GLUTAMATE IN CANCER-INDUCED BONE PAIN

CAPSAZEPINE ATTENUATES CANCER-INDUCED BONE PAIN BY INHIBITING GLUTAMATE RELEASE

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Abstract

Breast cancer has the highest incidence rate in women, accounting for more than 22% of all cancers and possessing a strong disposition to metastasize to bone. These skeletal metastases become a significant cause of morbidity and mortality in patients with the primary symptom being pain. Pain is a major concern in determining a patient's quality of life and there have been many attempts to understand and control bone pain with little success. Previous studies have shown that glutamate plays a role in bone cancer pain, with an excess in free glutamate able to cause pain either directly through excitotoxic pathways or indirectly though the dysregulation of osteoclasts and osteoblasts, causing bone dysregulation. TRPV-1 receptors have also has been implicated in the mechanisms of bone cancer pain, as osteoclasts release protons during bone remodeling which can elicit a TRPV-1-related nociceptive response from neurons in the surrounding periosteum. Capsazepine was identified during a high throughput screen of 30,000 compounds to be a potent inhibitor of breast cancer cell-mediated glutamate release, a neurotransmitter with known associations in neural signaling, bone homeostasis, and pain. Capsazepine also has antagonistic effects on transient receptor potential vanilloid type 1 (TRPV-1) receptors which act as key players in both heat and vanilloid-induced nociception. These findings suggest that Capsazepine may provide a multi-site effect for the treatment of cancer-induced bone pain. An animal model of breast cancer-induced bone pain involved intrafemorally injecting MDA-MB-231 cancer cells to measure pain. Behavioural tests are then performed measuring dynamic weight bearing and paw withdrawal thresholds. These measurements are used to demonstrate both movement-evoked and spontaneous pain-related behaviour of the affected limb. Using Capsazepine, we demonstrate a dose-dependent attenuation of pain behaviour in vivo, while confirming tumour presence using immunohistochemistry (IHC). We show that TRPV-1 and glutamate play an important role in the onset and severity of bone cancer pain and blocking these pain pathways provide relief from pain commonly associated with cancer in the bone.

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List of Abbreviations

AMPA……..…. α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AMPAR…….. α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor
AP……... Action Potential
AP-1……..……. Activator Protein 1
AREB……..…… Animal Research Ethics Board
ASC……... Alanine-Serine-Cysteine
ATP……... Adenosine Triphosphate
BBB……... Blood Brain Barrier
BDNF……... Brain-Derived Neurotrophic Factor
BK……...… Bradykinin
BMP…….. Bone Morphogenic Protein
cAMP……...…. Cyclic Adenosine Monophosphate
CFA…… Complete Freund's Adjuvant
CNS……... Central Nervous System
CPCCOEt……. Cyclopropan[b] Chromen-1a-Carboxylate
CSF……... Cerebrospinal Fluid
DAG…….. Diacylglycerol
DMEM……... Dulbecco's Modified Eagle Medium
DMSO……..….. Dimethyl Sulfoxide
DPA……. Dynamic Plantar Aesthesiometer
DRG…….. Dorsal Root Ganglion
DWB…… Dynamic Weight Bearing
EAAT…….. Excitatory Amino-Acid Transporter
ECM……. Extracellular Matrix
EPSP……..…….. Excitatory Postsynaptic Potential
FBS……... Fetal Bovine Serum
GPCR……. G-Protein Coupled Receptor
ICV……..…… Intracerebroventricular
iGluR…….. Ionotropic Glutamate Receptor
IP_3…….. Inositol 1, 4, 5-Trisphosphate
kDa…….. Kilodalton
M-CSF…….. Macrophage Colony-Stimulating Factor
MEGM……. Mammary Epithelial Cell Growth Medium
mGlur…… Metabotropic Glutamate Receptor
MMP…… Matrix Metalloproteinase
MPEP……...…… 2-Methyl-6-(Phenylethynyl) Pyridine

