*. 14:1091-1104.

Mach, D.B., S.D. Rogers, M.C. Sabino, N.M. Luger, M.J. Schwei, J.D. Pomonis, C.P. Keyser, D.R. Clohisy, D.J. Adams, P. O'Leary, and P.W. Mantyh. 2002. Origins of skeletal pain: sensory and sympathetic innervation of the mouse femur. *Neuroscience*. 113:155-166.

Maini, R.N., P.C. Taylor, J. Szechinski, K. Pavelka, J. Broll, G. Balint, P. Emery, F. Raemen, J. Petersen, J. Smolen, D. Thomson, and T. Kishimoto. 2006. Double-blind randomized controlled clinical trial of the interleukin-6 receptor antagonist, tocilizumab, in European patients with rheumatoid arthritis who had an incomplete response to methotrexate. *Arthritis Rheum*. 54:2817-2829.

Mancino, A.T., V.S. Klimberg, M. Yamamoto, S.C. Manolagas, and E. Abe. 2001. Breast cancer increases osteoclastogenesis by secreting M-CSF and upregulating RANKL in stromal cells. *J Surg Res*. 100:18-24.

Manfredi, P.L., G.R. Gonzales, R. Sady, S. Chandler, and R. Payne. 2003. Neuropathic pain in patients with cancer. *J Palliat Care*. 19:115-118.

Mantovani, A., F. Marchesi, C. Portal, P. Allavena, and A. Sica. 2008. Linking inflammation reactions to cancer: novel targets for therapeutic strategies. *Adv Exp Med Biol*. 610:112-127.

Mantovani, A., and M.A. Pierotti. 2008. Cancer and inflammation: a complex relationship. *Cancer Lett*. 267:180-181.

Mantyh, P.W. 2002. A mechanism based understanding of cancer pain. *Pain*. 96:1-2.

Mantyh, P.W. 2006. Cancer pain and its impact on diagnosis, survival and quality of life. *Nat Rev Neurosci*. 7:797-809.

Mantyh, P.W., D.R. Clohisy, M. Koltzenburg, and S.P. Hunt. 2002a. Molecular mechanisms of cancer pain. *Nat Rev Cancer*. 2:201-209.

Mantyh, P.W., D.R. Clohisy, M. Koltzenburg, and S.P. Hunt. 2002b. Molecular mechanisms of cancer pain. *Nat Rev Cancer*. 2:201-209.

Mantyh, W.G., J.M. Jimenez-Andrade, J.I. Stake, A.P. Bloom, M.J. Kaczmarska, R.N. Taylor, K.T. Freeman, J.R. Ghilardi, M.A. Kuskowski, and P.W. Mantyh. 2010. Blockade of nerve sprouting and neuroma formation markedly attenuates the development of late stage cancer pain. *Neuroscience*. 171:588-598.

Marz, P., J.G. Cheng, R.A. Gadient, P.H. Patterson, T. Stoyan, U. Otten, and S. Rose-John. 1998. Sympathetic neurons can produce and respond to interleukin 6. *Proc Natl Acad Sci U S A*. 95:3251-3256.

Marz, P., R.A. Gadient, and U. Otten. 1996. Expression of interleukin-6 receptor (IL-6R) and gp130 mRNA in PC12 cells and sympathetic neurons: modulation by tumor necrosis factor alpha (TNF-alpha). *Brain Res*. 706:71-79.

McBride, W.H. 1986. Phenotype and functions of intratumoral macrophages. *Biochim Biophys Acta*. 865:27-41.

McCarthy, T.L., W.Z. Chang, Y. Liu, and M. Centrella. 2003. Runx2 integrates estrogen activity in osteoblasts. *J Biol Chem*. 278:43121-43129.

McNicol, E., S.A. Strassels, L. Goudas, J. Lau, and D.B. Carr. 2005. NSAIDS or paracetamol, alone or combined with opioids, for cancer pain. *Cochrane Database Syst Rev*:CD005180.

Mercadante, S. 1997. Malignant bone pain: pathophysiology and treatment. *Pain*. 69:1-18.

Mercadante, S. 2002. The use of bisphosphonates in the management of bone metastatic prostate cancer. *Curr Urol Rep*. 3:244-249.

Mercadante, S. 2011. The use of rapid onset opioids for breakthrough cancer pain: The challenge of its dosing. *Crit Rev Oncol Hematol*.

Mercadante, S., F. Fulfaro, and A. Casuccio. 2002a. Pain mechanisms involved and outcome in advanced cancer patients with possible indications for celiac plexus block and superior hypogastric plexus block. *Tumori*. 88:243-245.

Mercadante, S., F. Fulfaro, and A. Casuccio. 2002b. A randomised controlled study on the use of anti-inflammatory drugs in patients with cancer pain on morphine therapy: effects on dose-escalation and a pharmacoeconomic analysis. *Eur J Cancer*. 38:1358-1363.

