Bivalent Ligand MMG22 Reduces Bone Cancer Pain without Tolerance or Sedation

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Acknowledgements

Dedication

I lovingly dedicate this work to my children, the apple of my eyes;
(Abdelhamed & Joud)
To my beloved husband (Adel Gumma)
To my parents Saleh Shueb, Suad Suliman and Franca Puddo
To my siblings (Ihab, Ahmad, Lina, Raghda, Raghib and Roya)
To the soul of my beloved brother Waeil
To my Beloved country; Libya

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Chapter 1

Introduction to Pain and the Somatosensory Pathways

Pain

As defined by the International Association of the Study of Pain (IASP), pain is "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage"[1]. Pain is categorized according to its time course as acute pain which is characterized by sudden onset associated with injury and subsides as the injury heals, and as chronic pain that is characterized by being prolonged beyond the healing time and lasts more than 3 months beyond healing of the injury[2–4].

According to the Center for Disease Control and Prevention, it is estimated that ~20.4% of the American population (about 50 million people) suffers from chronic pain that pain interferes with the daily life of nearly 20 million Americans[5]. Chronic pain has a negative impact on the quality of life and often contributes to decreased relationship satisfaction, increased relationship conflicts, higher divorce rates, and increased role strain in families. Moreover, mental status can also be affected, and chronic pain is often associated with depression and anxiety that aggravates symptoms. In addition to the effects on quality of life, chronic pain is associated with enormous costs. It has been estimated that in the United States alone, chronic pain costs up to $635 billion a year in medical costs and lost productivity, which is more than the yearly costs for cancer, heart disease and diabetes[6]. Importantly, the prevalence of chronic pain is expected to increase in the coming

years because of expected increases in diseases associated with pain such as diabetes and cancer[7].

Pain is often characterized as being nociceptive, inflammatory, and neuropathic[2–4]. Nociceptive pain is caused by acute stimulation of nociceptors (pain receptors on nerve endings) that innervate different tissues on the body[8]. Nociceptors are excited by intense mechanical and thermal stimuli, and by irritant chemicals. Inflammatory pain refers to pain and hypersensitivity that occurs in response to trauma, tissue damage, or inflammation. Inflammation is a natural biological reaction produced by the immune system to eliminate necrotic cells and initiate the repairing process[9,10]. Inflammatory pain may be a type of nociceptive pain that is caused by the acute stimulation of nociceptors following release of inflammatory mediators from activated immune cells[8]. Neuropathic pain results from damage or disease of the nervous system. Patients suffering from neuropathic pain may experience pain episodes that are electric-like jolts that occur spontaneously[11]. These patients also may experience numbness, ongoing pain and/or burning sensation, and hyperalgesia[12]. The neuropathic pain symptoms could be intermittent that lasts for seconds to minutes or continuous[13].

The Somatosensory System

Under normal conditions, cutaneous sensation begins with the activation of sensory receptors located on primary afferent nerve fibers that innervate the skin. These receptors can occur as encapsulated nerve endings or as free nerve endings. Their primary function is to transduce natural environmental stimuli into a neural signal that is transmitted to the brain. Activation of cutaneous receptors represent the initial and most elementary neural encoding of stimulus location, quality, and intensity. The cell body of primary

somatosensory neurons are located in dorsal root ganglia at spinal levels. A peripheral branch terminates in tissues such as skin and contains the sensory receptors, and a central branch of the dorsal root ganglion neuron terminates in the spinal cord. The axons may be classified as being thickly myelinated (Aβ), thinly myelinated (Aδ) or unmyelinated (C) fibers. Nerve axons also may be classified based on conduction velocities of action potentials: >30 m/sec for Aβ fibers, 2-30 m/sec for Aδ fibers, and <2 m/sec for C fibers.
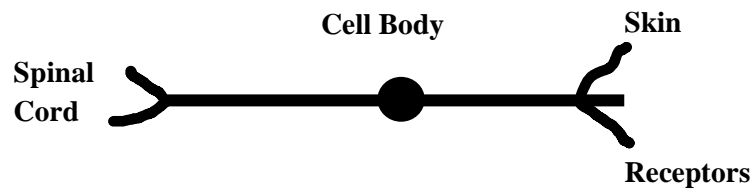
Figure 0.1. **Basic anatomical structure of primary somatosensory neurons.**

Mechanoreceptors

There are four types of cutaneous mechanoreceptors that detect light touch, pressure, texture, and vibration. These receptors are associated with fast-conducting Aβ nerve fibers and transmit non-noxious sensory information.

Pacinian corpuscles

Pacinian corpuscles (PCs) are encapsulated receptors and are widely distributed throughout the body, including skin, subcutaneous connective tissue, ligaments, joints capsules, the nipples, external genitalia, serous membranes, mesenteries, and viscera. PCs are sensitive to light pressure (mechanoreceptors) and vibration and are classified as type II rapidly adapting (RA) mechanoreceptors[14]. Depending on the peripheral location and the

central projections these receptors serve mainly non-noxious mechanical sensory functions. Subcutaneous receptors respond to pressure on the skin, while those in or near joint capsules serve proprioceptive functions. PCs also detect vibration in the skin with optimal sensitivity of 250Hz, while those in the viscera and mesentery contribute to the sensation of hollow organs fullness. Deformation of the corpuscle generates action potentials by allowing sodium ions to flow into the neuron [14].

Meissner's corpuscles

Meissner's corpuscles are encapsulated receptors that are found in the dermal papillae of the glabrous skin and are most dense at the distal ends of the fingers and toes[5]. These receptors are rapidly adapting and are most sensitive to the initial deformation of the skin. The action potential activity generated by sustained pressure decreases rapidly[14]. In addition to the detection of light touch and pressure, these receptors detect vibration and surface texture.

Merkel's discs

Merkel's discs are located in close proximity to cutaneous nerve endings in the basal layer of hairless and hairy skin and around hair follicles [14]. Merkel cells detect mechanical stimuli such as pressure, position, and steady deformation of the skin. They synapse with adjacent nerve endings and upon depolarization activate nerve endings to generate action potentials. The Merkel cells communicate with neurons via release of glutamate. Merkel cells exhibit a vigorous response to sustained pressure and are classified as slowly adapting (SA) receptors. In contrast to PCs, Merkel cells are most sensitive to vibration at much lower frequencies of 5Hz to 15Hz [14].

Ruffini endings

Ruffini endings are SA encapsulated mechanoreceptors located between dermal papillae and hypodermis and are sensitive to skin stretch and torque. Ruffini endings also contribute to kinesthetic sense such as finger movement and position[14].
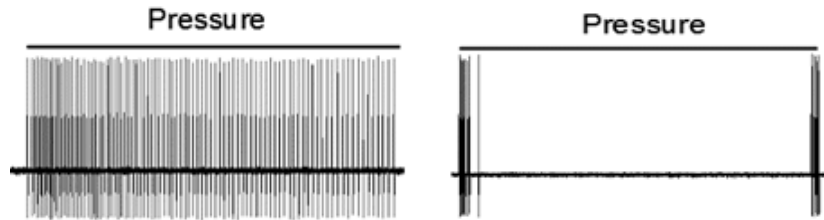
Figure 0.2. **RA and SA mechanoreceptors.** Examples of responses of a slowly adapting (left; Pacinian corpuscle) and rapidly adapting (right; Meissner's corpuscle) mechanoreceptors to sustained pressure.

Pain sensation and nociceptors

Nociceptors transduce noxious mechanical, thermal and chemical irritant stimuli to generate action potential activity in neurons[15]. Nociceptors are located on the peripheral nerve endings of unmyelinated C-fibers and thinly myelinated Aδ fibers. Based on differences in conduction velocities, Aδ nociceptors convey fast pain, such as pinprick, whereas C fiber nociceptors evoke a diffuse slow pain characterized as burning or aching[15,16]. Nociceptive afferent fibers are found in all tissues; however they have most thoroughly studied in cutaneous tissues[16].

Nociceptors are functionally diverse. While many are polymodal (responsive to multiple stimulus modalities, such as mechanical, heat and cold stimuli) others are more specific and respond to only one or two modalities. Sensitivity of the nociceptor afferent fiber depends on the density of different stimulus-transducing ion channels on each nerve

ending. Signal transduction in nociceptors is complex and involves several cation permeable ion channels that transduce specific stimulus modalities. The members of the family of Transient Receptor Potential (TRP) transmembrane proteins are best known and are grouped into those that are classified as vallinoid (TRPV), melastatin (TRPM), and ankyrin (TRPA)[17]. TRPV1 was the first recognized "pain channel" and is sensitive to noxious heat with a threshold of about 43° C (approximate pain threshold in humans) as well as capsaicin [18–20]. Other TRP channels are sensitive to intense heat (TRPV2), painful cold stimuli (TRPM8), and mechanical stimuli (TRPA1) [21,22].

Peripheral molecular mechanism of pain

Nociceptors express many ion channels and receptors to detect noxious stimuli. Tropomyosin kinase A (TrkA) signaling is necessary to initiate nociceptors' molecular and functional identity [23]. Runx1 is needed to activate a good portion of the nociceptor-specific ion channel/receptor [24]. Runx1 is particularly needed for thermal and not mechanical pain sensitivity [23,24]. In summary, intrinsic factors, e.g., Runx1 link with target-derived signal e.g., glial cell derived neurotrophic factor (GDNF), set up nociceptor diverseness.

Volage-gated sodium channels (VGSCs)

Tetrodotoxin-sensitive sodium channels, i.e., Nav1.1, 1.6, and 1.7, and the tetrodotoxin-resistant sodium channels, i.e., Nav1.8 and 1.9, are all expressed in somatosensory neurons. Studies have shown that some mutations of Nav1.7 increase pain in humans[25], whereas in patients with mutation that inactivates Nav1.7 fail to detect noxious stimuli and may suffer tissue injury due to a lack of a protective reflex. Patients with a gain-of-function mutation in Nav1.7 experience high excitability of the channel,

leading to pain disorders, such as erythromelalgia and paroxysmal pain disorders[26]. Studies with mice lacking C-fiber nociceptors showed a crucial role of Nav1.7 channel in thermal stimuli and noxious mechanical stimuli super-sensitivity following inflammation[27,28]. Unexpectedly, pain from nerve injury is unaffected which shows that different isotypes of Nav1.7 expressing afferent, contributes neuropathic pain[29].

The Nav1.8 channel also is expressed by C-fiber nociceptors and animals lacking Nav1.8 displayed insensitivity to innocuous or noxious heat or innocuous pressure but responded to noxious mechanical stimuli[28]. Nav1.8 is also required for the transmission of cold-evoked activity[30] since it is activated under low temperatures hence generate action potential under cold conditions.

In summary, VGSCs are putative targets for new analgesic drugs. Nav1.7 targets inflammatory pain syndrome because Nav1.7 inhibitors reduce pain sensation such as in treating extreme hypersensitivity to cold (Nav1.8)[30] without inhibiting critical normal physiological processes. Common antidepressants based on inhibiting 5-hydroxytryptamine and norepinephrine reuptake have been used to treat neuropathic pain and are thought to act by blocking voltage-gated sodium channels[31].

Voltage-gated calcium channels

Nociceptors express N-, P/Q-, and T-types of calcium channels [32]. Mutation of the P/Q- type channel has been linked to hemiplegic migraine and are expressed in substantia gelatinosa and nucleus proprius of the dorsal horn[33]. On the other hand, N- and T- types are expressed in C-fiber nociceptors and are upregulated after nerve injury and other pathophysiology states. Animal studies show that loss of Cav2.2 and 3.2 channels resulted in less sensitivity to mechanical stimuli following inflammation and thermal stimuli

following nerve injury[34,35]. The inhibitor of N-type channels, Conotoxin GVIA, when given intrathecally has been shown to relieve uncontrollable cancer pain in bone cancer model[36]. Considerable experimental and clinical data suggest that drugs that modify Voltage-gated calcium and sodium channel activity are possible targets for new analgesics.

Central pathways

Nociceptive primary afferent fibers terminate in the dorsal horn (DH) of the spinal cord where they release neurotransmitters, primarily glutamate, substance P and calcitonin gene-related peptide (CGRP) to excite DH neurons[15]. The activity of DH neurons is integrated, modulated, and relayed to brain areas involved in the discriminative and emotional aspects of pain.

Organization of the dorsal horn (DH)

The DH is part of the gray matter that is present at all levels of the spinal cord; it contains sensory neurons which receive somatosensory input from primary afferent fibers that innervate skin and deep tissues. The grey matter in the spinal cord is divided into ten layers, based on Bror Rexed's classification in the 1950s[37]. The DH is composed of six layers. Lamina I is referred to as the marginal

Figure 0.3. **Organization of the dorsal horn.** Lamina organization and primary afferent input to the dorsal horn of the spinal cord.
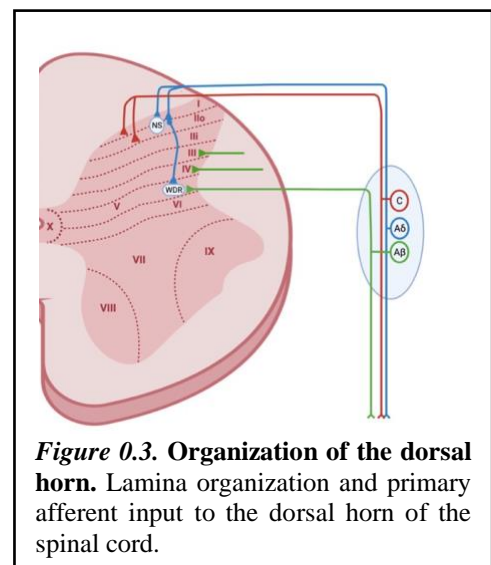
zone and Lamina II is referred to as the substantia gelatinosa of Rolando (collectively laminae I-II are referred to as the superficial DH). Most nociceptive primary afferent fibers terminate in the superficial DH. This area is fundamental to pain perception and is

recognized as a possible target for novel drugs for the treatment of chronic pain[38]. Laminae III and IV receive primarily low threshold mechanoreceptive input from primary afferents and are referred to as nucleus proprium[39,40,41]. Lamina V, or the neck of the dorsal horn, also receives input from nociceptive primary afferent fibers as well as input from low threshold mechanoreceptors[41]. Lamina V neurons receive afferent input from cutaneous, muscle and joint nociceptors as well as visceral nociceptors. Wide dynamic range neurons in lamina V receive cutaneous and visceral input that may contribute to referred pain[42]. Lamina V neurons also receive input from large-diameter fibers innervating muscles and joints and from muscle spindles which are sensitive to innocuous joint movement and muscle stretch and relay this information to the cerebellum to modulate muscle tone [43,44].

The DH is comprised of two main neuronal subtypes: interneurons (IN) and projection neurons (PN). The interneurons are the most abundant and play a significant role in integrating sensory input with effector outflow[45]. INs may be further categorized as either local intra-segmental interneurons or relay neurons that connect longitudinally with neurons in more distant spinal segments. Local IN generally have a short axon that contribute to the microcircuits that process noxious sensory information. Conversely, relay INs have relatively long axons that connects various other spinal segments including projections to the ventral horn, lateral horn, and intermediate column. Relay neurons provide for effective coordination and transmission of somatomotor information across multiple spinal segments.