NFκB…….. Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B-Cells
NGF…… Nerve Growth Factor
NMDA……. N-Methyl-D-Aspartate
NMDAR……... N-Methyl-D-Aspartate Receptor
NSAID……... Non-Steroidal Anti-Inflammatory Drug
OPG…… Osteopretegerin
PBS……. Phosphate Buffered Saline
PGE_2…….……. Prostaglandin E_2
PNS……... Peripheral Nervous System
PPP……... Pentose Phosphate Pathway
PTH…… Parathyroid Hormone
PTH1R……... Parathyroid Hormone Type-1 Receptor
PTHrP……. Parathyroid Hormone–Related Protein
RANK……. Receptor Activator of NFκB
RANKL…….. Receptor Activator of NFκB Ligand
RBC…… Red Blood Cells
ROS……. Reactive Oxygen Species
RPM……..……. Revolutions per Minute
Runx2……. Runt-Related Transcription Factor-2
(S)-4-CPG…….…….. (S)-4-Carboxyphenylglycine
SEM…….……. Standard Error of the Mean
SLC1……... Solute Carrier Family-1
SLC3……... Solute Carrier Family-3
SLC7A11…… Solute Carrier Family-7 Member 11
Smad3…….…… Mothers Against Decapentaplegic Homolog 3
SSZ…….. Sulfasalazine
TGF-β…….…… Transforming Growth Factor β
TRP……. Transient Receptor Potential
TRPC……. Transient Receptor Potential Canonical
TRPV…….. Transient Receptor Potential Vanilloid
TRPA…… Transient Receptor Potential Ankyrin
TRPM……... Transient Receptor Potential Melastatin
TRPP…… Transient Receptor Potential Polycystin
TRPML……. Transient Receptor Potential Mucolipin
TRPN…… Transient Receptor Potential No Mechanoreceptor Potential C
TRPV-1……. Transient Receptor Potential Cation Channel Vanilloid Subfamily Member-1
TRR1……..... Thioredoxin Reductase 1
VAS…….……. Visual Analogue Scale

VEGF…… Vascular Endothelial Growth Factor
VGLUT…… Vesicular Glutamate Transporter
WHO…… World Health Organization

Chapter 1: Introduction

Cancers of the bone can be divided into either primary or secondary tumours. Primary cancers arise from the bone itself whereas secondary bone tumours are a product of metastasis from distant sites. Most cancers of the bone are secondary in nature, occurring from primary tumours metastasizing from distant sites rather than through the growth of a primary cancer in the bone. Primary cancers involving bone metastasis are unevenly distributed, occurring more frequently in cancers of the lung, thyroid, kidney, lung, prostate, and breast having the highest likelihood of bone metastasis (Coleman, 2006). These cancers usually metastasize to bone with high blood supply such as the spine, ribs, pelvis, arms and thigh as blood supply is vital for the transportation of metastatic tumour cells from distal sites (Steenland et al., 1999).

This thesis will focus on breast cancer, which has the highest incidence of all women, accounting for 22.9% of diagnosed cancers (IARC, 2008) and is characterized by a strong predisposition to metastasize to bone (Coleman, 2006). Bone metastasis has an approximately 70% prevalence in patients with advanced breast cancers (Abdelaziz et al., 2014) and is seen in greater than 90% of patients who die from breast carcinoma (Clohlsy & Mantyh, 2003). Once tumours metastasize to the skeleton they are associated with a dramatic change of the bone microenvironment, altering osteoclast and osteoblast functions and regulation, resulting in a host of pathological consequences such as skeletal remodeling, fractures, anemia, hypocalcaemia (Coleman, 2006) and cancer-induced bone pain (Jimenez-Andrade, 2010). Bone metastasis is correlated with a sharp increase in patient morbidity and mortality (Jimenez-Andrade, 2010).

Pain is the most common symptom of bone cancer metastasis, ranging from 15% to over 75% of cancer patients depending on the severity of the disease (Portenoy et al., 2011). Once tumour cells have invaded the bone, pain is usually described as a constant dull sensation which gradually increases with time. Treatment of bone pain is commonly done through adherence to the World Health Organization's (WHO) analgesic ladder, a stepwise progression from non-opioid analgesics to strong opioids, increasing the medication strength as pain progresses past the analgesic barriers provided by the current treatment regimen (WHO, 1996). Adjuvant therapies such as radiotherapy, bisphosphonates, and corticosteroids can also be used in conjunction with the WHO analgesic ladder. However, it has been shown that at least 20-40% of cancer pain is not adequately relieved by application of the WHO analgesic ladder. Many of these secondary treatment strategies develop tolerance or have dose-dependent side effects that limit treatment options to short term pain relief (Weiss et al., 2001; Twycross et al., 1990). Limited success has been achieved at relieving pain long term, with more than 50% of patients experiencing pain reoccurring shortly after treatment and 63% of patients describing one or more episodes of pain breaking through their analgesic regimen daily (Clohlsy & Mantyh 2003).