Mercadante, S., L. Radbruch, A. Davies, P. Poulain, T. Sitte, P. Perkins, T. Colberg, and M.A. Camba. 2009. A comparison of intranasal fentanyl spray with oral transmucosal fentanyl citrate for the treatment of breakthrough cancer pain: an open-label, randomised, crossover trial. *Curr Med Res Opin*. 25:2805-2815.

Mercadante, S., P. Villari, P. Ferrera, M. Bianchi, and A. Casuccio. 2004. Safety and effectiveness of intravenous morphine for episodic (breakthrough) pain using a fixed ratio with the oral daily morphine dose. *J Pain Symptom Manage*. 27:352-359.

Mielke, S., A. Sparreboom, and K. Mross. 2006. Peripheral neuropathy: a persisting challenge in paclitaxel-based regimes. *Eur J Cancer*. 42:24-30.

Mikhailov, A., D. Patel, D.J. McCance, and C.L. Rieder. 2007. The G2 p38-mediated stress-activated checkpoint pathway becomes attenuated in transformed cells. *Curr Biol*. 17:2162-2168.

Mishra, S., S. Bhatnagar, P. Chaudhary, and S.P. Rana. 2009. Breakthrough cancer pain: review of prevalence, characteristics and management. *Indian J Palliat Care*. 15:14-18.

Mizushima, T., K. Obata, H. Yamanaka, Y. Dai, T. Fukuoka, A. Tokunaga, T. Mashimo, and K. Noguchi. 2005. Activation of p38 MAPK in primary afferent neurons by noxious stimulation and its involvement in the development of thermal hyperalgesia. *Pain*. 113:51-60.

Mori, K., B. Le Goff, C. Charrier, S. Battaglia, D. Heymann, and F. Redini. 2007. DU145 human prostate cancer cells express functional receptor activator of NFkappaB: new insights in the prostate cancer bone metastasis process. *Bone*. 40:981-990.

Morrissey, C., J.S. Lai, L.G. Brown, Y.C. Wang, M.P. Roudier, I.M. Coleman, R. Gulati, F. Vakar-Lopez, L.D. True, E. Corey, P.S. Nelson, and R.L. Vessella. 2010. The expression of osteoclastogenesis-associated factors and osteoblast response to osteolytic prostate cancer cells. *Prostate*. 70:412-424.

Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verastegui, and A. Zlotnik. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 410:50-56.

Mundy, G.R. 1991. Inflammatory mediators and the destruction of bone. *J Periodontal Res*. 26:213-217.

Nachbaur, D.M., M. Herold, A. Maneschg, and H. Huber. 1991. Serum levels of interleukin-6 in multiple myeloma and other hematological disorders: correlation with disease activity and other prognostic parameters. *Ann Hematol*. 62:54-58.

Nelson, J.B., S.P. Hedican, D.J. George, A.H. Reddi, S. Piantadosi, M.A. Eisenberger, and J.W. Simons. 1995. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med*. 1:944-949.

Neugebauer, V., P. Rumenapp, and H.G. Schaible. 1996. Calcitonin gene-related peptide is involved in the spinal processing of mechanosensory input from the rat's knee joint and in the generation and maintenance of hyperexcitability of dorsal horn-neurons during development of acute inflammation. *Neuroscience*. 71:1095-1109.

Neve, R.M., T. Holbro, and N.E. Hynes. 2002. Distinct roles for phosphoinositide 3-kinase, mitogen-activated protein kinase and p38 MAPK in mediating cell cycle progression of breast cancer cells. *Oncogene*. 21:4567-4576.

Nishimoto, N. 2004. [Anti-cytokine therapy for intractable hematologic diseases using monoclonal antibodies]. *Nippon Naika Gakkai Zasshi*. 93:390-396.

Nishimoto, N. 2006. [IL-6 targetting therapy for inflammatory immune diseases]. *Nippon Naika Gakkai Zasshi*. 95:1795-1800.

Nomura, H., A. Furuta, Y. Tanaka, and T. Iwaki. 2005. Forced retraction of spinal root injury enhances activation of p38 MAPK cascade in infiltrating macrophages. *Neuropathology*. 25:37-47.

Nordlind, K., L. Eriksson, A. Seiger, and M. Bakhiet. 2000. Expression of interleukin-6 in human dorsal root ganglion cells. *Neurosci Lett*. 280:139-142.

Oades, G.M., J. Coxon, and K.W. Colston. 2002. The potential role of bisphosphonates in prostate cancer. *Prostate Cancer Prostatic Dis*. 5:264-272.

Oka, T., K. Oka, M. Hosoi, and T. Hori. 1995. Intracerebroventricular injection of interleukin-6 induces thermal hyperalgesia in rats. *Brain Res*. 692:123-128.

Okada, S., T. Okusaka, H. Ishii, A. Kyogoku, M. Yoshimori, N. Kajimura, K. Yamaguchi, and T. Kakizoe. 1998. Elevated serum interleukin-6 levels in patients with pancreatic cancer. *Jpn J Clin Oncol*. 28:12-15.