Spinal neurons that relay somatosensory information to higher brain regions are densely located in superficial DH as well as in deeper lamina (III-VII) and the LSN[46]. Spinal PNs that terminate in supraspinal brain regions are involved in basic discriminative

aspects of somatosensations, such as stimulus location, quality, and intensity, as well as areas involved in affective aspects of pain. Furthermore, other PNs in the spinal cord have propriospinal collaterals. Notably, PNs are often large multipolar neurons characterized by radial dendritic arborization. The radial arrangement of the dendrites facilitates the integration of convergent inputs.

The Spinothalamic Tract (STT)

The STT, also referred to as the anterolateral system, is a primary pathway for ascending transmission of nociceptive information. STT neurons receive afferent directly or indirectly via interneurons. This ascending pathway consists of DH neurons whose axons ascend to the brain through the anterolateral aspect of the spinal cord and decussate at the level of the pyramids and then ascend to synapse in the ventral posterolateral thalamus via the lateral spinothalamic tract. STT neurons relay this information to the primary somatosensory cortex for the perception of basic discriminative aspects of pain (**Figure 1.5**).

Functional properties of neurons in the dorsal horn

Neurons in the DH of the spinal cord have been characterized according to the responses to natural stimuli applied to the receptive field (RF)[47] and may be classified as low-threshold mechanoreceptive (LTM), nociceptive-specific (NS), and wide-dynamic range (WDR) neurons. LTM neurons mainly receive information from mechanoreceptive Aβ fibers and respond only to non-noxious mechanical stimuli[48] and are located mainly in lamina III and IV[47]. NS neurons receive information from C and Aδ nociceptive fibers are located mainly in the superficial DH and are excited primarily by noxious stimulation[49].

WDR neurons receive information from afferent fibers that transmit innocuous and noxious sensory information and are located primarily in the deep dorsal horn (Laminae II, IV and V[15,50,51]). WDR neurons are excited in a graded fashion by both innocuous (touch) and noxious (pinch, heat, and/or cold) [47,52] .
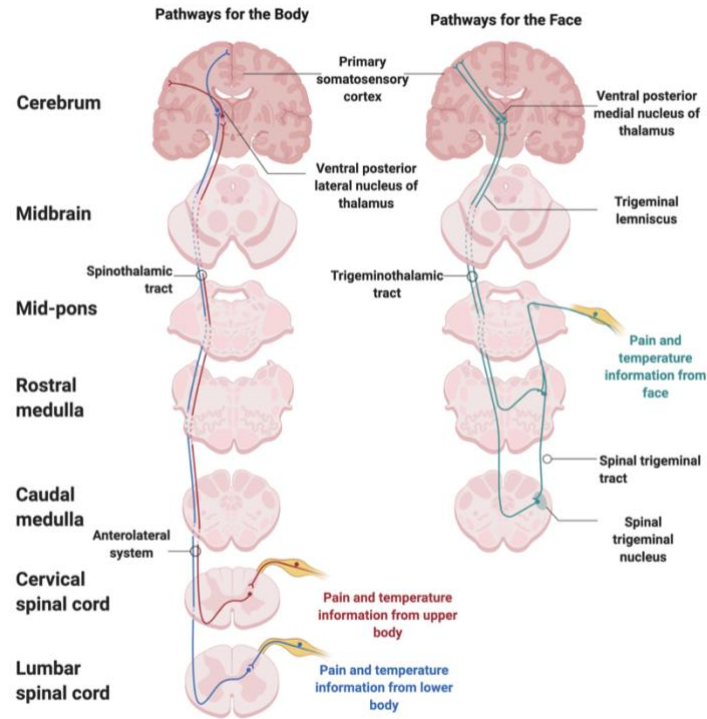
Figure 1.5. **Ascending Pain Pathways:** The two main ascending pathways of pain. (A) The Spinothalamic Tract (STT) and the (B) Trigeminothalamic tract.

Hyperalgesia

Hyperalgesia is defined as an increased sensitivity to painful stimuli. Operationally hyperalgesia may include a decrease in the pain threshold, increased pain to stimuli that are normally painful, and ongoing pain. Allodynia is often defined as pain produced by stimuli that are not normally painful, such as light touch or gentle warming or cooling. Allodynia is a common symptom following inflammation and or nerve injury such as diabetic neuropathy.

Hyperalgesia can be characterized as primary or secondary based on its spatial localization. Primary hyperalgesia refers to hyperalgesia at the site of injury (such as a burn), whereas secondary hyperalgesia refers to hyperalgesia induced from uninjured tissue surrounding an injury. Mechanisms of primary hyperalgesia include sensitization of nociceptors, whereas secondary hyperalgesia involves changes in the central nervous system, including the spinal cord[53–56]. Although hyperalgesia is protective to give the tissue time to heal, it can become pathological and chronic.
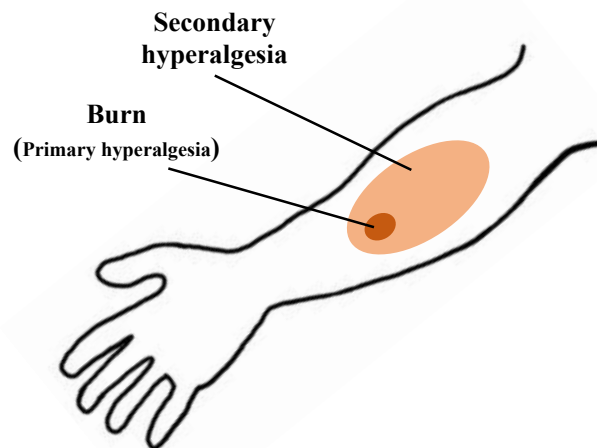
Figure 0.6. **Localization of primary and secondary hyperalgesia.** Schematic of an example of primary and secondary hyperalgesia following a localized burn injury to the skin. Mechanical hyperalgesia occurs within the injured area (primary hyperalgesia) and in a large surrounding area (secondary hyperalgesia) in a large area.

Peripheral Sensitization

Inflammation arises following tissue injury resulting in the release of factors such as substance P and CGRP that leads to a hyperexcitable state of nociceptors known as nociceptor sensitization. The inflammatory mediators also cause an increase in vascular permeability and edema and the release of prostaglandins, bradykinin and cytokines leading to further sensitization, reduction in firing threshold and ectopic discharge. Sensitized nociceptors exhibit a decreased activation threshold to thermal, chemical, and mechanical stimuli that may involve multiple mechanisms. First, proinflammatory molecules may be released from blood vessels and activated immune cells[57]. These molecules sensitize nociceptors resulting in the generation action potentials at thresholds. lower than expected as well as an increase in the firing rates.

Secondly, activated nociceptors also transmit action potentials back towards the nerve endings causing the peripheral release of neurochemicals such as substance P [57]. Peripheral release of neurochemicals from nociceptor nerve endings causes the further release of inflammatory mediators by stimulating immune cells in the affected tissue. Inflammatory mediators then activate receptors, ion channels, on nociceptor membranes to increase excitability. Neurochemicals that cause vasodilation and plasma extravasation results in a process termed neurogenic inflammation.

The input from nociceptors to the spinal cord may excite inhibitory as well as excitatory neurons, forming axonic synapses with the central endings of neighboring sensory nerve fibers[57]. Spinal inhibitory interneurons release chemicals such as GABA to depolarize adjacent sensory nerve fibers to the extent to which the action potentials are fired back to PNS endings[58]. Neurochemicals such as substance P are then released from

activated nerve endings to the affected tissue, further releasing chemical mediated substances.

Many studies in humans and animals have shown that both Aδ and C nociceptors located at the site of an injury become sensitized after injury and contribute to primary hyperalgesia[53–56]. Nociceptor sensitization is characterized by a decrease in threshold for activation, increased responses to suprathreshold stimuli, and spontaneous activity. Sensitization may result from mechanical, thermal, and chemical stimuli. Following injury, inflammation, and inflammatory mediators, including bradykinin, ATP, prostaglandins, cytokines, and chemokines, are released from activated immune cells or injured tissue and activate and/or sensitize nociceptors[59–61]. Many inflammatory mediators, acting through intracellular pathways, sensitize ion channels involved in nociceptor activation and transduction, such as protons (H+) and ATP[62,63]. In addition, the neuropeptides SP and CGRP are released from the peripheral terminals of activated C nociceptors, causing increased vascular permeability (redness or flare) and edema, which is referred to as neurogenic inflammation[64,65].

Central sensitization

Central sensitization refers to increased sensitivity of nociceptive neurons in the spinal cord. Increased sensitivity of spinal neurons after injury was first described by Woolf and colleagues[66,67]. Neural plasticity is vital in cellular changes with increased membrane excitability and synaptic efficacy, resulting in the widespread, non-anatomical distribution of pain[68].

There are two types of central sensitization: windup and long-term potentiation (LTP). Windup is referred to a progressive increase in the number of action potentials

evoked by repeated low frequency (1 Hz) activation of C fiber nociceptors[69]. This is demonstrated using electrical stimulation of a peripheral nerve at an intensity sufficient to activate C fibers. Although input (stimulation intensity) does not change, responses of the DH neurons gradually increase along with after discharges. Windup is due to temporal summation of C fiber-evoked synaptic potentials[70] and activation of NMDA receptors[69,71–73]. Importantly, temporal summation of pain evoked repetitive stimulation occurs in humans[74,75]. Thus, windup due to repetitive nociceptive input into the spinal cord at low frequency is sufficient to cause central sensitization.

Central sensitization also occurs following a high frequency barrage of C fiber activity[56,76] and is considered similar to long term potentiation (LTP)[77]. This form of central sensitization results in a decrease in response threshold, increased activity to suprathreshold stimuli, and increased receptive field size[78]. This type of sensitization was shown to occur in STT neurons in monkeys and to correlate well with hyperalgesia and allodynia in humans[54]. The mechanisms of this sensitization are complex and involve the release of glutamate and neuropeptides (such as SP and CGRP) from C fibers (see below).

Central sensitization involves various mechanisms. First, central sensitization reduces the threshold of glutamate receptor activation kinetics that involve both NMDA and AMPA receptors, which in turn, increases the neuronal excitability[68]. Secondly, there is an increase in cell membrane glutamate receptor number which produces a greater response by increasing presynaptic membrane excitability[68]. Also, there is an alteration in axonal ion channels, thus increasing inward flow and decreasing outward flow, which facilitates neuronal depolarization. Altered ion flow results in increased excitability and

reduced inhibition by limiting the release of inhibitory neurotransmitters such as GABA and glycine [58].

NMDA and AMPA receptors

Glutamate released by primary afferent nociceptors interacts with several types of ionotropic glutamate receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and N-methyl-D-aspartate (NMDA) receptors on spinal cord neurons. Activation of AMPA receptor channels (calcium-permeable and calcium impermeable) allows for rapid excitatory synaptic transmission[79]. Peptidergic primary afferent neurons also release neuropeptides such as substance P and CGRP in response to a stimulus following an increase in firing rate by nociceptors. Second order neurons in the spinal cord express NK-1 receptors that bind substance P. AMPA receptor activation is necessary for the removal of the Mg2+ block which then allows NMDA receptors on the postsynaptic membrane to be triggered resulting in a more prolonged and postsynaptic depolarization and increased calcium influx in second-order neurons[80].

The N-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor, which may be activated by glutamate and/or glycine. Peripheral noxious stimulation activates NMDAR and calcium influx and is closely linked to neuronal sensitization and hyperalgesia, and decreased functionality of opioid receptors. Hyperalgesia often persists due to maladaptive neuroplasticity-induced central sensitization[81]. In models of inflammatory, neuropathic and cancer pain, central sensitization of nociceptive dorsal horn neurons has been demonstrated to be necessary for persistent hyperalgesia[82,83,84] and requires NMDAR activation [85,86]. Central sensitization of DH neurons have been reported in studies utilizing several models of bone cancer pain[87,88].

The NR2B subunit of the NMDAR is highly expressed in the forebrain and the superficial dorsal cord[89–91] which is the location of the central terminals of primary afferent nociceptive neurons[91,92]. Studies have shown that the number of NR2B-positive neurons in the superficial DH and DRG ipsilateral to the tumor-bearing hind limb and NR2B mRNA were considerably enhanced in tumor-bearing animals, according to immunohistochemical staining and RT-PCR assay[84,93,94]. Blocking NR2B subunit of NMDA receptor decreases hyperalgesia and sensitization in models of bone cancer[84,93].

Typically, receptor binding of the ligands is not sufficient to open the channel as it blocked by Mg^{2+} and Zn ions. However, upon sufficient depolarization, the Mg and Zn ions are dislodged from the pores, allowing Na^+ and Ca^{+2} ions into the cell and K^+ ions out of the cell[95]. The NMDA receptor is thought to be necessary for long-term increases in synaptic plasticity, as many studies have shown that NMDA receptor antagonists block sensitization and reduce hyperalgesia in several animal models and in humans [75,96–99]. However, because of the wide distribution of NMDARs throughout the brain, NMDA-related drugs are often associated with significant side effects, such as hallucinations, lightheadedness, dizziness, fatigue, headache, out-of-body sensation, nightmares, and sensory changes[100,101].

Neurokinin-1 (NK-1) receptors

Substance P (SP) belongs to the tachykinin family of peptides and the neurokinin 1 receptor (NK-1R), is the primary receptor for SP[102]. SP is released by the endings of nociceptor primary afferent fibers and binds to NK-1 receptors located on second-order DH neurons. Substance P and the activation of NK-1 receptors are important for the development of central sensitization[103,104]. Chemically ablating NK-1+ neurons has been

shown to prevent the formation of hyperalgesia in neuropathic and inflammatory pain models[105,106] and the activation of NK-1R causes sustained depolarization and calcium mobilization within the cell[107]. Intraspinal injection of an NK-1 receptor inhibitor reduces wind-up and the second-phase response to intradermal formalin injection[108].

Direct monosynaptic responses to primary afferent stimulation are mediated by glutamate release and AMPA receptor activation. This initial response then causes NMDA receptor activation, which sets off a series of complex cascades that initiate the processes that "sensitize" the postsynaptic dorsal horn neuron[108,109,110,111]. It has been shown that NK-1 receptor antagonists block the development of sensitization, but not its maintenance[111].

Glial interaction

The brain comprises more than 100 million nerve cells (neurons) and many more glial cells. Glial cells offer support, protection to the neurons and participate in neural activity, neural nutrition, and the central nervous system's defense processes. Glial cells exist in many different cell types: oligodendrocytes, Schwann cells, astrocytes, ependymal cells, and microglia. Glial cells are ten times more abundant in the mammalian brain than neurons. Since nerve tissue has only a minimal extracellular matrix, glial cells furnish a microenvironment suitable for neuronal activity.

Astrocytes are numerous and exhibit a unique morphology and functional diversity. Astrocytes bind neurons to capillaries and pia matter, and structurally, are divided into fibrous astrocytes, which have few processes located in the white matter, and protoplasmic astrocytes, which have many short processes and are found in the gray matter. Their primary functions include structural support, repair processes, blood-brain, barrier, and metabolic exchanges with CNS neurons. Astrocytes are able to respond to several stimuli

due to the expression of multiple receptors such as adrenergic receptors, amino acid receptors, e.g., GABA and peptide receptors like natriuretic peptide, angiotensin II, endothelin, vasoactive intestinal peptide, and thyrotropin-releasing hormone[112].