Non-steroidal anti-inflammatory drugs (NSAIDs) are most often the first line of response to pain, but as with other treatments, they are associated with several dose-limiting side effects such as toxic renal, haematological, gastrointestinal, and cardiovascular effects (Portenoy et al., 2011). The limited effectiveness of current pain control proves to be an important area of research as current therapies are either ineffective, or have side-effect profiles that limit long term use. The investigation of novel targets and pain management strategies are critical to

maintaining patient quality of life and these deficiencies in the current practice of medicine have shaped this thesis project towards finding an alternative route or mechanism for the treatment of cancer-induced bone pain. My project follows glutamate, a common neuro-signaling molecule in the nervous system with known associations with pain and the regulation of proper bone homeostasis (Seidlitz et al., 2010b).

Glutamate

Glutamate is the deprotonated form of glutamic acid, a non-essential amino acid used as a fundamental building block of proteins. It makes up more than 10% of all amino acids found in all three domains of life (Eukaryotes, Prokaryotes and Archaea), making it one of the most abundant amino acids known, with $_{L}$-glutamate being the dominant form (Burkhard Rost, 2002). In humans, $_{L}$-glutamate is mainly produced through the action of glutaminase, a mitochondrial enzyme which readily synthesizes glutamate from its precursor molecule, glutamine (Newsholme et al., 2002). Glutamate can also be derived from α-ketoglutarate, an intermediate of the Krebs cycle by transamination (Nedergaard et al, 2002) (see Figure 1.1 for glutamate synthesis pathways). This second reaction however is far less common in mammals as the reaction equilibrium favours ammonia and α-ketoglutarate. In humans, glutamate has been shown to have an expanded functional repertoire beyond its use as a neurotransmitter, acting as a signalling molecule for non-neuronal cells, an essential cellular nutrient, a reactionary intermediate, and a pain producing signal.

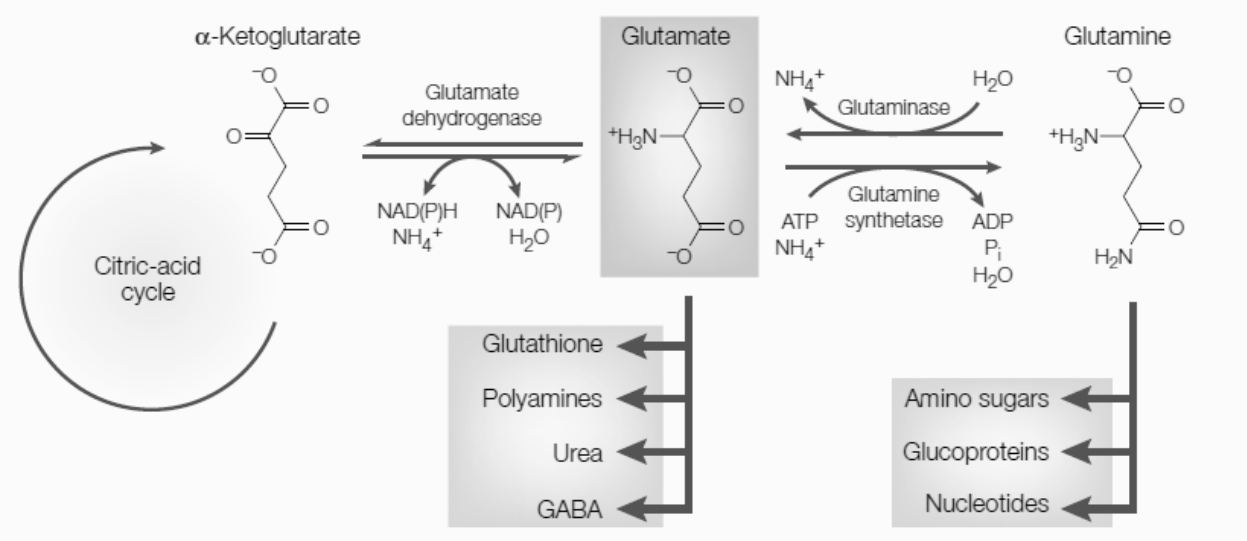


Figure 1.1: Common glutamate synthesis pathways from glutamine and α-ketoglutarate (Nedergaard et al, 2002)**.**