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

Olsen, N.K., P. Pfeiffer, K. Mondrup, and C. Rose. 1990. Radiation-induced brachial plexus neuropathy in breast cancer patients. *Acta Oncol*. 29:885-890.

Oneschuk, D., and M.Z. al-Shahri. 2003. The pattern of gabapentin use in a tertiary palliative care unit. *J Palliat Care*. 19:185-187.

Ono, K., and J. Han. 2000. The p38 signal transduction pathway: activation and function. *Cell Signal*. 12:1-13.

Opree, A., and M. Kress. 2000. Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. *J Neurosci*. 20:6289-6293.

Ossipov, M.H., J. Lai, T. King, T.W. Vanderah, and F. Porreca. 2005. Underlying mechanisms of pronociceptive consequences of prolonged morphine exposure. *Biopolymers*. 80:319-324.

Ossipov, M.H., J. Lai, T.P. Malan, Jr., and F. Porreca. 2000. Spinal and supraspinal mechanisms of neuropathic pain. *Ann N Y Acad Sci*. 909:12-24.

Pages, G., E. Berra, J. Milanini, A.P. Levy, and J. Pouyssegur. 2000. Stress-activated protein kinases (JNK and p38/HOG) are essential for vascular endothelial growth factor mRNA stability. *J Biol Chem*. 275:26484-26491.

Pavlakis, N., R. Schmidt, and M. Stockler. 2005. Bisphosphonates for breast cancer. *Cochrane Database Syst Rev*:CD003474.

Perl, E.R. 2011. Pain Mechanisms: A Commentary on Concepts and Issues. *Prog Neurobiol*.

Peters, C.M., J.R. Ghilardi, C.P. Keyser, K. Kubota, T.H. Lindsay, N.M. Luger, D.B. Mach, M.J. Schwei, M.A. Sevcik, and P.W. Mantyh. 2005. Tumor-induced injury of primary afferent sensory nerve fibers in bone cancer pain. *Exp Neurol*. 193:85-100.

Platanias, L.C. 2003. Map kinase signaling pathways and hematologic malignancies. *Blood*. 101:4667-4679.

Polomano, R.C., and J.T. Farrar. 2006. Pain and neuropathy in cancer survivors. Surgery, radiation, and chemotherapy can cause pain; research could improve its detection and treatment. *Am J Nurs*. 106:39-47.

Pomonis, J.D., S.D. Rogers, C.M. Peters, J.R. Ghilardi, and P.W. Mantyh. 2001. Expression and localization of endothelin receptors: implications for the involvement of peripheral glia in nociception. *J Neurosci*. 21:999-1006.

Poole, A.R. 1999. An introduction to the pathophysiology of osteoarthritis. *Front Biosci*. 4:D662-670.

Portenoy, R.K., D.S. Bennett, R. Rauck, S. Simon, D. Taylor, M. Brennan, and S. Shoemaker. 2006. Prevalence and characteristics of breakthrough pain in opioid-treated patients with chronic noncancer pain. *J Pain*. 7:583-591.

Portenoy, R.K., M.A. Southam, S.K. Gupta, J. Lapin, M. Layman, C.E. Inturrisi, and K.M. Foley. 1993. Transdermal fentanyl for cancer pain. Repeated dose pharmacokinetics. *Anesthesiology*. 78:36-43.

Purohit, A., M.W. Ghilchik, L. Duncan, D.Y. Wang, A. Singh, M.M. Walker, and M.J. Reed. 1995. Aromatase activity and interleukin-6 production by normal and malignant breast tissues. *J Clin Endocrinol Metab*. 80:3052-3058.

Quasthoff, S., and H.P. Hartung. 2002. Chemotherapy-induced peripheral neuropathy. *J Neurol*. 249:9-17.

Raghavendra, V., F. Tanga, and J.A. DeLeo. 2003. Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *The Journal of pharmacology and experimental therapeutics*. 306:624-630.

Raingeaud, J., S. Gupta, J.S. Rogers, M. Dickens, J. Han, R.J. Ulevitch, and R.J. Davis. 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem*. 270:7420-7426.

Recio, J.A., and G. Merlino. 2002. Hepatocyte growth factor/scatter factor activates proliferation in melanoma cells through p38 MAPK, ATF-2 and cyclin D1. *Oncogene*. 21:1000-1008.

Reddi, A.H. 1994. Bone and cartilage differentiation. *Curr Opin Genet Dev*. 4:737-744.

Rice, A.S. 2008. Should cannabinoids be used as analgesics for neuropathic pain? *Nat Clin Pract Neurol*. 4:654-655.

Ricote, M., I. Garcia-Tunon, F. Bethencourt, B. Fraile, P. Onsurbe, R. Paniagua, and M. Royuela. 2006. The p38 transduction pathway in prostatic neoplasia. *J Pathol*. 208:401-407.

Rosenbaum, E., and M.A. Carducci. 2003. Pharmacotherapy of hormone refractory prostate cancer: new developments and challenges. *Expert Opin Pharmacother*. 4:875-887.