Astrocytes undergo reactive astrogliosis after noxious stimuli and nerve damage (morphological and functional alterations) in association with chronic pain. In this process, naïve astrocytes differentiate into scar-forming astrocytes and reactive astrocytes. Reactive astrocytes are classified according to their function as toxic A1 astrocytes and neuroprotective A2 astrocytes. A1 astrocytes release neurotoxin, which rapidly induces death to oligodendrocytes and neurons. At the same time, A2 astrocytes, upon nerve injury, influence neuronal survival and activity through their ability to regulate constituents of the extracellular environment, absorb local excess of neurotransmitters, and release metabolic and neuroactive molecules, e.g., peptides of angiotensinogen family, vasoactive endothelins, enkephalins and somatostatin[113].

Reactive astrogliosis is a mechanism for repairing damage by increasing neuroprotection and nutritional support for injured neurons[112]. The expanded processes of the scar-forming astrocytes form a continuous layer at the external surface of the CNS, which increases to form cellular [112] scar tissue when the central nervous system is damaged. Some astrocytes develop processes with expanded end-feet linked to endothelial cells, through which they transfer molecules such as energy-rich compounds and ions from the vascular system to the neurons. They metabolize glucose to lactate, which is then supplied to the neurons. Astrocytes communicate with one another via gap junctions, forming a network through which information can flow from one point to another to reach distant sites.

By contrast, activated astrocytes also can contribute to chronic pain by releasing proinflammatory signaling molecules, e.g., interleukins, leukemia inhibitory factor, ciliary neurotrophic factor, transforming growth factor, and tumor necrosis factor. Signaling molecules are also released by neurons, microglia, inflammatory cells, and oligodendrocytes[112]. Although the reactive astrocytes help in repair function, they also may contribute to chronic pain.

Microglia cells are mononuclear phagocytic cells and are derived from precursor cells in the bone marrow[114]. They are activated by neuroinflammatory mechanisms and act to repair injured neurons in the adult CNS. Upon activation, microglia produce and release neutral proteases and oxidative radicals. Activated microglia also display structural changes and retract their processes similar to the morphologic characteristics of macrophages, thereby becoming phagocytic and acting as antigen-presenting cells. Microglia also secrete several immunoregulatory cytokines and dispose of unwanted cellular debris caused by CNS lesions[114]. In AIDS, dementia complex microglia are affected by HIV-1 and cytokines such as interleukin-1 and tumor necrosis factor alpha.

Oligodendrocytes and Schwann cells provide electrical insulation for neurons in the CNS and PNS, respectively. In contrast, ependymal cells line the brain's ventricles and the central canal of the spinal cord. In some brain areas, ependymal cells are ciliated, thus facilitating the movement of cerebrospinal fluid. Studies show that glial cells, especially astrocytes and microglia, release cytokines, chemokines and other neuroactive substances which disrupt the excitatory and inhibitory amino acid homeostasis, resulting in elevating and prolonging pain. In addition, various substances released by glial cells may enhance pain by reducing efficacy of endogenous opioids[115].

Chapter 2

Cancer Pain

In 2018, 1,806,590 new cancer cases were diagnosed in the US, and 606,520 cancer cases were estimated to cause morality[116]. According to WHO, the most common cancers occur in the breast, lungs, prostate gland, bronchus, colon, rectum, skin, bladder, kidney, renal pelvis, and pancreas in that descending order. In men, 43% of all the cases diagnosed in the United States are prostate, lung, and colorectal cancer, while breast, lung, and colorectal cancers made up more than 50% of new cancer cases in women[116]. At the global level, 18.1 million new cases and 9.5 million deaths were caused by cancer in 2018. The numbers are projected to increase every year by 29.5 million cases by 2040, with 16.4 million deaths being caused by cancer annually[116].

Many of the types of cancer mentioned above metastasize to bones. Bones are the third most common tissue site for metastases, and almost all patients with end-stage cancer report pain[117,118–120]. Pain, particularly associated with metastasis to bone, can be severe and is among the most common symptoms in patients diagnosed with cancer[121]. Indeed, over half of cancer patients have been shown to have tumor-related pain, and about two-thirds of patients with advanced disease report ongoing pain[122,123]. Pain is often associated with emotional distress and decreased function, which negatively affects the patient's quality of life [124–127].

There are a variety of factors that contribute to bone cancer pain. These include inflammation, nerve compression resulting in neuropathic pain, bone fractures, bone destruction through the activation of osteoclasts, bone resorption, and substances released

from the cancer cells[128–130]. Each of these factors may produce a microenvironment that, in turn, produce factors that further sensitize nociceptors[131,132].

Opioids are considered the gold standard for treatment of patients with bone cancer pain; however, opioids are often associated with side effects including itching, constipation, analgesic tolerance, respiratory depression, addiction, and overdose-related death due to respiratory depression[133]. The National Health Institute reports that patients who require opioids for pain reduction, such that at least one in ten patients show opioid dependency[133]. Therefore, there is need to develop novel treatments for palliative care with fewer side effects and a better analgesia and safety profile. The mechanisms underlying cancer pain are not well defined and will require greater knowledge in order to develop new therapies.

Pre-clinical Models to Examine Mechanisms of Bone Cancer Pain

A variety of animal models of cancer pain have been developed to identify underlying pain mechanisms. A recent meta-analysis for models for bone cancer pain revealed at least 38 different animal models[134]. The most common bone cancer model in rodents involves surgically implanting or injecting different types of cancer cells into certain bones such as the femur, humorous[135], tibial or calcaneus bone[134,136–140]. Implantation into the femur or tibia produced hyperalgesia that was measured on the plantar of the ipsilateral hind paw[136–140], suggesting that this might correspond to a referred hyperalgesia. Tumor growth in the humorous bone evoked a deep tissue hyperalgesia, measured as a decrease in forelimb grip force[129].

In our studies, we used a reliable mouse model of bone cancer pain developed by researchers at the University of Minnesota[141]. In this model, osteolytic fibrosarcoma cells

(NCTC clone 2472 cells derived from a spontaneous connective tissue tumor found in C3H mice) are implanted into and around the calcaneus bone in C3H mice. Within days, these mice exhibit hyperalgesia (increased withdrawal responses) to mechanical, heat, and cold stimuli applied to the tumor-bearing paw[142] Electrophysiological studies in vivo from our lab showed that hyperalgesia was associated with sensitization of C-fiber nociceptors in the skin overlying tumor growth [143] and sensitization of nociceptive dorsal horn neurons[144]. This bone cancer pain model was used in our studies to investigate the efficacy and mechanisms underlying the unprecedented antinociception produced by MMG22.

Sensitization of nociceptors in bone cancer

The tumor microenvironment contains many molecules and ions that can excite and sensitize nociceptors. Cancer cells release protons that lead to a decrease in the tissue's pH[145], which can sensitize nociceptors through TRPV1 channels[146,147] and through the acid-sensitive channel, ASIC-3[148]. The acidic microenvironment provides a favorable environment for the osteoclast to resorb bone[149]. As bone resorption progresses, the bone becomes fragile and eventually fractures.

Bone cancer cells also produce prostaglandins and endothelin which can sensitize nociceptors[149,150]. Peripheral sensitization of nociceptors also results from the release of cytokines such as interleukin-1 and tumor necrosis factor by macrophages; these polypeptides cause pain as well as tissue destruction and bone resorption, which lead to the release of growth factors that activates nerve fibers in the bony tissue[149,150]. The growth and expansion of tumor cells also may contribute to cancer pain by mechanical mechanisms by physical encroachment on bone marrow neurons [149,150]

Sensitization of Dorsal Horn Neurons Contribute to Bone Cancer Pain

We have reported that nociceptive neurons in the spinal cord, particularly WDR neurons, are sensitized to mechanical, heat and cold stimuli applied to the tumor-bearing paw during tumor development [144]. Sensitization of DH neurons was evidenced by a decrease in response threshold and increased responses to suprathreshold stimuli.

Several mechanisms likely contribute to central sensitization of spinal neurons in bone cancer. Bone cancer induced an elevation in several mediators in the spinal cord involved in central sensitization, such as and substance P, CGRP, and dynorphin, as well as elevation in the levels of activation transcription factor 3 (ATF3)[149]. It has also been accompanied by spinal astrocyte enlargement associated with the reduction of glutamate reuptake transporter expression, leading to an increase in the excitatory neurotransmitter glutamate[151,152].

Activation of spinal astrocytes and increased expression of aromatase has suggested that endogenous production of estrogens also may play a role in thermal and mechanical hyperalgesia associated with bone cancer[113]. Activation of astrocytes was detected at days 10, 14, and 21 following cancer cell implantations in a bone cancer model, and there was a 14-fold increase in the activation of astrocytes, depicted by an increase in GFAP labeling, particularly in laminae V and VI of the dorsal horn of the spinal cord. Astrocytes are thought to contribute to pain by secreting molecules such as NO and prostaglandins[113] which can enhance the release of neurotransmitters at the presynaptic terminal or increase the excitability of postsynaptic neurons. Astrocytic activation is also associated with a reduction in the expression of glutamate reuptake transporters that lead to the accumulation of glutamate in the synaptic cleft[153] which also contributes to central sensitization.

Chapter 3

MMG22, a novel bivalent ligand for the treatment of chronic pain

Current and future treatments for Cancer Pain

With advancements in diagnosing and treating cancer, there is increasing demand for developing new therapeutic options to decrease pain from cancer and its treatments. Bone cancer has been characterized by worsening of pain symptoms as the disease progress, which could be related to the fact that different mechanisms are involved at different stages. Therefore, various therapies are used at different stages of the disease depending on the severity of the pain.

The World Health Organization (WHO) proposed the WHO analgesic ladder in 1986 as a way to offer effective pain treatment for cancer patients[154,155]. The treatment of cancer pain is dependent on the magnitude and quality of pain the patient is experiencing. Non-opioids (NSAIDs), e.g., ibuprofen, naproxen, and paracetamol, fall under step one in the ladder. Their side effects include gastric ulcers, increased bleeding risk due to gastric ulcers, and reduced renal blood flow. NSAIDs are contraindicated in people with kidney disease, heart failure, cirrhosis, and severe asthma. Paracetamol is contraindicated in patients with liver damage or disease and severe malnutrition[156,157].

Weak opioids fall under step two in the ladder. They include Codeine, tramadol, and co-codamol. Co-codamol is a combination of codeine and paracetamol (30mg of codeine combined with 500mg of paracetamol). It is vital to monitor the patient's dosage. Typically, 1 gram of paracetamol every 4hours and a maximum of 4 grams in 24hours.

Codeine most commonly causes constipation, while tramadol most commonly causes agitation plus other side effects[158].

The third step is the use of non-pharmacological therapies. This step differs largely from the older ladder. Therapies such as acupuncture, massage, and yoga can be used at any step of the ladder, but nerve block, radiofrequency, and disc decompression fall under step three. They can be considered after the first and the 2nd step has failed. Authors emphasize this step due to the opioid's crisis[158]. The modified ladder is mostly applicable to chronic non-cancer pain, whereas physical therapies for cancer pain is not often considered. Strong opioids comprise the fourth step. They include morphine sulfate (found in oramorph), IV morphine, oxycodone, and fentanyl patches (used in renal impairment). Adjuvants such as antidepressants are often given along with opioid analgesics.

There may be a breakthrough pain where a patient experiences pain despite ongoing drug therapy[118–120,159]. In this case, doctors usually describe a sixth of the total daily opioid dose with monitoring. Opioids often cause constipation because mu opioid receptors are highly expressed in gut, and their activation causes decreased transit, which is the reason why stimulant laxatives must be administered together with opioids[158] .Other side effects include nausea, sedation, depression, addiction, and respiratory problems.

As mentioned earlier, bone cancer pain starts before bone destruction. At this early stage of the disease, analgesics such as COX1 & 2 inhibitors and endothelin antagonists can effectively treat pain associated with bone cancer[160]. As the bone destruction continues and nerve fibers become injured, Na+2 channel blockers such as gabapentin and pregabalin are used. Bone cancer leads to osteoclast activation and hypertrophy and bone destruction, and at this stage, the use of anti-osteoclastogenic medications as bisphosphonates or

osteoprotegerin were shown to reduce pain[161]. The acidic environment created by the cancer cells leads to the activation of both TRP and ASIC channels that leads to nociceptor hypersensitivity; therefore, the use of TRPV1 and ASIC antagonists have been shown to reduce bond cancer pain[160].

Mu Opioid Receptors (MOR)

Opium has been used for pain relief for hundreds of years[162,163]. Natural opioids, including morphine are derived from opium. Opioid receptors are divided into three main subtypes: mu (μ), kappa (κ), and delta (δ), and their endogenous ligands include β-endorphin, enkephalins and dynorphins, respectively[164]. All three receptor types are G-protein coupled receptors and activate inhibitory G-proteins[165] which inhibit adenylyl cyclase[166]. This causes a decrease in the production of cyclic adenosine monophosphate (cAMP), and a decrease in the activity of PKA, which in turn decreases the ion channel conductance including TRPV1 and ASIC[167,168]. The $\beta\gamma$ subunit of the G protein opens G protein-gated inwardly rectifying K+ (GIRK) channels[169,170], and inhibits N-type, P/Q-type and L-type calcium channels[169,171–175]. Together these actions decrease neuronal excitability and neurotransmitter release.

The opioids used in pain management, including cancer pain, are mainly Mu (μ) opioid receptor agonists, i.e., Codeine, tramadol, hydromorphone, and morphine, among others. Mu opioid receptors are expressed on peripheral nerves (nociceptors) and are widely distributed in the CNS. In the periphery, MOR is expressed on the peripheral and central terminals of approximately 20-30% of primary afferent neurons[176,177,178]. Activation of MOR in the periphery decreases responses of nociceptors[179–181], inhibits release of neuropeptides from peripheral nerve endings[182,183] and decreases TRPV1 activity[168].

Indeed, activation of MOR in the periphery has been shown to reduce hyperalgesia in rodent models of inflammatory pain[181,184,185] and to reduce arthritic pain in humans[186,187].

Localization of MOR in the CNS includes the spinal cord and other areas involved in pain processing. In the spinal cord, MOR is expressed by interneurons and projection neurons, but the majority is expressed presynaptically on the central terminals of primary afferent nociceptors[188]. Activation of these receptors decreases the release of neurotransmitters (e.g., glutamate and SP) from nociceptive primary afferents[179,189–195] and decreases the excitability of Aδ and C fibers . In addition, activation of MOR on dorsal horn neurons increases GIRK channel potassium conductance to hyperpolarize dorsal horn neurons[196–199] thereby decreasing their excitability[184,200].

MOR is also expressed on neurons that are part of descending pain modulating pathways originating in the PAG and RVM. In the RVM, a major output of descending pathways to the spinal cord, activation of MOR inhibits pain-facilitation ON cells, and excites pain-inhibitory OFF cells by inhibiting GABAergic cells[201–204]. Together, this results in decreased excitability of nociceptive neurons in the spinal cord and contributes to the analgesic effect of opioids.

Although pain management using opioids is effective, it has also been challenging due to the their side effects, including nausea, constipation, tolerance, addiction, opioid-induced hyperalgesia, respiratory depression and death due to overdose[165]. These side effects underscore the need for alternative approaches to pain management that are effective but without the side effects of traditional opioids.