Glutamate is most widely known for its role in the CNS, acting as an excitatory neurotransmitter and cell signaling molecule. Each neuron in the CNS contains roughly 5mM of intracellular glutamate (Fitzpatrick et al., 1988) stored in synaptic vesicles which will become the primary source of extracellular glutamate in the CNS. The release of these vesicles is dependent upon calcium channels, as Ca^{2+} influx initiates vesicular release of glutamate into the synapse (Shoudai et al., 2010). After exocytosis of the glutamatergic vesicles occurs, the now extracellular glutamate rapidly binds to one of several receptor types on the postsynaptic neuron with surrounding astrocytes waiting to reuptake the excess. Glutamate is an excitatory neurotransmitter, meaning that binding of this ligand to its receptor initiates excitatory post-

synaptic potential (EPSP) in the postsynaptic neural dendrites. EPSP, buildup in the dendrites of the receiving neuron and if the signal is of sufficient strength, will lead to AP propagation.

Glutamate Receptors

Glutamate receptors are divided into two groups based on the mechanism by which their activation will lead to signal propagation. These two groups are ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) (Figure 1.5).

Ionotropic Glutamate Receptors

The iGluR family consists of three main types of glutamate receptor: N-methly-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate receptors. The receptor names are derived from selective agonists that bind each receptor type. iGluRs are chemically gated ion channels which allow ions such as Ca^{2+} to pass through the membrane in response to glutamate binding.

NMDA receptors (NMDARs) are a specific type of glutamate receptor that predominately consist of a heterotetramer between two GluN1 and two GluN2 subunits which are necessary for functional control of NMDARs in humans (Retchless et al., 2012). NMDARs are permeable to the influx of both Na^{+} and Ca^{2+} and the efflux of K^{+}, but initially are blocked at an allosteric site by resident Mg^{2+}. This property is unique to NMDARs and removal of Mg2+ is

necessary for the pore to open (Mayer et al., 1984). Freeing the channel from Mg2+ is achieved through membrane depolarization commonly caused by other glutamate receptors such as AMPA receptors (AMPAR). This initial inhibition means that the early stages of glutamate binding are driven solely by AMPAR while NMDARs are slower to act (Rosenzweig & Breedlove, 2007).

AMPARs mainly consist of four subunit types (GluR1, GluR2, GluR3, and GluR4) but can exclude GluR2 (Song et al., 2014). These receptors are responsible for rapid synaptic responses from glutamate due to its specific binding properties. Glutamate can bind any of the four subunits, with the ion channel becoming permeable to Na^+ and Ca^{2+} and the efflux of K^+ after two or more sites are bound. If more than two sites are ligand-bound, the current increases, driving more ion exchange with the extracellular environment (Rosenmund et al., 1999). The permeability of calcium ions is governed by GluR2, as its presence can make the channel impermeable to Ca^{2+}. This is due to ribonucleic acid (RNA) editing of the subunit, substituting a glutamine for a positively-charged arginine, changing its structure and making calcium entry energetically unfavourable (Wight & Vissel, 2012).

The kainate receptors, originally identified as a distinct receptor type for its selective affinity for kainate, is the least understood of the ionotropic receptors. There are five types of kainate receptor subunits (GluR5, GluR6, GluR7, KA1 and KA2) which, similarly to AMPAR composition, are arranged into tetramers (Dingledine et al., 1999). These receptors also require

the binding of glutamate to two or more subunits in order to open the ion channel, and similarly to AMPAR, respond quickly to synaptic glutamate (Schauder et al., 2013).