Ross, J.R., K. Goller, J. Hardy, J. Riley, K. Broadley, R. A'Hern, and J. Williams. 2005. Gabapentin is effective in the treatment of cancer-related neuropathic pain: a prospective, open-label study. *J Palliat Med*. 8:1118-1126.

Ross, J.R., C. Waight, J. Riley, and K. Broadley. 2001. Gabapentin: resistant neuropathic pain and malignancy. *Palliat Med*. 15:348-349.

Rousseau, S., F. Houle, and J. Huot. 2000. Integrating the VEGF signals leading to actin-based motility in vascular endothelial cells. *Trends Cardiovasc Med*. 10:321-327.

Rowbotham, M.C. 2005. Mechanisms of neuropathic pain and their implications for the design of clinical trials. *Neurology*. 65:S66-73.

Sabino, M.A., and P.W. Mantyh. 2005. Pathophysiology of bone cancer pain. *J Support Oncol*. 3:15-24.

Saccani, S., S. Pantano, and G. Natoli. 2002. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat Immunol*. 3:69-75.

Safieh-Garabedian, B., S. Poole, A. Allchorne, J. Winter, and C.J. Woolf. 1995. Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. *Br J Pharmacol*. 115:1265-1275.

Saito, O., T. Aoe, and T. Yamamoto. 2005. Analgesic effects of nonsteroidal antiinflammatory drugs, acetaminophen, and morphine in a mouse model of bone cancer pain. *J Anesth*. 19:218-224.

Salvador, J.M., P.R. Mittelstadt, G.I. Belova, A.J. Fornace, Jr., and J.D. Ashwell. 2005a. The autoimmune suppressor Gadd45alpha inhibits the T cell alternative p38 activation pathway. *Nat Immunol*. 6:396-402.

Salvador, J.M., P.R. Mittelstadt, T. Guszczynski, T.D. Copeland, H. Yamaguchi, E. Appella, A.J. Fornace, Jr., and J.D. Ashwell. 2005b. Alternative p38 activation pathway mediated by T cell receptor-proximal tyrosine kinases. *Nat Immunol*. 6:390-395.

Sanz-Rodriguez, F., A. Hidalgo, and J. Teixido. 2001. Chemokine stromal cell-derived factor-1alpha modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. *Blood*. 97:346-351.

Sarantopoulos, C., B. McCallum, W.M. Kwok, and Q. Hogan. 2002. Gabapentin decreases membrane calcium currents in injured as well as in control mammalian primary afferent neurons. *Reg Anesth Pain Med*. 27:47-57.

Sarantopoulos, C.D. 2007. Advances in the therapy of cancer pain: from novel experimental models to evidence-based treatments. *SIGNA VITAE*. 2:23-41.

Schafers, M., C.I. Svensson, C. Sommer, and L.S. Sorkin. 2003a. Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *J Neurosci*. 23:2517-2521.

Schafers, M., C.I. Svensson, C. Sommer, and L.S. Sorkin. 2003b. Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *J Neurosci*. 23:2517-2521.

Schaible, U.E., S. Gay, C. Museteanu, M.D. Kramer, G. Zimmer, K. Eichmann, U. Museteanu, and M.M. Simon. 1990. Lyme borreliosis in the severe combined immunodeficiency (scid) mouse manifests predominantly in the joints, heart, and liver. *Am J Pathol*. 137:811-820.

Schibler, K.R., Y.C. Yang, and R.D. Christensen. 1992. Effect of interleukin-11 on cycling status and clonogenic maturation of fetal and adult hematopoietic progenitors. *Blood*. 80:900-903.

Schobitz, B., F. Holsboer, R. Kikkert, W. Sutanto, and E.R. De Kloet. 1992a. Peripheral and central regulation of IL-6 gene expression in endotoxin-treated rats. *Endocr Regul*. 26:103-109.

Schobitz, B., D.A. Voorhuis, and E.R. De Kloet. 1992b. Localization of interleukin 6 mRNA and interleukin 6 receptor mRNA in rat brain. *Neurosci Lett*. 136:189-192.

Schwarze, M.M., and R.G. Hawley. 1995. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-xL. *Cancer Res*. 55:2262-2265.

Schwei, M.J., P. Honore, S.D. Rogers, J.L. Salak-Johnson, M.P. Finke, M.L. Ramnaraine, D.R. Clohisy, and P.W. Mantyh. 1999. Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. *J Neurosci*. 19:10886-10897.

Serafini, A.N. 2001. Therapy of metastatic bone pain. *J Nucl Med*. 42:895-906.

Sevcik, M.A., J.R. Ghilardi, C.M. Peters, T.H. Lindsay, K.G. Halvorson, B.M. Jonas, K. Kubota, M.A. Kuskowski, L. Boustany, D.L. Shelton, and P.W. Mantyh. 2005.

Anti-NGF therapy profoundly reduces bone cancer pain and the accompanying increase in markers of peripheral and central sensitization. *Pain*. 115:128-141.