Metabotropic Glutamate Receptor-5 (mGluR5) and pain

Metabotropic glutamate receptors (mGluRs) are G protein-coupled glutamate receptors that are classified into three groups. Group I include mGluR1 and mGluR5, Group II includes mGluR2, and Group III includes mGluR4, mGluR7, and mGluR8. Here we will focus on mGluR5 since this has the most studied metabotropic glutamate receptor in relation to pain. Several lines of evidence clearly show a role for mGluR5 in pain processing. First, Intraplantar[205,206] or intrathecal[82,207,208] administration of mGluR5 receptor agonists produced pain behaviors and hyperalgesia to mechanical and thermal stimuli. Second, mGluR5 is expressed by nociceptive primary afferent fibers[206,209–212], by nociceptive dorsal horn neurons[211,213–217] and is upregulated in models of inflammatory[211,218], neuropathic pain[212,219–221] and bone cancer pain[222]. Interestingly, blocking mGluR5 with selective antagonists did not alter acute withdrawal responses to noxious stimuli[206,223], but reduced hyperalgesia in models of inflammatory[206,224,225], neuropathic pain[224–227], and bone cancer pain[222]. This suggests that mGluR5 is primarily involved in hyperalgesia rather than acute pain. This is further supported by studies showing that mGluR5 KO mice had normal withdrawal responses, but decreased hyperalgesia produced by inflammation[228–231].

Summary of metabotropic glutamate receptors:

- Group I: these include mGluR1 and mGluR5, coupled to G_q alpha protein subunit alpha ($G_{\alpha q}$). Group I mGluRs are linked to phosphatidylinositol hydrolysis, and stimulation of these receptors introduces phospholipase-C (PLC) catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). DAG leads to the translocation and triggering of PKC, while IP3

activates the discharge of Ca2+ from the endoplasmic reticulum (ER)[232]. Ca2+ also leads to the translocation and activation of PKC. IN addition, it leads to the formation of NO via Ca2+/calmodulin activation of NOS.

- Group II: this consists of mGluR2 and mGluR3, which have an inhibitory effect on adenylyl cyclase through G_i/G_o and leads to inhibition of neurotransmitter release, including GABA Glutamate. Group II metabotropic glutamate (mGlu) receptors are involved in pain processing and are located presynaptically on peripheral and spinal neurons.

- Group III: comprises mGluR4, mGluR7, and mGluR8, which inhibit adenyl cyclase activity from forming cAMP and pyrophosphate. They are also involved in presynaptic inhibition, inhibiting toxic neural transmission. However, their effect can prevent normal synaptic transmission. mGluR6 is involved in vision transmission.

mGluR5 signaling

mGluR5 is coupled to the Gq/11 trimeric G protein and activates several pathways including phospholipase C (PLC). This promotes the hydrolysis of phosphoatidylinositol-4,5-bisphosphate (PIP2) to form inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG)[233]. The reduction in PIP2 disinhibits TRPV1 channel, and thereby increases its excitability[234]. IP3 can also bind calcium release channels on the endoplasmic reticulum to open these channels and promote an increase in cytosolic calcium. Indeed, mGluR5 activation causes calcium transients in cultured DRG neurons[235]. The increases in cytosolic calcium and DAG activate protein kinase C (PKC) which phosphorylates AMPA and NMDA channels, increasing their conductance[96,236,237] and increases cellular excitability.

PKC also phosphorylates TRPV1 receptors, causing decreased thresholds and increased membrane insertion[238–243], also contributing to increased cellular excitability. mGluR5 activation also leads to the activation of ERK (via PKC), which decreasesKv4.2 A-type potassium currents, also contributing to enhanced cellular excitability[244,245].

Importantly, mGluR5 interacts with the NMDA receptor to produce central sensitization[236,246]. mGluR5 is structurally linked to the NMDA receptor via a protein scaffold [247] and functional interactions have been demonstrated between the two receptors[248]. The interaction of mGluR5 with NMDA occurs through a covalent link with the NR2 subunit of the NMDAR, and the NR2 subunit has been shown to modulate neuronal excitability[97,249,250]. Thus, mGluR5 may contribute to central sensitization by activating NMDA receptors, and blocking mGluR5 may block sensitization by blocking activation of the NMDA receptor[236,246].
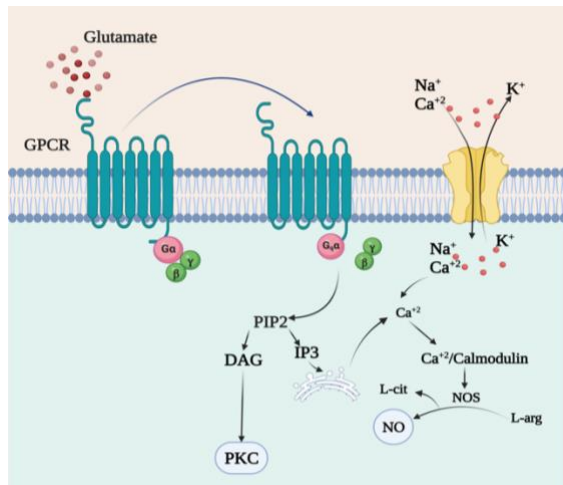
Figure 0.1. mGluR5 receptor activation.
Activation of Group I mGluRs (mGluR1 and mGluR5) induces phospholipase C (PLC)–catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). IP3 enhances the release of Ca2+ from endoplasmic reticulum (ER) storage, while DAG promotes PKC translocation and activation. Ca2+ also stimulates PKC translocation and activation, as well as NO generation via NOS activation by Ca2+/calmodulin.

Functional interactions between MOR and mGluR5

Opioid receptors and mGluR5 have been shown to form heteromers with several different receptor subtypes[251], and receptor dimerization can alter receptor function, ligand pharmacology, signal transduction, and cellular trafficking[252–255]. Bivalent ligands targeting GPCR dimers may result in more potent and selective compounds that act only on cells that express both receptors[256], minimizing potential off-target effects[257]. It has also been proposed that the proclivity of different GPCRs to form heteromers may be modulated in pathological states[258]. GPCR heteromers can be targeted not only to individual cells, but also to certain disease states. Significantly, heteromer formation may be modulated in pathological conditions[259].

Several lines of evidence suggest that MOR interacts functionally with the metabotropic glutamate receptor 5 (mGluR5). Like MOR, mGluR 5 is essential for the development and modulation of pain. mGluR5 is upregulated during pain[260–263], and mGluR5 antagonists decreased hyperalgesia[208]and responses of dorsal horn neurons in models of neuropathic pain[264,265], increased the analgesic efficacy of opioids[264,266,267], and decreased place preference and morphine self-administration[268].

Importantly, pain and opioid administration have been shown to increase mGluR5 expression in the spinal cord dorsal horn[260–263], and mGluR5 upregulation is thought to contribute to the development of analgesic tolerance to opioids[269,270]. *In vivo*, MOR and mGluR5 are both found on the peripheral and central[260,262,263,271] terminals of primary afferent nociceptors and post-synaptically on neurons in the superficial dorsal horn[260–263,272–274]. Importantly we found mRNA for these receptors to be co-localized on the same

cell[275]. In cultured cells, phosphorylation, internalization, and MOR desensitization are reduced following mGluR5 antagonism[276]. Collectively, evidence suggests that combining a MOR agonist with a mGluR5 antagonist may effectively treat chronic pain conditions, including cancer pain, with fewer serious side effects attributed to opioids as analgesic tolerance[265,269,277,278].

Bivalent Ligand MMG22

The interactions between MOR and mGluR5, their expression on glial cells and neurons[279], and reports suggesting co-expression of MOR/mGluR5 receptors in cultured cells associate as a heteromer [279] led to the development of MMG22.

The functional interactions between MOR and mGluR5 described above, including the enhanced antinociception and reduced tolerance when MO agonists and mGluR5 antagonists when co-administered, led to the development of MMG22 by the Portoghese lab[280]. MMG22 is a bivalent ligand that consists of a mu-opioid receptor agonist, oxymorphone, and the metabotropic glutamate receptor-5 (mGluR5) antagonist MPEP[281], that are separated by a 22-atom spacer. MMG22 is believed to target a MOR-mGluR5 heteromer because its analgesic potency was dependent on the 22-atom spacer in models of inflammatory pain (increases or decreases in length were less effective). Homologues with shorter or longer spacers than 22 atoms have been reported to have 3 orders of magnitude lower potency with respect to antinociception in LPS inflamed mice[281]. Furthermore, MMG22 was more potent than simply co-administration of the two drugs. MMG22 has been shown to be highly potent when given i.t. in the LPS model, but not when given into the brain (icv), suggesting that the spinal cord is the primary target site.

MMG22 also reduced neuropathic pain in a nerve injury model soon after injury, when there is significant inflammation.

Intrathecal administration of MMG22 was highly efficacious in reducing hyperalgesia in a model of bone cancer pain and was orders of magnitude more potent than morphine[279]. Moreover, the potency of MMG22 increased with tumor growth of over 21 days. The potency increase was 572-times greater on cancer post-implantation day 21 (PID21) than on PID3. Even though the MMG22 was given in some experiments with a much higher dose than its ED50, no side effects were detected, suggesting a high safety profile. MMG22 may be useful for cancer pain because it may target heteromers located on astrocytes (and perhaps neurons as well) which are known to be a mediator of cancer pain.

The mechanisms underlying antinociception following MMG22 includes the inhibition of the NMDAR via the known link between mGluR5 and NMDAR[94] via the NR2B subunit of the NMDAR as described above[99]. In a study by Akgün et. al[282], mice were pretreated with the NMDAR blocker, MK801, and this reduced MMG22 antinociception by 2700-fold, implying that MMG22 antinociception is attributable, at least in part, to NMDAR inhibition via MMG22 antagonism of mGluR5. Further, based on the known connection of mGluR5 to the NR2B subunit[283], it was shown that the selective NR2B antagonist, Ro 25-6981, decreased the potency of MMG22 4600-fold in LPS-treated mice[282]. Thus, MMG22 antinociception is attributed, at least in part, to NMDAR inhibition via MMG22 antagonism of mGluR5[284]. This is consistent with the fact that MMG22 is only effective when the NMDAR is activated by inflammation, as MMG22 was ineffective in naive mice[281]. MOR opioid receptors also contribute to antinociception

produced by MMG22. Administration of the MOR irreversible antagonist, β-FNA, decreased antinociception produced by MMG22[284].

A major problem with opioids is their associated side effects, including tolerance and respiratory depression. MMG22 was found to lack these side effects, and MMG22 did not produce conditioned place preference[275,279], suggesting it is not rewarding. However, systemic administration of MMG22 produced constipation[275], like opioids, which is due to activation of opiate receptors in the periphery
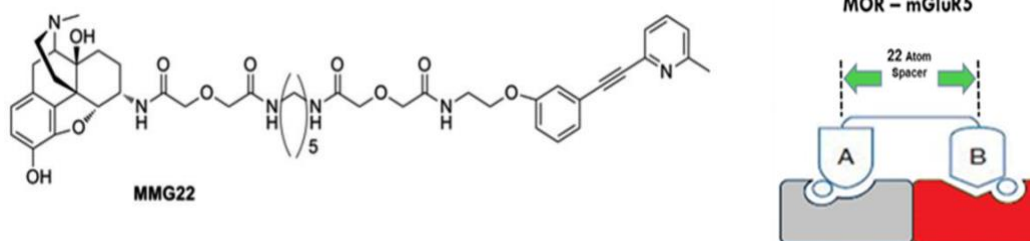
Figure 0.2. **Structure and binding of MMG22.** Left: The chemical structure of MMG22. Right: a schematic illustration of the binding of MMG22 to the putative MOR-mGluR5 heteromers.

Specific Aims and Research Hypotheses

The treatment of cancer pain is a significant clinical problem. Although opioids can be effective, they are associated with serious side effects, including tolerance, addiction, and respiratory depression. The bivalent ligand MMG22, which consists of a mu opioid receptor agonist linked toa mGluR5 antagonist, has been shown to produce potent antihyperalgesia when administered intrathecally in a mouse model of bone cancer pain, and lacks many of the side effects of traditional opioids. However, intrathecal administration is not as attractive for clinical use as is systemic administration. Therefore, these studies determined whether systemic administration of MMG22 inhibited tumor-evoked ongoing pain, and mechanical and thermal hyperalgesia and if systemic administration of MMG22 produced some of the side effects associated with opioids. Because MMG22 is believed to act in the spinal cord, and because nociceptive spinal neurons are sensitized in our model of cancer pain, we determined if systemic administration of MMG22 reduced responses of nociceptive neurons in the spinal neurons in the spinal cord.

Specific Aim I: Determine the effect of systemic administration of MMG22 on tumor-evoked mechanical and thermal hyperalgesia, and if MMG22 produced side effects associated with traditional opioids.

MMG22 was administered subcutaneously, intramuscularly, and orally. Dose-response functions for reducing hyperalgesia were determined, and comparisons were made with morphine, the individual pharmacophores, and MMG with other spacer lengths (10-atom; MMG10). Following cancer cell implantation, behavioral testing was done at

various times after cancer cell implantation (day 3-21) We also determined if systemic MMG22 produced analgesic tolerance, depressed motor function, produced naloxone-precipitated withdrawal, and was rewarding.

Hypothesis: Systemic administration of MMG22 will produce potent antinociception without tolerance and without motor deficits. In addition, MMG22 will not be rewarding and will not produce signs of withdrawal.

Specific Aim II: Determine if MMG22 decreases sensitization of nociceptive neurons in the spinal cord.

Nociceptive neurons in the spinal cord of mice are sensitized after cancer cell implantation and exhibit greater responses to stimulation of the tumor-bearing hind paw as compared to control mice. Electrophysiological recordings will be made from single nociceptive neurons in the lumbar spinal cord with receptive fields located on the plantar surface of the tumor-bearing hind paw. Spontaneous activity and responses evoked by controlled mechanical stimuli applied to the receptive field will be determined before and after s.c. injection of vehicle or MMG22 (dose was determined from behavioral studies).

Hypothesis: MMG22, but not vehicle, will decrease spontaneous and evoked activity of nociceptive dorsal horn neurons in tumor-bearing mice with hyperalgesia.

Chapter 4

Analgesic effect of MMG22 when administered systematically

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Shueb SS, Erb SJ, Lunzer MM, et al. Targeting MOR-mGluR5 heteromers reduces bone cancer pain by activating MOR and inhibiting mGluR5. Neuropharmacology. 2019 Dec;160:107690. DOI: 10.1016/j.neuropharm.2019.107690

Overview

Pain is among the most common symptoms in cancer and approximately 90% of patients experience end-stage cancer pain. The management of cancer pain is challenging due to the significant side effects associated with opioids, and novel therapeutic approaches are needed. MMG22 is a bivalent ligand containing MOR agonist and mGluR$_5$ antagonist pharmacophores joined by a 22-atom spacer. MMG22 exhibited extraordinary analgesia following intrathecal administration in a mouse model of bone cancer pain. Here, we assessed the effectiveness of systemic administration of MMG22 in reducing cancer pain and evaluated whether MMG22 displays side effects associated with opioids. Fibrosarcoma cells were injected into and around the calcaneus bone in C3H mice. Mechanical hyperalgesia was defined as an increase in the paw withdrawal frequencies (PWFs) evoked by application of a von Frey monofilament (3.9 mN bending force) applied to the plantar surface of the hind paw.