Metabotropic Glutamate Receptors

The metabotropic glutamate receptors, or mGluRs are a part of the C-group family of G-Protein coupled receptors (GPCRs) which respond to GTP activation and other metabotropic processes, such as secondary messenger systems. Similarly to other metabotropic receptors, mGluRs have seven transmembrane domains flanked on either side by an intracellular C-terminal and an extracellular N-terminal (Kunishima et al., 2000). There are eight variations of mGluRs which are further divided into three groups (I, II, and III). This secondary grouping is based upon physiological activity (Kunishima et al., 2000). Group I mGluRs (mGluR 1 and 5) are associated with phospholipase C activity to hydrolyze lipids in the plasma membrane leading to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) production. IP_3, in mammalian cells, induces endoplasmic release of calcium, increasing cytosolic levels and overall proliferative ability of the cell. The role of DAG is to act as a co-factor in the activation of protein kinase C, which has a multitude of functions depending on the cell type (Hannan et al., 2001).

Group II mGluRs (mGluR2 and mGluR3) and group III mGluRs (mGluRs 4, 6, 7, and 8) are often categorized together, based on their similar physiological responses and the fact that they appear on the presynaptic site, as opposed to group I which are postsynaptic. The main mechanism of group II and III is the inhibition of cAMP creation from ATP by activating an

inhibitory G-protein which inhibits adenylyl cyclase (Kunishima et al., 2000). While Group I mGlurs act to increase EPSP, group II and III mGluRs reduce overall cAMP, causing a reduction in glutamate release and opposing the function of group I mGluRs (Niswender & Conn, 2010).

Glutamate Transporters

There are two main classes of glutamate transporters, the excitatory amino-acid transporters (EAATs), which are dependent on an electrical gradient and the vesicular glutamate transporters (VGLUTs), which are electrically independent. There is also an amino acid antiporter in the family of heteromeric amino acid transports called system x_c^-, which is heavily upregulated in cancer cells (Lewerenz et al, 2014) and thought to be responsible for glutamate overproduction in MDA-MB-231 cancer cells, the primary cell type of this thesis (Figure 1.5).

EAATs

The EAATs are sodium and potassium-dependant members of the solute carrier family-1 (SLC1). There are five EAAT subtypes (EAAT1-5), each with a primary cellular distribution: EAAT1 (glia), EAAT2 (glia), EAAT3 (cortical neurons), EAAT4 (Purkinje neurons) (Anderson & Swanson, 2000) and EAAT5 (retina) (Pow & Barnett, 2000). These transporters range from six to eight transmembrane domains in length and are highly sensitive to glutamate. In addition to glutamate transport, EAATs are also able to transport aspartic acid. During transport of either

amino acid, co-transport of three sodium ions and one hydrogen ion and antiport of one potassium ion is required to overcome concentration gradients (Kanai & Hediger, 2004).

VGLUTs

The VGLUTs consist of four different transporters, VGLUT1-3, (Naito & Ueda, 1983) and sialin, a novel transporter thought to play a role in Salla disease (Verheijen et al., 1999). VGLUT family transporters use synaptic vesicles to exocytose their contents into the synapse and are dependent on proton gradients that form in the secretory system. These gradients are maintained by ATPase proton pumps. Compared to EAATs, VGLUTs have a very low affinity for glutamate and also unlike EAATs (with the exception of sialin) VGLUTs do not transport aspartate (Shigeri, 2004).

System x_c^-

First described in 1980, system x_c^- is a sodium-independent and chloride-dependant amino acid transporter which exchanges L-cystine for L-glutamate in a 1:1 ratio (Bannai & Kitamura, 1980). System x_c^- belongs to the family of heterodimeric amino acid transporters, which consist of a heavy polypeptide chain and a specific light chain subunit coupled via a disulfide bridge (Figure 1.2) (Lewerenz et al., 2011). The heavy chain of system x_c^-, 4F2hc, is a member of the solute carrier family-3 (SLC3) and is a membrane glycoprotein common to many amino acid transporters (Torrents et al., 1998). It has a molecular weight of approximately 85

kilodaltons (kDa) and plays a role in trafficking the complete transporter to the membrane. The light chain subunit of system x_c^- is a 502 amino acid protein termed xCT or solute carrier family-7 member A11 (SLC7A11) (Lewerenz et al., 2011). The xCT light chain subunit has a molecular weight of approximately 40kDa and is comprised of twelve transmembrane domains with both N-terminal and C-terminal being intracellular (Sato et al., 1999). xCT is entirely responsible for the transport capability of system x_c^- (Giacometti, 1979). Although system x_c^- has been shown to operate in both directions (Bannai, 1986) it generally favours the import of cystine for the export of glutamate, as system x_c^- is gradient driven and intracellular cystine is rapidly reduced into useable cysteine leaving intracellular concentrations of cystine negligibly small (Bannai, 1986).