Sevcik, M.A., N.M. Luger, D.B. Mach, M.A. Sabino, C.M. Peters, J.R. Ghilardi, M.J. Schwei, H. Rohrich, C. De Felipe, M.A. Kuskowski, and P.W. Mantyh. 2004. Bone cancer pain: the effects of the bisphosphonate alendronate on pain, skeletal remodeling, tumor growth and tumor necrosis. *Pain*. 111:169-180.

Seybold, V.S. 2009. The role of peptides in central sensitization. *Handb Exp Pharmacol*:451-491.

Silva, G., A. Cunha, I.P. Gregoire, M.P. Seldon, and M.P. Soares. 2006. The antiapoptotic effect of heme oxygenase-1 in endothelial cells involves the degradation of p38 alpha MAPK isoform. *J Immunol*. 177:1894-1903.

Silverman, S.M. 2009. Opioid induced hyperalgesia: clinical implications for the pain practitioner. *Pain Physician*. 12:679-684.

Simi, A., M. Porsmyr-Palmertz, A. Hjerten, M. Ingelman-Sundberg, and N. Tindberg. 2002. The neuroprotective agents chlomethiazole and SB203580 inhibit IL-1beta signalling but not its biosynthesis in rat cortical glial cells. *J Neurochem*. 83:727-737.

Simon, C., H. Goepfert, and D. Boyd. 1998. Inhibition of the p38 mitogen-activated protein kinase by SB 203580 blocks PMA-induced Mr 92,000 type IV collagenase secretion and in vitro invasion. *Cancer Res*. 58:1135-1139.

Simonet, W.S., D.L. Lacey, C.R. Dunstan, M. Kelley, M.S. Chang, R. Luthy, H.Q. Nguyen, S. Wooden, L. Bennett, T. Boone, G. Shimamoto, M. DeRose, R. Elliott, A. Colombero, H.L. Tan, G. Trail, J. Sullivan, E. Davy, N. Bucay, L. Renshaw-Gegg, T.M. Hughes, D. Hill, W. Pattison, P. Campbell, S. Sander, G. Van, J. Tarpley, P. Derby, R. Lee, and W.J. Boyle. 1997. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*. 89:309-319.

Smolen, J.S., A. Beaulieu, A. Rubbert-Roth, C. Ramos-Remus, J. Rovensky, E. Alecock, T. Woodworth, and R. Alten. 2008. Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. *Lancet*. 371:987-997.

Smolen, J.S., and R.N. Maini. 2006. Interleukin-6: a new therapeutic target. *Arthritis Res Ther*. 8 Suppl 2:S5.

Sommer, C., and M. Kress. 2004. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neurosci Lett*. 361:184-187.

Sommer, C., M. Marziniak, and R.R. Myers. 1998. The effect of thalidomide treatment on vascular pathology and hyperalgesia caused by chronic constriction injury of rat nerve. *Pain*. 74:83-91.

Sommer, F., G. Faller, M. Rollinghoff, T. Kirchner, T.W. Mak, and M. Lohoff. 2001. Lack of gastritis and of an adaptive immune response in interferon regulatory factor-1-deficient mice infected with Helicobacter pylori. *Eur J Immunol*. 31:396-402.

Sora, I., C. Wichems, N. Takahashi, X.F. Li, Z. Zeng, R. Revay, K.P. Lesch, D.L. Murphy, and G.R. Uhl. 1998. Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. *Proc Natl Acad Sci U S A*. 95:7699-7704.

Sorkin, L.S., D.L. Boyle, D. Hammaker, D.S. Herman, E. Vail, and G.S. Firestein. 2009. MKK3, an upstream activator of p38, contributes to formalin phase 2 and late allodynia in mice. *Neuroscience*. 162:462-471.

Sorkin, L.S., K.N. Westlund, K.A. Sluka, P.M. Dougherty, and W.D. Willis. 1992. Neural changes in acute arthritis in monkeys. IV. Time-course of amino acid release into the lumbar dorsal horn. *Brain Res Brain Res Rev*. 17:39-50.

Strand, V., and J.A. Singh. 2010. Newer biological agents in rheumatoid arthritis: impact on health-related quality of life and productivity. *Drugs*. 70:121-145.

Strassmann, G., M. Fong, J.S. Kenney, and C.O. Jacob. 1992. Evidence for the involvement of interleukin 6 in experimental cancer cachexia. *J Clin Invest*. 89:1681-1684.

Sueoka, N., E. Sueoka, S. Okabe, and H. Fujiki. 1996. Anti-cancer effects of morphine through inhibition of tumour necrosis factor-alpha release and mRNA expression. *Carcinogenesis*. 17:2337-2341.

Sung, V., J.T. Stubbs, 3rd, L. Fisher, A.D. Aaron, and E.W. Thompson. 1998. Bone sialoprotein supports breast cancer cell adhesion proliferation and migration through differential usage of the alpha(v)beta3 and alpha(v)beta5 integrins. *J Cell Physiol*. 176:482-494.