Subcutaneous (s.c.), intramuscular (i.m.), and oral (p.o.) administration of MMG22 produced robust dose-dependent antihyperalgesia, whose ED$_{50}$ was orders of magnitude lower than morphine. Moreover, the ED$_{50}$ for MMG22 decreased with disease progression. Importantly, s.c. administration of MMG22 did not produce acute (24 h) or long-term (9 days) tolerance, was not rewarding (conditioned place preference test), and did not produce naloxone-induced precipitated withdrawal or alter motor function. A possible mechanism of action of MMG22 is discussed in terms of inhibition of spinal NMDAR via antagonism of its co-receptor, mGluR$_5$, and concomitant activation of neuronal MOR. We suggest that MMG22 may be a powerful alternative to traditional opioids for managing cancer pain.

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Introduction

Pain is among the most common symptoms in cancer patients and is estimated to affect 90% of patients with end-stage cancer[285]. Of the millions of patients diagnosed with cancer, approximately 58% suffer from intolerable pain, which increases to 85% of the population as the disease becomes terminal[285].

Pain is usually associated with emotional distress and decreased function, and negatively affects the patient's quality of life[172]. Although opioids are the primary therapeutic used to treat severe cancer pain, these analgesics have many adverse side effects including nausea, sedation, constipation, tolerance, dependence, respiratory depression, and overdose-related death that limit their use[287]. Therefore, there is an urgent need to develop new and effective treatments for cancer pain that lack the serious side effects associated with opioids.

Earlier studies showed that co-administration of a mu opioid receptor (MOR) agonist and metabotropic glutamate receptor 5 (mGluR$_5$) antagonist reduced morphine analgesic tolerance and dependence, and augmented its antinociceptive properties[269,288]. The interaction between MOR and mGluR$_5$, their expression in astrocytes and neurons, and evidence that MOR/mGluR$_5$ heteromers exist in cultured cells,[289,290]led to the development of MMG22. MMG22 is a bivalent ligand consisting of an oxymorphone-derived MOR agonist and the mGluR$_5$ antagonist, M-MPEP, tethered by a 22-atom spacer[281]. Significantly, intrathecal (i.t.) administration of MMG22 exhibited thousands of folds greater potency based on ED50 in murine models of LPS-induced inflammatory pain relative to naïve mice. The necessity of inflammation as a condition for efficacy

also, was observed in a murine model of cancer pain in which osteolytic fibrosarcoma cells were implanted into and around the calcaneus bone[291].

Intrathecal (i.t.) administration of MMG22 afforded antinociception that was three orders of magnitude more effective than morphine, a gold standard for reducing tumor-evoked hyperalgesia[292]. That MMG22 exhibited 38,000-times greater potency than a mixture of the individual monovalent ligands containing MOR agonist and mGluR$_5$ antagonist pharmacophores supports the notion that MMG22 interacts with a MOR-mGluR$_5$ heteromer[281]. The exceptional potency of MMG22 may be a result of optimal bridging of protomers to a putative MOR-mGluR$_5$ heteromer.

In the present study, we show that systemic administration of MMG22 is highly effective at reducing cancer pain. The extraordinary potency of MMG22 and lack of side effects typically associated with opioids, suggests that MMG22 is an attractive alternative to morphine in managing cancer pain.

Methods

Subjects

Adult male C3H/HeNCr MTV mice (Charles River; 25–30 g) were used. Mice were housed four per cage, allowed free access to food and water, and maintained on a 12-hour light/dark schedule. All protocols and procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee and were conducted according to the guidelines established by the International Association for the Study of Pain[293].

Cancer cell implantation

NCTC clone 2472 fibrosarcoma cells (American Type Culture Collection, Manassas, VA, USA) were maintained as described previously[294]. This clone was derived from a connective tissue tumor in a C3H mouse, thus the fibrosarcoma cells are syngeneic with C3H/He mice[143]. Mice were anesthetized with isoflurane (2%) and fibrosarcoma cells (2×10^5 cells in 10 μL PBS, pH 7.3) were injected into and around the calcaneus bone of the animal's left hind paw using a 29-g needle. This approach produces a tumor with bone osteolysis[141].

Drug preparation and administration

All bivalent ligands were synthesized as described previously (Akgün et al., 2013). Compounds and Morphine sulfate (Mallinckrodt Inc., Hazelwood, MO) were all dissolved in 1.0% DMSO. Homologs of MMG22 with spacer lengths of 10 and 21 atoms were compared.

Behavioral studies of mechanical hyperalgesia

Mice were placed on an elevated wire mesh platform, covered individually with glass containers, and allowed to habituate to their surroundings for 30 min before testing. A calibrated von Frey monofilament (3.9 mN) was used to measure mechanical sensitivity of the hind paw and was applied to the plantar surface of each hind paw ten times. Mechanical hyperalgesia is defined as a significant increase in the paw withdrawal frequency (PWF), which is calculated as the (number of withdrawal responses/total stimuli) X 100% for each paw[291].

Mice were tested for three consecutive days before cancer cell implantation to confirm the absence of hyperalgesia and to acclimate to the testing procedure. PWFs were determined on post-implantation day (PID) 3-17 during which time mechanical hyperalgesia is maximal. After baseline testing, mice were divided randomly into groups of 6-8 mice per group. On the test day, animals received MMG22 administered s.c., i.m., or p.o. at escalating doses until the percent maximum possible effect(%MPE) was achieved. The %MPE was calculated from paw withdrawal frequencies using the following formula.

$$\%Maximum\ possible\ effect = \frac{(Predrug\ response - postdrug\ response)}{(Predrug\ response - max.\ postdrug\ response)} \times 100\%,$$

These values were adjusted to 100 and 0, respectively in order to address only the antihyperalgesic drug effects[295,296]. A separate group of tumor-bearing mice received morphine (5 mg/kg, s.c.) for comparison. PWFs were determined before and every 30 min for 4h following drug administration. The experimenter conducting the behavioral experiments was blinded to the treatment for all experiments, and at least two drug groups were tested in each session. Data were analyzed using multivariate analysis of variance (MANOVA) to compare differences in the PWF between groups and post-hoc comparisons were made using Bonferroni t-tests. ED_{50} values with 95% confidence intervals (C.I.) were obtained using nonlinear regression in GraphPad Prism v4.

Conditioned place preference

The conditioned place preference (CPP) test[297] was used to determine whether MMG22 was rewarding in naïve mice. The CPP apparatus consists of two chambers with walls containing either horizontal or vertical black lines (visual stimuli). Naïve mice (n=8/group) were given access to the entire compartment for 30 min/day for 3 days before conditioning in order to acclimate to the testing environment. Baseline measures were taken one day before conditioning, where animals received six counter-balanced conditioning trials (3 drug and 3 vehicle). Conditioning consisted of mice receiving vehicle and MMG22 once per day (morning and afternoon) respectively for three consecutive days where they were restricted to one side (vertical vs. horizontal black lines on the walls) for 30 min and paired with drug or vehicle. On the test day, mice were given access to both compartments and the time spent in each compartment was determined for 30 min. The mean amount of time spent in each compartment (drug-paired vs. vehicle-paired) was compared between drug and vehicle using one- way ANOVA.

Naloxone-induced jumping response

Naloxone-induced jumping[298,299] was used to determine whether MMG22 produces physical dependence. Testing of tumor-bearing mice began on PID17, and the mean PWF was determined. Mice were given three s.c. injections of morphine (n=5) or MMG22 (n=6) per day four hours apart. Morphine doses were escalated on day 1 to day 4 (5, 10 and 20 mg/kg). On the fifth day, mice received a single injection of 20 mg/kg. Similarly, MMG22 was given in escalating doses of 0.1, 0.2, and 0.4 mg/kg for the first four days, and mice received 0.4 mg/kg on the fifth day. At 3h after the final injection of morphine or MMG22, all mice received a single bolus of naloxone (50 mg/kg, s.c.).

Mice were placed in a Plexiglas observation cylinder and the number of jumps counted for 10 min by two independent investigators. Differences in the mean number of jumps were analyzed by student's t-test.

Effects of MMG22 on motor coordination

The rotarod test was used to determine whether MMG22 alters motor function. Naïve C3H mice (n=8/group) were acclimated for 3 days before drug administration. The treadmill was gradually accelerated from 3.75 to 5 rpm, with a maximum cutoff time of 300 sec. On the test day, mice received one s.c. injection of the 1 mg/kg MMG22, or the 5 mg/kg of clonidine as positive control[300,301].

Testing was done 60 min after drug administration and the time when the mouse fell off the treadmill was recorded. Mean times spent on the treadmill were compared between groups using two-way ANOVA with post-hoc Bonferroni analysis (p<0.05) considered significant.

Results

MMG22 dose-dependently reduces tumor-evoked hyperalgesia

Systemic administration of MMG22 potently reduced tumor-evoked mechanical hyperalgesia as defined by MPE% derived from the formula above. Subcutaneous, intramuscular, and oral administration each reduced hyperalgesia dose dependently. Depending on the dose, the antihyperalgesia peaked at 30-60 minutes after administration and persisted for at least 4 hours. Hyperalgesia returned to baseline levels by 24 hours. Interestingly, s.c. administration not only produced potent antihyperalgesia, but also became more potent with continued tumor growth as evidenced by a profound leftward shift in the dose response curve with increasing PID. This is best illustrated by the ED_{50} values at various times after tumor cell implantation (**Table 1**). For example, at PID3, the ED_{50} (confidence interval) for MMG22 was 1.16 (0.15-8.90) mg/kg, and this decreased to 0.00096 (0.0003-0.003) mg/kg at PID17. At this time, the ED_{50} for morphine was 2.37 mg/kg (CI=1.93-2.9). The ED_{50} values for MMG22 given i.m. and p.o. decreased over time, but not as dramatically as for s.c. administration. The antihyperalgesic effect of morphine given at a dose of 2.5 mg/kg s.c. was tested at different PIDs (**Figure 1**). Although it reduced hyperalgesia, it did not show potentiation over time (1-way ANOVA with repeated measures, $F_{(2, 11)} = 0.206$, P= 0.82). Compared to this dose of morphine, which consistently reduced hyperalgesia by approximately 50% at all PIDs (estimated ED_{50}), the efficacy of the ED_{50} dose for MMG22 was approximately 250 times more potent at PID 17 (**see Figure 4.1**).

To determine if shorter (MMG10, MMG21) spacer lengths had effects similar to that of MMG22, tumor-bearing mice (PID4) were injected with each and were tested for acute tolerance the following day. The short spacer MMG10 resulted in acute tolerance for all routes of administration, s.c. (1-way ANOVA with repeated measures, $F_{(1, 10)} = 6.98$, $p<0.05$), o.p (1-way ANOVA with repeated measures, $F_{(1, 10)} = 6.52$, $p<0.05$) and i.m. (1-way ANOVA with repeated measures, $F_{(1, 8)} = 8.09$, $p<0.05$).
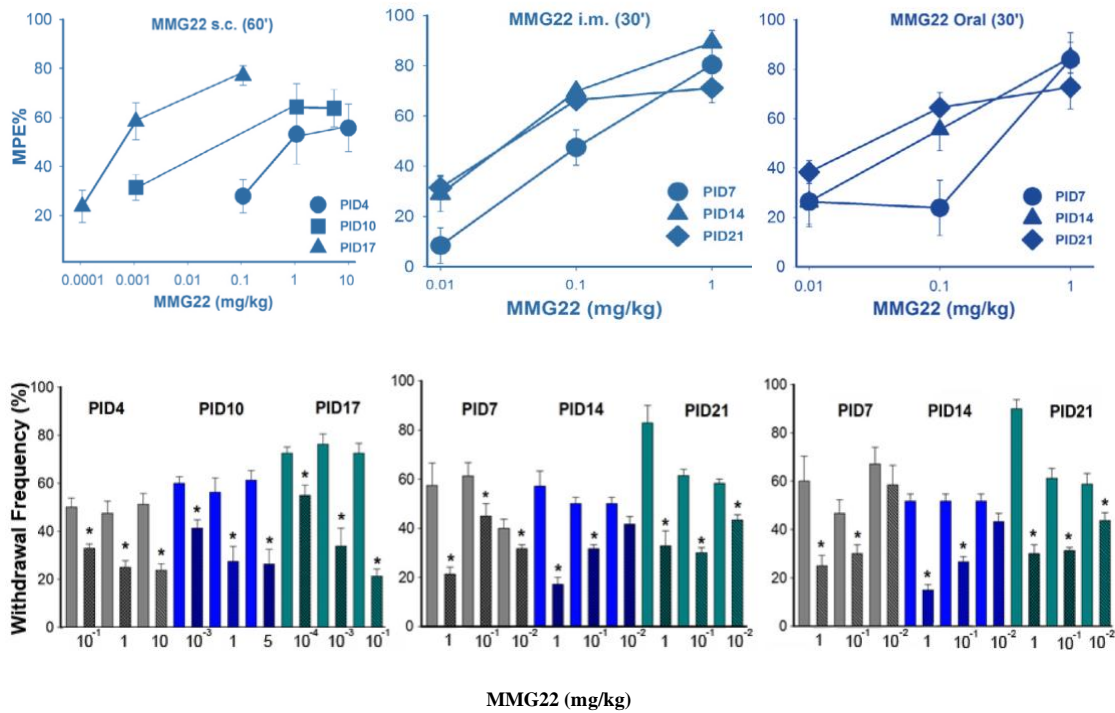
Figure 0.1. **Effect of MMG22 on tumor-evoked hyperalgesia.** Dose-response curves illustrating the reduction in tumor-evoked mechanical hyperalgesia following systemic administration of MMG22 (s.c., i.m., and oral) on different post-implantation days (PID). Data in upper panels show mean (±SEM) % maximum possible effect. A) Antinociception s.c. administration of MMG22 increased from PID4–17. The increase in potency occurred at all subsequent time points tested. B) Reduction in mechanical hyperalgesia on different PIDs following i.m. administration of MMG22. C) Reduction in mechanical hyperalgesia on different PIDs following oral administration of MMG22. The X-axis scale in A differs from those in B and C because of the greater number of doses used. Lower panels show mean paw withdrawal frequencies that correspond to the MPE% in the panels above.

MMG22 did not produce acute or chronic tolerance

To determine whether MMG22 produced acute tolerance, the antihyperalgesic effects of MMG22 were determined for two consecutive days beginning on PID4. As shown in (**Figure 4.2**), MMG22 (10 mg/kg, s.c., n=8) reduced mechanical hyperalgesia similarly on each day. The decrease in paw withdrawal frequency at 120 min after injection did not differ between the two days (1-way ANOVA with repeated measures, $F_{(4, 7)} = 61.2$, $p < 0.001$). Mice were then given a second injection of MMG22 (10 mg/kg, s.c.) 24hrs (PID5) after the first injection and produced a similar antihyperalgesic effect MMG22 peaked at 60-120 min after injection for PID5. Hyperalgesia returned to baseline values at 4 hours after injection on both days, indicating that the time course of antihyperalgesia produced by MMG22 was not altered following the second administration. These data show that MMG22 produces long-lasting anti-hyperalgesia without acute tolerance.
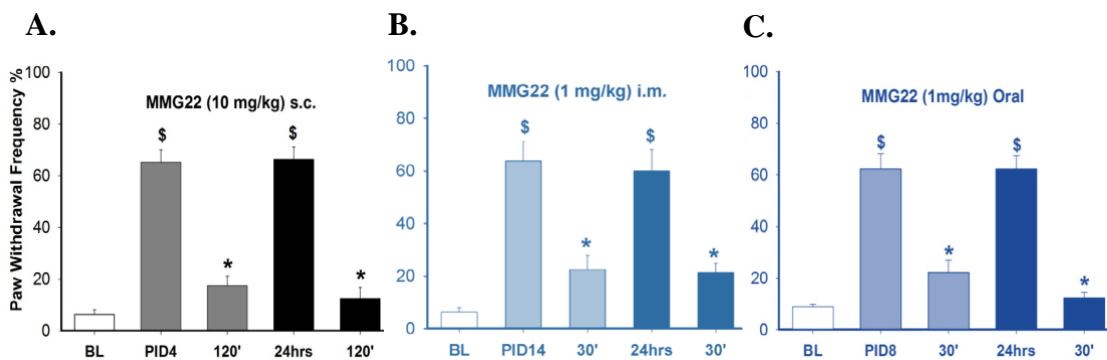
Figure 0.2. **MMG22 did not produce tolerance.** MMG22 did not produce acute tolerance to its analgesic effects when administered s.c. (A), i.m. (B). or orally (C). Anti-hyperalgesia peaked at 60–120 min after MMG22 and was just as effective when administered 24 h after the first administration. $ indicates a significant difference from baseline. * Indicates a significant difference from pre-drug values. All comparisons were made using Bonferroni t-tests, (p < 0.001).