Svensson, C.I., B. Fitzsimmons, S. Azizi, H.C. Powell, X.Y. Hua, and T.L. Yaksh. 2005. Spinal p38beta isoform mediates tissue injury-induced hyperalgesia and spinal sensitization. *J Neurochem*. 92:1508-1520.

Svensson, C.I., X.Y. Hua, A.A. Protter, H.C. Powell, and T.L. Yaksh. 2003a. Spinal p38 MAP kinase is necessary for NMDA-induced spinal PGE(2) release and thermal hyperalgesia. *Neuroreport*. 14:1153-1157.

Svensson, C.I., M. Marsala, A. Westerlund, N.A. Calcutt, W.M. Campana, J.D. Freshwater, R. Catalano, Y. Feng, A.A. Protter, B. Scott, and T.L. Yaksh. 2003b. Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. *J Neurochem*. 86:1534-1544.

Svensson, C.I., M. Marsala, A. Westerlund, N.A. Calcutt, W.M. Campana, J.D. Freshwater, R. Catalano, Y. Feng, A.A. Protter, B. Scott, and T.L. Yaksh. 2003c. Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. *J Neurochem*. 86:1534-1544.

Svensson, C.I., S. Medicherla, S. Malkmus, Y. Jiang, J.Y. Ma, I. Kerr, J. Brainin-Mattos, H.C. Powell, Z.D. Luo, S. Chakravarty, S. Dugar, L.S. Higgins, A.A. Protter, and T.L. Yaksh. 2008. Role of p38 mitogen activated protein kinase in a model of osteosarcoma-induced pain. *Pharmacol Biochem Behav*. 90:664-675.

Takekawa, M., M. Adachi, A. Nakahata, I. Nakayama, F. Itoh, H. Tsukuda, Y. Taya, and K. Imai. 2000. p53-inducible wip1 phosphatase mediates a negative feedback regulation of p38 MAPK-p53 signaling in response to UV radiation. *Embo J*. 19:6517-6526.

Takekawa, M., and H. Saito. 1998. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell*. 95:521-530.

Tanoue, T., M. Adachi, T. Moriguchi, and E. Nishida. 2000. A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat Cell Biol*. 2:110-116.

Temeles, D.S., H.E. McGrath, E.L. Kittler, R.K. Shadduck, V.K. Kister, R.B. Crittenden, B.L. Turner, and P.J. Quesenberry. 1993. Cytokine expression from bone marrow derived macrophages. *Exp Hematol*. 21:388-393.

Thacker, M.A., A.K. Clark, F. Marchand, and S.B. McMahon. 2007. Pathophysiology of peripheral neuropathic pain: immune cells and molecules. *Anesth Analg*. 105:838-847.

Thomas, J., M. Gupta, Y. Grasso, C.A. Reddy, W.D. Heston, C. Zippe, R. Dreicer, P.A. Kupelian, J. Brainard, H.S. Levin, and E.A. Klein. 2002. Preoperative combined nested reverse transcriptase polymerase chain reaction for prostate-specific antigen and prostate-specific membrane antigen does not correlate with pathologic stage or biochemical failure in patients with localized prostate cancer undergoing radical prostatectomy. *J Clin Oncol*. 20:3213-3218.

Timoshenko, A.V., C. Chakraborty, G.F. Wagner, and P.K. Lala. 2006. COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. *Br J Cancer*. 94:1154-1163.

Tsuda, M., K. Inoue, and M.W. Salter. 2005. Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. *Trends Neurosci*. 28:101-107.

Tsuda, M., A. Mizokoshi, Y. Shigemoto-Mogami, S. Koizumi, and K. Inoue. 2004. Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. *Glia*. 45:89-95.

Ueda, T., E. Shimada, and T. Urakawa. 1994. Serum levels of cytokines in patients with colorectal cancer: possible involvement of interleukin-6 and interleukin-8 in hematogenous metastasis. *J Gastroenterol*. 29:423-429.

Urch, C.E., T. Donovan-Rodriguez, R. Gordon-Williams, L.A. Bee, and A.H. Dickenson. 2005. Efficacy of chronic morphine in a rat model of cancer-induced bone pain: behavior and in dorsal horn pathophysiology. *J Pain*. 6:837-845.

Vachon, P.H., C. Harnois, A. Grenier, G. Dufour, V. Bouchard, J. Han, J. Landry, J.F. Beaulieu, A. Vezina, A.B. Dydensborg, R. Gauthier, A. Cote, J.F. Drolet, and F. Lareau. 2002. Differentiation state-selective roles of p38 isoforms in human intestinal epithelial cell anoikis. *Gastroenterology*. 123:1980-1991.

van der Pluijm, G., B. Sijmons, H. Vloedgraven, C. van der Bent, J.W. Drijfhout, J. Verheijen, P. Quax, M. Karperien, S. Papapoulos, and C. Lowik. 2001. Urokinase-receptor/integrin complexes are functionally involved in adhesion and progression of human breast cancer in vivo. *Am J Pathol*. 159:971-982.

Vanderkerken, K., I. Vande Broek, D.L. Eizirik, E. Van Valckenborgh, K. Asosingh, I. Van Riet, and B. Van Camp. 2002. Monocyte chemoattractant protein-1 (MCP-1),

secreted by bone marrow endothelial cells, induces chemoattraction of 5T multiple myeloma cells. *Clin Exp Metastasis*. 19:87-90.

Vardanyan, M., O.K. Melemedjian, T.J. Price, M.H. Ossipov, J. Lai, E. Roberts, T.L. Boos, J.R. Deschamps, A.E. Jacobson, K.C. Rice, and F. Porreca. 2010. Reversal of pancreatitis-induced pain by an orally available, small molecule interleukin-6 receptor antagonist. *Pain*. 151:257-265.

Vera-Portocarrero, L.P., M.H. Ossipov, T. King, and F. Porreca. 2008. Reversal of inflammatory and noninflammatory visceral pain by central or peripheral actions of sumatriptan. *Gastroenterology*. 135:1369-1378.

Vera-Portocarrero, L.P., E.T. Zhang, M.H. Ossipov, J.Y. Xie, T. King, J. Lai, and F. Porreca. 2006. Descending facilitation from the rostral ventromedial medulla maintains nerve injury-induced central sensitization. *Neuroscience*. 140:1311-1320.

Vierck, C.J., P.T. Hansson, and R.P. Yezierski. 2008. Clinical and pre-clinical pain assessment: are we measuring the same thing? *Pain*. 135:7-10.

Wacnik, P.W., L.J. Eikmeier, T.R. Ruggles, M.L. Ramnaraine, B.K. Walcheck, A.J. Beitz, and G.L. Wilcox. 2001. Functional interactions between tumor and peripheral nerve: morphology, algogen identification, and behavioral characterization of a new murine model of cancer pain. *J Neurosci*. 21:9355-9366.

Wagner, E.F., and A.R. Nebreda. 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer*. 9:537-549.

Walther, M.M., B. Johnson, D. Culley, R. Shah, J. Weber, D. Venzon, J.C. Yang, W.M. Linehan, and S.A. Rosenberg. 1998. Serum interleukin-6 levels in metastatic renal cell carcinoma before treatment with interleukin-2 correlates with paraneoplastic syndromes but not patient survival. *J Urol*. 159:718-722.

Wang, G., S. Haile, B. Comuzzi, A.H. Tien, J. Wang, T.M. Yong, A.E. Jelescu-Bodos, N. Blaszczyk, R.L. Vessella, B.A. Masri, and M.D. Sadar. 2009. Osteoblast-derived factors induce an expression signature that identifies prostate cancer metastasis and hormonal progression. *Cancer Res*. 69:3433-3442.

Watkins, D.N., D.J. Peroni, J.C. Lenzo, D.A. Knight, M.J. Garlepp, and P.J. Thompson. 1999. Expression and localization of COX-2 in human airways and cultured airway epithelial cells. *Eur Respir J*. 13:999-1007.

Watkins, L.R., L.E. Goehler, J. Relton, M.T. Brewer, and S.F. Maier. 1995. Mechanisms of tumor necrosis factor-alpha (TNF-alpha) hyperalgesia. *Brain Res*. 692:244-250.

Watkins, L.R., E.D. Milligan, and S.F. Maier. 2001a. Glial activation: a driving force for pathological pain. *Trends Neurosci*. 24:450-455.

Watkins, L.R., E.D. Milligan, and S.F. Maier. 2001b. Spinal cord glia: new players in pain. *Pain*. 93:201-205.

Watkins, L.R., E.P. Wiertelak, L.E. Goehler, K.P. Smith, D. Martin, and S.F. Maier. 1994. Characterization of cytokine-induced hyperalgesia. *Brain Res*. 654:15-26.

Wen, Y.R., M.R. Suter, R.R. Ji, G.C. Yeh, Y.S. Wu, K.C. Wang, T. Kohno, W.Z. Sun, and C.C. Wang. 2009. Activation of p38 mitogen-activated protein kinase in spinal microglia contributes to incision-induced mechanical allodynia. *Anesthesiology*. 110:155-165.

Wiffen, P.J., J.E. Edwards, J. Barden, and H.J. McQuay. 2003. Oral morphine for cancer pain. *Cochrane Database Syst Rev*:CD003868.

Winding, B., H. Misander, C. Sveigaard, B. Therkildsen, M. Jakobsen, T. Overgaard, M.J. Oursler, and N.T. Foged. 2000. Human breast cancer cells induced angiogenesis, recruitment, and activation of osteoclasts in osteolytic metastasis. *J Cancer Res Clin Oncol*. 126:631-640.

Woolf, C.J., A. Allchorne, B. Safieh-Garabedian, and S. Poole. 1997. Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. *Br J Pharmacol*. 121:417-424.