Similarly, acute tolerance for 1mg/kg of MMG22 (ED_{90}) was measured when the drug was administered i.m. and p.o. No acute tolerance to the analgesic effect was detected for i.m. administration (1-way ANOVA with repeated measures, $F (1, 7) = 0.046$, $p = 0.836$) or p.o. administration (1-way ANOVA with repeated measures, $F (1, 8) = 4.00$, $p=0.081$).

Tolerance usually develops with repeated administration of morphine resulting in the need for higher doses. We therefore examined whether tolerance occurred after recurrent administration of MMG22 and compared it with morphine. We compared the antihyperalgesia produced by repeated administration of the ED_{80} dose for MMG22 (0.1 mg/kg) with that produced by repeated administration of the ED_{80} dose for morphine (5 mg/kg) in separate groups of C3H mice. Beginning on PID10, mice were given twice daily s.c. injections of either MMG22 or morphine for nine consecutive days.

Withdrawal response frequencies were determined before and at 60 minutes after injection of MMG22 or morphine on day 1, 3, 6, and 9 of treatment. Tolerance was not observed after MMG22 which produced maximal antihyperalgesia over the 9-day time course (1-way ANOVA with repeated measures, $F (3, 21) = 0.605$, $P = 0.62$) (**Figure 4.3**). However, morphine demonstrated tolerance as early as day 6 (1-way ANOVA with repeated measures, $F (3, 21) = 37.658$, $p <0.001$), and did not produce any antihyperalgesia by day 9 of treatment (Bonferroni t-test, $t=9.453$, $P=<0.001$).
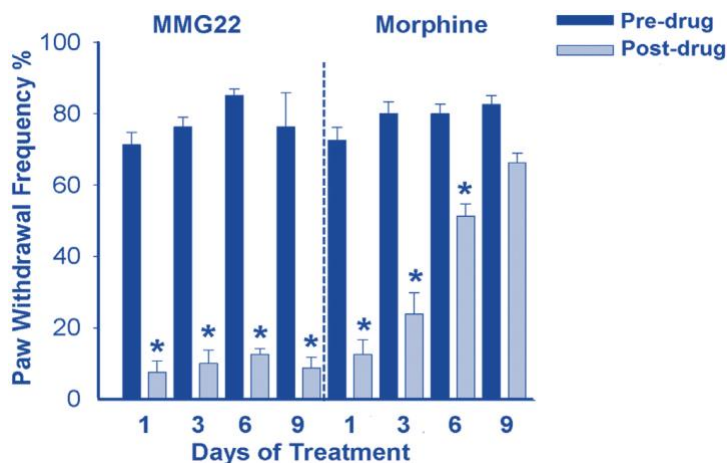
Figure 0.3. **MMG22 does not produce tolerance to its analgesic effect following prolonged administration.** Tumor-bearing mice were treated twice daily with s.c. injections of MMG22 (0.1 mg/kg; n=8) (left panel) or morphine (5 mg/kg; n=8) (right panel) for nine consecutive days, and paw withdrawal frequency was determined before and at 60 min after the injection on days 1, 3, 6, and 9. No analgesic tolerance occurred in mice given MMG22. Black bars are paw withdrawal frequencies before injection and grey bars are withdrawal frequencies after injection. * Indicates a significant difference from pre-injection values (Student-Newman Keuls test, $p < 0.001$.

MMG22 did not produce conditioned place preference

Conditioned place preference test was used to determine whether MMG22 is rewarding in naïve mice. Vehicle or MMG22 (10 mg/kg, s.c.) did not increase the amount of time mice spent on the drug-paired side of the chamber (1-Way ANOVA, $F_{(3, 35)} = 1.58$, $p = 0.21$). The mean amount of time (min) spent in the vehicle-paired chamber was 14.9 ±1.4 before conditioning and 12.9 ±1.2 min. Similarly, the amount of time spent in the MMG22-paired chamber was 15.0 ±1.4 before conditioning and 17.0 ±1.2 min.

MMG22 did not alter motor function

The rotarod test was used to assess whether MMG22 produced sedation and/or motor deficits. Rotarod testing revealed differences between the groups (MMG22 vs. clonidine) in the amount of time mice remained on the treadmill. Whereas mice

remained on the treadmill for less time following clonidine (5 mg/kg) as compared to baseline values, MMG22 (1mg/kg) did not alter the amount of time spent on the treadmill (2-way ANOVA with repeated measures $F_{(1, 31)} = 9.74$, $p = 0.004$). These data indicate that MMG22 did not cause sedation or impair motor function (**Figure 4.4**).
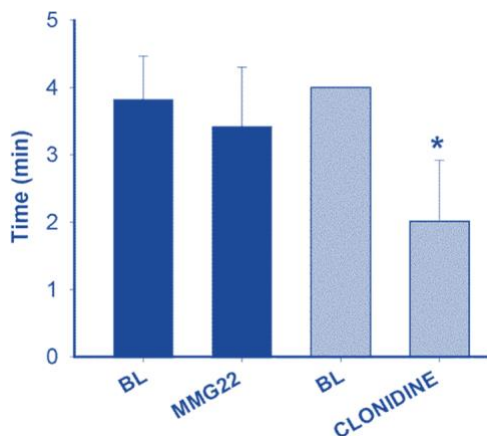
Figure 0.4. **MMG22 did not produce sedation or motor impairment.** Naïve mice were given either MMG22 (1 mg/kg, s.c.) or Clonidine (5 mg/kg, s.c.) and then place on a treadmill before and at 1 h after injection. Data show the amount of time mice spent on the treadmill. MMG22 did not alter the amount of time spent on the treadmill whereas this was reduced following clonidine. ∗ Indicates the difference between time spent on the treadmill before and after clonidine (Bonferroni t-test, p < 0.001).

Naloxone did not precipitate withdrawal following MMG22

We determined whether naloxone produced precipitated withdrawal by determining naloxone-induced jumping in mice treated with morphine or MMG22 for four consecutive days. Naloxone was given 3 hours after the final dose of MMG22 or morphine and produced jumping responses in mice treated with morphine (Mean ±SEM = 32.2 ±10.1 jumps), but not in those that received MMG22 (Mean = 0.0; t= 45.0, P=0.004).

Discussion

The design of the bivalent ligand MMG22 was based on studies showing that opioid receptors can form heteromers with multiple types and classes of GPCRs[251], and

on evidence indicating that MOR and mGluR5 interact functionally[290]. Receptor dimerization can alter receptor function, ligand pharmacology, signal transduction, and cellular trafficking[302]. Importantly, the formation of heteromers may be modulated in pathological states[259]. Thus, MMG22 consisting of pharmacophores derived from oxymorphone (MOR agonist) and M- MPEP (mGluR5 antagonist) and linked through a 22-atom spacer was developed in an effort to target putative MOR-mGluR5 heteromers[303]. The presence of MOR-mGluR5 heteromers has been suggested in cultured cells[290], where phosphorylation, internalization, and desensitization of MOR is reduced following mGluR5 antagonism[290]. Importantly, pain and opioid administration have both been shown to be associated with increased mGluR5 expression in the spinal cord dorsal horn[176,215,260,304]. In this regard, mGluR5 upregulation is thought to contribute to the development of analgesic tolerance of opioids[211,227,305,306].

However, the increased expression of mGluR5 in the inflammatory state alone does not explain the ultra-high efficacy of MMG22 in reducing hyperalgesia, given that a mixture of the monovalents of oxymorphone and MPEP did not enhance antinociception[281,307]. Another possibility is induction of heteromer formation by a bivalent ligand[308,309]. Regardless of how heteromers are formed, a spacer of specific length (22-atom) that links the pharmacophores plays a crucial role in the enhancement of antinociceptive potency of MMG22. Homologues with shorter or longer spacers than 22 atoms were reported to have 3 orders of magnitude lower potency with respect to antinociception in LPS in- flamed mice as compared to the 22-atom spacer[303], suggesting interaction with a heteromer.

In prior studies[291] the efficacy of MMG22 administered i.t. in mice with fibrosarcoma increased with respect to tumor growth and was 572-times greater on PID21 relative to PID3 (ED50: 5.7 to 0.01 fmol/mouse). Moreover, MMG22 was 23,000 times more potent than morphine on PID10 and 3.6 million times more potent on PID21[291]. Furthermore, i.t. administration of MMG22 did not cause tolerance or respiratory depression[303].The present study extends these findings and reveals that systemic administration (subcutaneous, intramuscular, and oral) is highly efficacious in producing progressive, potent antihyperalgesia without tolerance in tumor-bearing mice.

Although the precise mechanisms by which MMG22 reduces hyperalgesia is not clear, recent studies[307] suggest that spinal astrocytes are one of the likely targets of i.t. MMG22, given that the specific astrocyte toxin, L-α aminoadipipic acid (LAA), selectively reduced antinociception of MMG22 in inflamed mice. Both MOR and mGluR5 are found on the peripheral and central terminals of primary afferent nociceptors[215,249,260,304], post-synaptically on neurons in the superficial dorsal horn, and on astrocytes[176,211,215,260,304,305,274]. It has been re- ported that antagonism of cancer-mediated pain associated with upregulated mGluR5 is decreased by the administration of a selective mGluR5 antagonist[310]. Moreover, such treatment[223,266,306] decreases place preference and morphine self-administration[311]. In considering the reported linkage of mGluR5 to the NR2 subunit of the N-methyl-D-aspartate receptor (NMDAR)[99], it was determined whether MMG22 selectively inhibits this ionotropic receptor via antagonism of the mGluR5 co-receptor. Significantly, pre- treatment of mice with the specific NMDAR ion channel blocker, MK801, reduced the antinociception of MMG22 by 2700-fold, suggesting that the antinociception produced by MMG22 is due, at least in part, to

NMDAR inhibition via MMG22 antagonism of mGluR5[307]. This is consistent with the necessity for inflammation- induced activation of the NMDAR in order for MMG22 to be effective, as MMG22 was not effective in naïve mice. Opioid receptors involvement in MMG22 antinociception was established by irreversible blockage of antinociception using the selective MOR irreversible antagonist, β-FNA[307]. The antinociception following systemic administration of MMG22 was not associated with sedation or motor impairment when compared to clonidine as a positive control. Unlike morphine, MMG22 did not produce tolerance or naloxone-precipitated withdrawal, nor did it exhibit rewarding properties as suggested by the lack of conditioned place preference. Interestingly, MMG22 given to mice without bone cancer at a dose that potently reduced hyperalgesia, did not produce analgesic place preference. In addition, high doses of MMG22 given systemically did not cause respiratory depression (unpublished observations). Consistent with earlier studies using i.t. administration of MMG22[291] ,we found that longer and shorter spacer lengths (data not shown) were less potent than MMG22 and caused acute tolerance to its antihyperalgesic effect, further demonstrating the im- portance of the optimal 22-atom spacer length. In summary, the present study shows that systemic administration of MMG22 potently reduces cancer pain without adverse effects. Moreover subcutaneous, intramuscular, and oral routes of administration are substantially more potent and effective compared to that of morphine. The use of bivalent ligands offers a novel and effective approach to treat pain and these data suggest that MMG22 may be advantageous for long-term clinical usage. Given the effectiveness and ED50 dose ranges for MPE suggest that despite its relatively high molecular weight, systemic bioavailability of MMG22 does not appear to be a problem.

Chapter 5

Effects of MMG22 on response properties of nociceptive neurons in the spinal cord

Overview

Primary or metastatic bone cancer is severely painful and often poorly managed. Although opioids are used to treat bone cancer pain, they are associated with several serious side effects, including tolerance, addiction and respiratory depression, thus new and effective medications that are devoid of these side effects are needed. MMG22 consists of a mu-opioid receptor agonist and an mGluR-5 antagonist that are linked through a 22-atom spacer. Intrathecal and systemic administration of MMG22 potently reduced hyperalgesia in a mouse model of bone cancer pain. The goal of this study was to investigate the effects of MMG22 on the sensitization of nociceptive spinal neurons in model for bone cancer. Using a well-established mouse model of bone cancer pain, electrophysiological recordings were made from identified wide dynamic range neurons in the spinal cord. Responses evoked by mechanical and heat stimuli were determined before and after subcutaneous administration of vehicle or 0.1 mg/kg MMG22. MMG22, but not vehicle, dramatically reduced evoked responses.   These results show that the potent antihyperalgesia produced by MMG22 in a model of one cancer pain occurs, at least in part, by decreasing responsiveness of nociceptive dorsal horn neurons.

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Introduction

Pain is one of the most common symptoms reported by patients with cancer[121] and nearly 90% of patients with end-stage cancer report pain[121,159,312]. The most severe pain occurs when bone is involved, such as in primary bone cancer or metastatic bone cancer. Metastasis of tumor cells to bone is particularly common in patients with lung, breast, and prostate cancer[313] and patients with bone metastasis are more likely to experience severe pain[118,120,314–316]. The gold standard for treatment of severe cancer pain is opioids, but it is associated with multiple side effects including constipation, tolerance, addiction, and death caused by respiratory depression. Thus, there is a significant need for the development of new and effective analgesics for cancer pain that do not possess the side effects of traditional opioids.

Rodent models of cancer pain in mice[128,317–325] and rats[326] have been developed. In these models, implantation of osteolytic cancer cells into bone such as the femur or calcaneus, produced signs of spontaneous pain, and mechanical and thermal hyperalgesia on the ipsilateral hind paw. Electrophysiological studies showed that primary afferent nociceptors[143] and WDR neurons in the dorsal horn neurons[144] are sensitized during tumor growth. Sensitization was characterized as an increase in ongoing discharge rate, and increased responses evoked by mechanical, heat and/or cold stimuli.

Earlier studies showed that co-administration of morphine and a mGluR5 antagonist increased the antinociceptive effects of morphine and reduced the development of analgesic tolerance[269,270]. These functional interactions between MOR and mGluR5, and evidence that MOR and mGluR5 can form heteromers[251] provided the rationale to develop MMG22. The bivalent ligand, MMG22, which consists of a mu opioid receptor (MOR)

agonist agonist (oxymorphone) coupled to a metabotropic glutamate receptor-5 (mGluR5) antagonist (MPEP) by a 22-atom spacer, was designed to target a MOR-mGluR5 heteromer. The relation between the spacer length and the potency of MMG22 supported the notion that MMG22 targeted a MOR-mGluR5 heteromer[279]. MMG22 showed extraordinary efficacy and potency for reducing hyperalgesia in a variety of models, including bone cancer pain[307,327]. Based on its extreme potency when given intrathecally[327] suggested that the spinal cord is a primary site of action for MMG22 .