Woolf, C.J., and M.W. Salter. 2000. Neuronal plasticity: increasing the gain in pain. *Science*. 288:1765-1769.

Xu, L., Y. Huang, X. Yu, J. Yue, N. Yang, and P. Zuo. 2007. The influence of p38 mitogen-activated protein kinase inhibitor on synthesis of inflammatory cytokine tumor necrosis factor alpha in spinal cord of rats with chronic constriction injury. *Anesthesia and analgesia*. 105:1838-1844, table of contents.

Xu, X.J., J.X. Hao, S. Andell-Jonsson, V. Poli, T. Bartfai, and Z. Wiesenfeld-Hallin. 1997. Nociceptive responses in interleukin-6-deficient mice to peripheral inflammation and peripheral nerve section. *Cytokine*. 9:1028-1033.

Yaccoby, S., R.N. Pearse, C.L. Johnson, B. Barlogie, Y. Choi, and J. Epstein. 2002. Myeloma interacts with the bone marrow microenvironment to induce

osteoclastogenesis and is dependent on osteoclast activity. *Br J Haematol*. 116:278-290.

Yaksh, T.L., G. Kokotos, C.I. Svensson, D. Stephens, C.G. Kokotos, B. Fitzsimmons, D. Hadjipavlou-Litina, X.Y. Hua, and E.A. Dennis. 2006. Systemic and intrathecal effects of a novel series of phospholipase A2 inhibitors on hyperalgesia and spinal prostaglandin E2 release. *J Pharmacol Exp Ther*. 316:466-475.

Yasuda, H., N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, E. Tsuda, T. Morinaga, K. Higashio, N. Udagawa, N. Takahashi, and T. Suda. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A*. 95:3597-3602.

Yeomans, J., and M. Baptista. 1997. Both nicotinic and muscarinic receptors in ventral tegmental area contribute to brain-stimulation reward. *Pharmacol Biochem Behav*. 57:915-921.

Yi, B., P.J. Williams, M. Niewolna, Y. Wang, and T. Yoneda. 2002. Tumor-derived platelet-derived growth factor-BB plays a critical role in osteosclerotic bone metastasis in an animal model of human breast cancer. *Cancer Res*. 62:917-923.

Yin, J.J., K.S. Mohammad, S.M. Kakonen, S. Harris, J.R. Wu-Wong, J.L. Wessale, R.J. Padley, I.R. Garrett, J.M. Chirgwin, and T.A. Guise. 2003. A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. *Proc Natl Acad Sci U S A*. 100:10954-10959.

Yin, J.J., K. Selander, J.M. Chirgwin, M. Dallas, B.G. Grubbs, R. Wieser, J. Massague, G.R. Mundy, and T.A. Guise. 1999. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest*. 103:197-206.

Yokoe, T., Y. Iino, and Y. Morishita. 2000. Trends of IL-6 and IL-8 levels in patients with recurrent breast cancer: preliminary report. *Breast Cancer*. 7:187-190.

Yonou, H., A. Ochiai, M. Goya, N. Kanomata, S. Hokama, M. Morozumi, K. Sugaya, T. Hatano, and Y. Ogawa. 2004. Intraosseous growth of human prostate cancer in implanted adult human bone: relationship of prostate cancer cells to osteoclasts in osteoblastic metastatic lesions. *Prostate*. 58:406-413.

Yuen, K.K., M. Shelley, W.M. Sze, T. Wilt, and M.D. Mason. 2006. Bisphosphonates for advanced prostate cancer. *Cochrane Database Syst Rev*:CD006250.

Yun, I.A., K.T. Wakabayashi, H.L. Fields, and S.M. Nicola. 2004. The ventral tegmental area is required for the behavioral and nucleus accumbens neuronal firing responses to incentive cues. *J Neurosci*. 24:2923-2933.

Zeppetella, G. 2008. Opioids for cancer breakthrough pain: a pilot study reporting patient assessment of time to meaningful pain relief. *J Pain Symptom Manage*. 35:563-567.

Zeppetella, G., and M.D. Ribeiro. 2006. Opioids for the management of breakthrough (episodic) pain in cancer patients. *Cochrane Database Syst Rev*:CD004311.

Zhang, F.E., J.L. Cao, L.C. Zhang, and Y.M. Zeng. 2005. Activation of p38 mitogen-activated protein kinase in spinal cord contributes to chronic constriction injury-induced neuropathic pain. *Sheng Li Xue Bao*. 57:545-551.

Zhou, W.D., H.M. Yang, Q. Wang, D.Y. Su, F.A. Liu, M. Zhao, Q.H. Chen, and Q.X. Chen. 2010. SB203580, a p38 mitogen-activated protein kinase inhibitor, suppresses the development of endometriosis by down-regulating proinflammatory cytokines and proteolytic factors in a mouse model. *Human reproduction*. 25:3110-3116.

Zudaire, E., A. Martinez, and F. Cuttitta. 2003. Adrenomedullin and cancer. *Regul Pept*. 112:175-183.