Because the spinal cord appears to be an important site of action for MMG22, and because sensitization of dorsal horn neurons contributes to cancer pain, this study examined the effect of systemic administration (s.c.) of MMG22 on response properties of nociceptive spinal cord neurons in tumor-bearing hyperalgesic mice. Extracellular recordings were made from wide dynamic range (WDR) neurons in the spinal cord of tumor-bearing mice with hyperalgesia. Responses evoked by mechanical and heat stimuli were determined before and after subcutaneous (s.c.) administration of MMG22. These results revealed that MMG22, but not vehicle, dramatically decreased the responsiveness of WDR neurons to mechanical and thermal stimuli in tumor-bearing mice.

Methods

Subjects

Adult male C3H/HeNCr MTV mice (Charles River; 25–30 g) were used. Mice were housed four per cage, allowed free access to food and water, and maintained on a 12-hour light/dark schedule. All protocols and procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee and were

conducted according to the guidelines established by the International Association for the Study of Pain[328].

Cancer cell implantation

NCTC clone 2472 fibrosarcoma cells (American Type Culture Collection, Manassas, VA, USA) were maintained as described previously[329]. This clone was derived from a connective tissue tumor in a C3H mouse, thus the fibrosarcoma cells are syngeneic with C3H/He mice[325]. Mice were anesthetized with isoflurane (2%) and fibrosarcoma cells (2×10^5 cells in 10 µL PBS, pH 7.3) were injected into and around the calcaneus bone of the animal's left hind paw using a 29-g needle. This approach produces a tumor growth with bone osteolysis[325].

Drug preparation and administration

MMG22 was synthesized as described previously[330] and was dissolved in a vehicle of 1.0% DMSO.

Measurement of mechanical hyperalgesia

Mice were placed on an elevated wire mesh platform, covered individually with glass containers, and allowed to habituate to their surroundings for 30 min before testing. A calibrated von Frey monofilament (3.9 mN) was used to measure mechanical sensitivity of the hind paw and was applied to the plantar surface of each hind paw ten times. Mechanical hyperalgesia was defined as a significant increase in the paw withdrawal frequency (PWF), which is calculated as the (number of withdrawal responses/total stimuli) X 100% for each paw. Mice were tested for three consecutive

days before cancer cell implantation and immediately prior to electrophysiological recording after cell implantation to confirm that mice developed mechanical hyperalgesia.

Electrophysiological recording

Extracellular recordings were made from lumbar dorsal horn neurons in tumor-bearing hyperalgesic mice PID (10-18)[144],[331]. Mice were initially anesthetized with 2.5-4% isoflurane in an induction chamber and anesthesia was maintained with 1-2% isoflurane delivered via a nosecone. Core temperature maintained at 37ºC using a feedback-controlled heating pad (Physitemp Instruments, Inc, Clifton, NJ). A laminectomy was performed at L4 and L5 spinal segments to expose the lumbar enlargement. Mice were secured in a spinal frame, the dura was removed, and the spinal cord was bathed in mineral oil. Extracellular recordings were made from dorsal horn neurons using glass microelectrodes (~1 mΩ; Kation Scientific, Minneapolis, MN) that were lowered into the spinal cord in 3-µm steps using a hydraulic microdrive (Kopf, Tujunga, CA). Action potential activity was amplified, audio-monitored, displayed on a storage oscilloscope, and sent to a PC computer. Action potentials were discriminated according to shape and amplitude using Spike II data acquisition program (LabView, National Instruments Co., Austin, TX). Neuronal activity, discriminated impulses, time of application of mechanical stimuli, and stimulus temperatures were collected and stored for off-line analyses.

Classification of nociceptive neurons

The receptive field (RF) areas of dorsal horn neurons were identified by lightly stroking the skin with a soft brush, and by applying mild pressure and mild pinch of the skin with the experimenter's fingers on the plantar surface of the tumor-bearing paw. Neurons were classified as low threshold (LT), wide dynamic range (WDR) or high threshold (HT). Only single, nociceptive WDR neurons with well discriminated action potentials were studied.

Experimental design

Following identification and characterization of a WDR neuron, the level of spontaneous activity was recorded for 3 minutes. Subsequently, responses evoked by mechanical and heat stimuli were determined. Mechanical stimuli consisted of applying calibrated von Frey monofilaments (26 and 60 g; 255 and 588mN) to the RF 3 times for 5 s each with an interstimulus interval of approximately 60 s. The number of evoked impulses were averaged for each stimulus intensity. Next, responses evoked by a heat ramp (30-50°C maintained for 1s) were determined using a custom-made Peltier thermode (0.5 cm^2) that was placed on the RF. A within groups design was used and each cell received all treatments (vehicle and MMG22). Spontaneous activity (3 minutes) and responses to mechanical and heat stimuli were obtained before any injection, and at 30 and 60 minutes after s.c. injection of vehicle, and at 30 and 60 minutes following s.c. administration of 0.1 mg/kg MMG22 which was the ED80 dose obtained from our previous published studies[307] (**See Chapter 4 above**).

Data analyses

Responses to mechanical stimuli were determined by subtracting the average ongoing discharge rate during 10 seconds before the stimulus from the response that occurred during the stimulus (5 s) and for 5 s following stimulus cessation. For each neuron, the mean number of impulses evoked by each stimulus was obtained from 3 trials. Similarly, the ongoing discharge rate just prior to heat stimulation was subtracted from responses evoked during heat stimuli (during the heat ramp and for 10 sec after to include any after discharge). The number of impulses evoked by mechanical and heat stimuli were compared before and after vehicle or MMG22 by one-way ANOVA with repeated measures. Post-hoc comparisons were made using Bonferroni t-tests.

Results

Mice (n=8) were tested for mechanical sensitivity just prior to the recording experiments and all mice exhibited mechanical hyperalgesia, defined as an increase in the frequency of withdrawal evoked by a von monofilament with a bending force of g (0.3mN) (**Figure 5.1**).
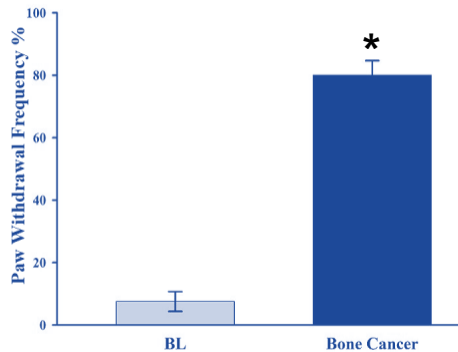
Figure 0.1. **Tumor-bearing mice exhibited mechanical hyperalgesia.** Prior to electrophysiological recording, tumor-bearing mice exhibited robust hyperalgesia (P = <0.001).

Electrophysiological recordings were made from the lumbar enlargement of tumor-bearing mice. Eight neurons were studied from 8 mice. The depth of the recording site measured from the dorsal surface of the spinal cord ranged from 159-620 µm, indicating that recorded neurons were located in the superficial and the deep dorsal horn. All neurons were classified as WDR (**see Figure 5.2**) according to their responses evoked by graded mechanical stimuli (brush, pressure, and pinch for 5 sec each) and had RF areas that included the plantar surface of the hind paw. Of the 8 neurons that were studied, 3 exhibited ongoing spontaneous activity that ranged from 0.5 – 1.1 m/s and 6 responded to heat.
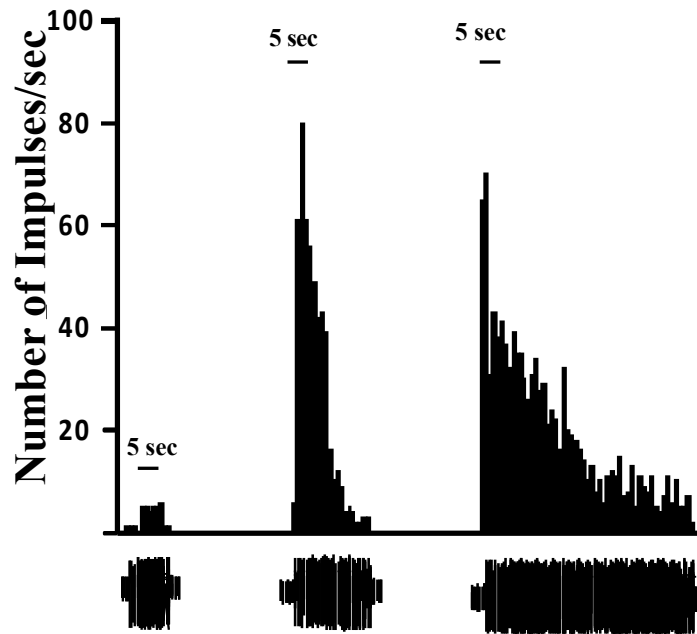
Figure 0.2. **Functional characterization if WDR neurons.** Response of a single WDR neuron evoked by brush (left), pressure (middle), and pinch (right) for 5 seconds. Response histograms show discharge rates/1-second bin width. Evoked action potentials are shown below each histogram.

MMG22 decreased responses of dorsal horn neurons to mechanical and heat stimuli

Earlier studies from our lab showed that responses of WDR neurons to mechanical and heat stimuli were increased in tumor-bearing mice as compared to naïve mice[144]. Therefore, we examined the effect of MMG22 on responses evoked by mechanical (26 g and 60 g applied for 5 s) and heat stimuli (50°C for 1 s) applied to the RF. Responses were obtained before any drug administration, 30 and 60 min after s.c. administration of vehicle, and 30 and 60 min after 0.1 mg/kg MMG22. (**Figure 5.3. A & B)** shows a representative example of the effect of vehicle and MMG22 on responses to mechanical stimuli for a single WDR neuron. In this example, MMG22 reduced the number of neuronal impulses evoked by 60 g and nearly eliminated responses evoked by

the 26 g monofilament at 30 and 60 min post-drug. A one-way ANOVA with repeated measures indicated that MMG22 reduced the number of impulses evoked by mechanical stimuli for 26 g (F $(2, 13)$ = 11.839, P=0.001) and 60 g F $(2, 8)$ = 10.598, P= 0.002) at 30 minutes. A one-way ANOVA with repeated measures indicated that MMG22 reduced the number of impulses evoked by mechanical stimuli for 26 g (F $(2, 13)$ = 10.598, P=0.002) and 60 g F $(2, 13)$ = 8.827, P= 0.004) at **30 minutes**. A one-way ANOVA with repeated measures indicated that MMG22 reduced the number of impulses evoked by mechanical stimuli for 26 g (F $(2, 13)$ = 10.598, P=0.002) and 60 g F $(2, 13)$ = 8.827, P= 0.004) at **60 minutes**. As shown in (**Figure 5.3. B**), s.c. administration of the vehicle did not alter responses to the 26 g monofilament at 30 or 60 min. In contrast, responses evoked by the 26 g were significantly reduced at 30 min (P< 0.05) and 60 min (P<0.05) after administration of MMG22. Similarly, responses evoked by the 60 g monofilament were also reduced at 30 min (P<0.001) and at 60 min (P<0.002) after MMG22. Subcutaneous injection of 0.1 mg/kg of MMG22 produced a 50% and 42.1% reduction in the number of action potentials produced by the 26 g von Frey filament at 30 and 60-minutes after injection, respectively. MMG22 decreased the number of action potentials produced by the of 60 g von Frey filament 54.5% and 45.5% 30 and 60minutes after injection, respectively.

A.

B.
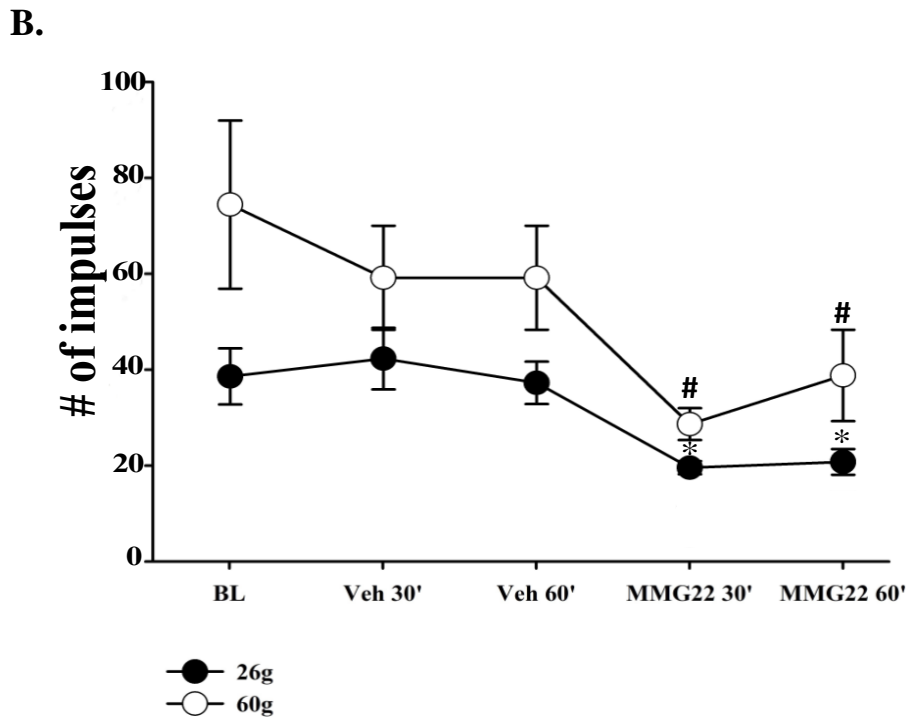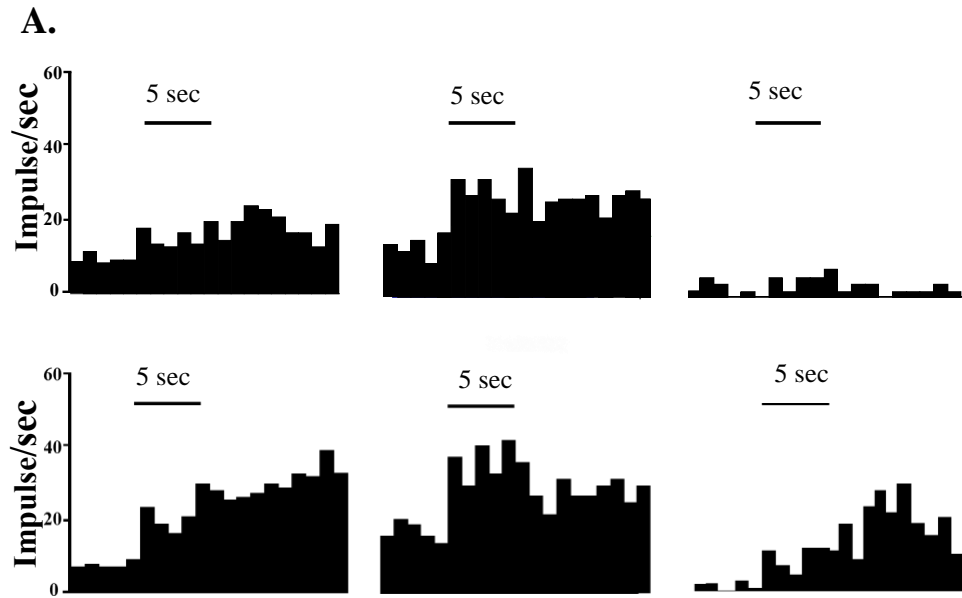
Figure 0.3. **MMG22 reduced responses evoked by mechanical stimuli.** A. Representative example of responses of a WDR neuron evoked by 26 g (above) and 60 g (below) at baseline (left), 60 minutes after vehicle (middle) and 60 minutes after MMG22 (0.1 mg/kg) (right). Response histogram shows discharge rates per one-second bin. **B**). Mean number of impulses for all neurons. ***** and **#** indicates a significant difference from baseline (*P < 0.05), #P<0.002; Bonferroni t-tests)

Responses to heat were also reduced after MMG22, but not after vehicle (F (4, 19) = 5.923, *P* =0.003). **Figure 5.4a.** shows a representative example of the responses of a single WDR neurons to the heat ramp to 50°C before injection, at 60 min after vehicle, and at 60 min following MMG22. These neurons exhibited similar robust responses to heat before injection and after vehicle, whereas the response was dramatically decreased after MMG22. The mean number of impulses for all neurons did not change after vehicle, whereas MMG22 caused a 86% at 30 minutes and 92% at 90 minutes post-drug.

A.

B.
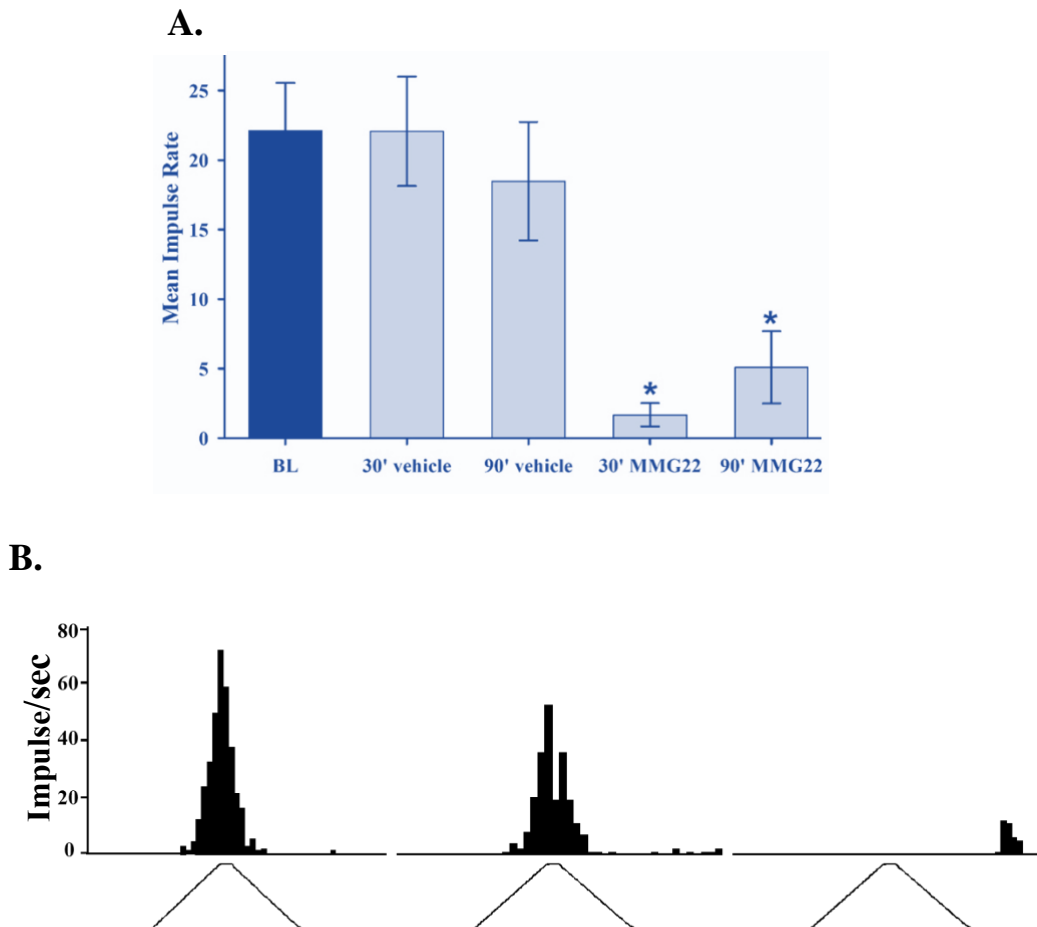
Figure 0.4. **Effects of MMG22 on WDR responses to heat stimuli.** (A) Mean (±SEM) number of impulses for all neurons before any injection (BL), at 30 and 60 minutes after vehicle, and at 30 and 60 minutes after MMG22 (*P<0.05; Bonferroni t-tests). (B) Representative responses of a single WDR neuron to heat before any injection (left panel), 60 minutes after injection of vehicle (middle panel) and 60 minutes after MMG22 (0.1mg/kg s.c.) (right panel). MMG22, but not vehicle, reduced the number of impulses evoked heat.

Discussion

Consistent with earlier studies of bone cancer pain[143,144,307,325,327,332], implantation of fibrosarcoma cells into and around the calcaneus bone in C3H mice produced robust mechanical and heat hyperalgesia. The mechanisms underlying pain and hyperalgesia in this model of bone cancer pain include both peripheral and central mechanisms. C-fiber nociceptors were shown to exhibit spontaneous activity and sensitization to heat, but not to mechanical or cold stimuli[143], suggesting the peripheral nociceptor sensitization contributes to spontaneous pain and thermal hyperalgesia. Dorsal horn neurons were also sensitized in this model[144]; however, unlike peripheral nociceptors, dorsal horn WDR neurons in tumor-bearing mice exhibited increased responses to mechanical, cold and heat stimuli[144,333], suggesting that central sensitization plays a role in pain and hyperalgesia from bone cancer. Results of the present study show that systemic administration of 0.1 mg/kg MMG22, which reduced hyperalgesia in tumor-bearing mice[307], decreased responses of WDR neurons to mechanical and heat stimuli in mice with tumor-evoked hyperalgesia.

MMG22 was designed to target a putative MOR-mGluR5 heteromer. MMG22 was designed to activate MOR and to inhibit mGluR5. mGluR5 was chosen as a target because of its known involvement in pain and its interactions with MOR. For example, mGluR5 antagonists increased the potency of opioids[264,266,267] and prevented the development of analgesic tolerance[211,227,305,306]. Activation of mGluR5 produces hyperalgesia[93-97] while mGluR5 antagonists reduce hyperalgesia in various pain models[94,112-115]. mGluR5 is expressed by dorsal horn neurons, as well as nociceptive primary afferent fibers[94,98–106]. Studies have shown that mGluR5 is upregulated in the dorsal horn in models of inflammatory[206,224,225], neuropathic[224–227], and bone cancer pain[222]. mGluR5 was also

upregulated in primary afferent fibers in models of neuropathic pain[219,220]. Importantly, mRNA for MOR and mGluR5 has been shown to be co-expressed by the same neurons in the DRG and spinal cord [275], suggesting that MOR and mGluR5 may form heteromers both in the periphery and in the spinal cord. If confirmed, this would suggest that MMG22 targets neurons in the periphery as well as spinal cord.

Although MMG22 reduced the sensitization of dorsal horn neurons, it is still unclear if this reflects a peripheral or central site of action, or both. A peripheral site of action is supported by electrophysiological studies showing that systemic administration of MMG22 reduced activity of C-fiber nociceptors in the spared nerve injury of neuropathic pain (Speltz thesis, paper in preparation). In this model, MMG22 was more potent in reducing hyperalgesia than morphine early after injury (first 10 days) but equipotent at 30 days after injury. It was proposed that this may be related to the degree of inflammation. After nerve injury, there is an early inflammatory response that resolves after 2 to 3 weeks[334–336]. whereas the inflammatory response associated with tumor growth remains elevated over time[337–339,340].

A spinal site of action for MMG22 is supported by the extreme potency of MMG22 in reducing inflammatory and cancer pain following intrathecal administration[292]. In the spinal cord, activation of NMDA receptors[236,246] and specifically its NR2B subunit, are important for the development of central sensitization and hyperalgesia. Hind paw inflammation increased the phosphorylation of NR2B in the spinal cord[236]. Systemic application of NR2B-selective antagonists had antinociceptive effects in models of inflammatory and neuropathic pain[341] and blocked central sensitization[342,343]. However, the role of NMDA receptors in MMG22-induced anti-nociception is not well defined.

Astrocytes have been implicated as another factor in the mechanism of MMG22; blocking the activation of astrocytes lead to the reduction of its analgesic effects[284]. This notion is consistent with the fact that astrocytes are upregulated in a bone cancer pain model[151] and may help explain the increase in potency of MMG22 as the bone cancer progresses[307,327,344].

Although MMG22 is extremely potent following intrathecal administration, and the link between mGluR5 and NMDA receptors supports a spinal site of action, it is unclear if MMG22 penetrates the spinal cord following systemic administration because of its high molecular weight (926 Da). However, it should be noted that the blood brain barrier can be disrupted in certain condition allowing greater passage[345]. Further studies are needed to determine if MMG22 gains access to the spinal cord in the cancer pain model.

Given that a combination of the monovalents, the mGluR5 antagonist, MPEP and oxymorphone delivered to mice provides no enhancement of antinociception[330], enhanced expression of mGluR5 in the inflammatory state alone cannot explain the ultra-high efficacy of MMG22 in lowering hyperalgesia. In this regard, a spacer of a specific length (22 atoms) that connects the pharmacophores is critical in optimizing MMG22's antinociceptive effectiveness. In LPS-inflamed mice[330], homologues with shorter or longer spacers than 22 atoms were observed to have 3 orders of magnitude lower antinociceptive potency. The introduction of a mGluR5 antagonist has been shown to reduce of cancer pain accompanied with elevated mGluR5 receptor expression[151]. Furthermore, this treatment was associated with reduction in place preference and morphine self-administration[223,266,268,346]

In conclusion, systemic administration of MMG22 greatly reduced the sensitization of dorsal horn neurons in our model of cancer pain, which coincided with its potent effect at reducing hyperalgesia in tumor-bearing mice. Based on evidence that MOR-mGluR5 heteromers may act on DRG and spinal neurons, suggests that the potent antinociceptive effects of MMG22 result from decreased excitability of both peripheral nociceptors and spinal dorsal horn neurons.

Chapter 6

General Discussion

Metastatic bone cancer is extremely painful and is associated with many other common cancers, including breast and prostate cancer. Cancer pain treatment in general is challenging due to its severity and the variety of side effects associated with treatments, and often requires a multidisciplinary approach[279]. Pain from cancer must be assessed correctly, and the efficacy of management must be carefully evaluated and consider side effects of treatment and improvement in patients' outcomes. Opiates, although often effective until tolerance develops, continue to be the mainstay for treating severe cancer pain but they are associated with many significant side effects, ranging from constipation to addiction and to death resulting from respiratory depression. The side effects of opiates underscore the need to develop novel treatment with high analgesic potency and minimal side effects.

The novel drug MMG22, developed by Dr. Portoghese and colleges[330], is based on the relatively new concept of receptor dimerization. The drug is composed of an mGluR5 antagonist and MOR agonist connected with a 22-atom linker; the concept of this structure is based on the current drug development strategy that hypothesizes that different G protein-coupled receptors form heterodimers. Indeed, there is evidence that mGluR5 and MOR can form a heteromer. Furthermore, it is now known that there are different physiological and functional effects between targeting a single receptor and targeting heteromers. The function of receptors, ligand pharmacology, signal transduction, and cellular transport can all be affected by receptor dimerization[347]. In the case of MMG22,

its efficacy and potency of MMG22 was more potent than morphine when administered intrathecally or systemically in a variety of rodent pain models. Moreover, it appears to lack the side effects of traditional opiates such as tolerance and respiratory depression.

Although multiple types and classes of GPCRs have been shown to form heteromers with opioid receptors[251], and the formation of heteromers can be influenced by certain pain states[258]. Two major issues remain regarding the mechanism of action of MMG22. The first is whether MMG22 specifically targets a MOR-mGluR5 heteromer. Although the components of MMG22 each produce antinociceptive effects, one line of evidence that MMG22 targets a heteromer is that MMG22 was more effective than administration of a combination of the monovalent[281]. Importantly, the antinociception produced by MMG22 occurs, at least in part, by inhibiting the NMDA receptor. Thus, since expression of mGluR5 is increased during inflammation, it is possible that this accounts for the high analgesic efficacy in inflammatory pain conditions. However, this alone cannot explain the ultra-high efficacy of MMG22 because of the relation of spacer length (22 atoms) that connects the pharmacophores to MMG22's antinociceptive effectiveness. For example, in LPS-inflamed mice[281], homologues with shorter or longer spacers than 22 atoms had approximately 3 orders of magnitude less antinociceptive potency than MMG22. The importance of the spacer length further supports the notion that MMG22 targets a heteromer. Finally, for a heteromer to develop, both receptors must be located on the same cells. The existence of MOR-mGluR5 heteromers was reported in cultured cells, where mGluR5 antagonism reduced MOR phosphorylation, internalization, and desensitization[276], and it was recently shown that

that mRNAs for both receptors were co-expressed in neurons in the lumbar spinal cord and DRG early after nerve injury[275].

Although the above studies support the notion that MMG22 acts on a MOR-mGluR5 heteromer, targeting of a heteromer as defined as by the importance of linker length to the potency of MMG22 may depend on the pain model used, the time after injury, and the route of administration. For example, in the spared nerve injury model of neuropathic pain, MMG22 and its shorter spacer analog were equipotent in reducing hyperalgesia[280]. Furthermore, it was demonstrated that co-administration of oxymorphone and MPEP exhibited analgesic synergism, suggesting that the two pharmacophores of MMG22 and related compounds may target MOR and mGluR5 as separate receptor monomers. Additional studies are needed to determine under which pathological pain conditions MMG22 targets a heteromer or targets their separate receptors to produce synergy.

A second major question regarding MMG22 is its site of action. In the model of bone cancer pain used in this study, hyperalgesia is associated with sensitization of WDR neurons in the spinal cord[348]. Because of MMG22's extraordinary potency following intrathecal administration, we sought to determine if systemic administration of MMG22 also reduced the activity of WDR neurons. However, these data need to be interpreted with caution since in the spared nerve injury model, systemic administration of MMG22 reduced evoked activity of C-fiber nociceptors by about 30% (Speltz, unpublished). This, in addition to the finding that mRNAs for both receptors were found on DRG neurons, suggests that MMG22 acts on peripheral nociceptors and on WDR neurons in the spinal cord. Further studies are needed to determine the contribution of peripheral mechanisms to

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the antinociception produced by systemic administration of MMG22 in the cancer pain model.

In summary, targeting multiple receptors offers the possibility of profound antinociceptive potency by either targeting heteromers or by synergy of the individual components. Both mechanisms may require very low doses which alone may minimize side effects. Moreover, targeting receptors in the periphery or spinal cord is particularly attractive since this approach may result in fewer side effects compared to receptor activation in the brain. For example, Wilcox and colleagues[184,349] showed that intraplantar injection or topical application of the peripherally-restricted MOR agonist loperamide and the delta opiate receptor agonist oxymorphindole synergized to produce potent antinociception to heat and mechanical stimuli which occurred by reducing the excitability of peripheral nociceptors. It is not yet known if peripheral (i.e. topical) administration of MMG22 is effective as an analgesic, although the concept of targeting multiple receptors may lead to the development of novel analgesics without serious side effects.

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