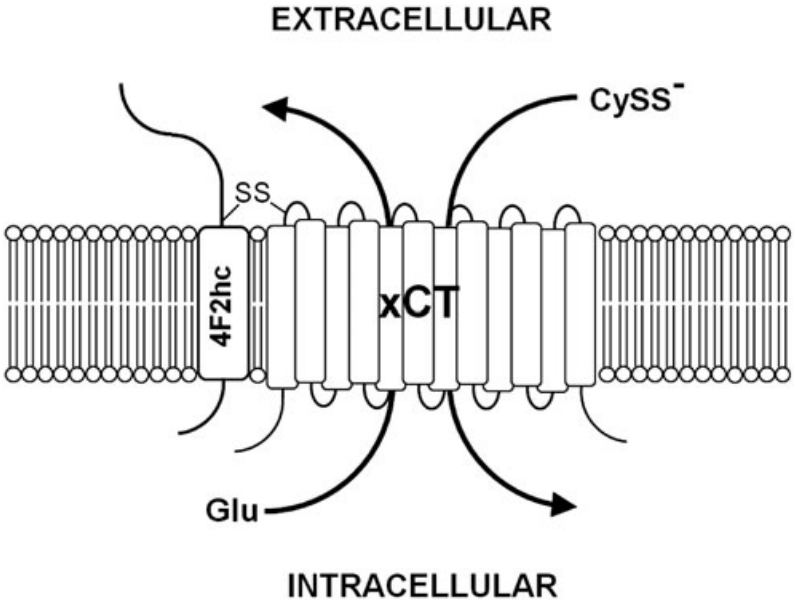


Figure 1.2: The Structure of system x_c^- showing both the heavy chain (4f2hc) and the light chain (xCT) which is responsible for the amino acid exchange (Lewerenz et al., 2011).

Tissue Distribution of System x_c^-

The tissue distribution of system x_c^- has been widely explored, being isolated in fibroblasts (Bannai & Kitamura, 1980), macrophages (Rimaniol et al., 2001), lung (Taguchi et al., 2007), spleen (Taguchi et al., 2007), glial cells (Kato et al., 1993), retinal cells (Tomi et al., 2003), osteoclasts (Takarada & Yoneda, 2008), osteoblasts (Uno et al., 2007) and the brain, which has the highest xCT expression of the organs studied (Bassi et al., 2001). It should also be stated that many cancer cell lines retain functional system x_c^- including lymphoma (Gout et al., 2001), glioma (Chung et al., 2005; Lyons et al., 2007), sarcoma (Kaleeba and Berger, 2006), and cancers of the prostate (Doxsee et al., 2007), lung (Guan et al., 2009; Huang et al., 2005; Baek et al., 2012), colon (Bassi et al., 2001) pancreas (Lo et al., 2006), ovaries (Huang et al., 2005) and breast (Narang et al., 2003; Baek et al., 2012).

Transporter Activity of System x_c^- and Intracellular Cystine Maintenance

Under normal physiological conditions, the role of system x_c^- is to exchange extracellular cystine for intracellular glutamate in an effort to increase cytosolic cystine concentrations. Cystine is a dimer consisting of two oxidized cysteine molecules linked covalently via a disulfide bond. Cysteine can either be created intracellularly from methionine through the transsulfuration pathway or imported into the cell. Typically, creation of cysteine is done in hepatocytes and epithelial cells (Belalcázar et al., 2014), whereas other cell types are left importing cysteine or cystine, via system x_c^- (Ishii et al., 2004). Once inside the cell, cystine is rapidly reduced to cysteine where it then becomes the rate limiting substrate for the production of glutathione (GSH), a powerful antioxidant and ROS scavenger (Dringen, 2000). Cystine is reduced into

cysteine either by intracellular GSH via the formation of a mixed disulfide intermediate or by thioredoxin reductase 1 (TRR1) (Lewerenz et al., 2011). The next step is the attachment of a free glutamate to cysteine to form a dipeptide of cysteine and glutamate, which is mediated by γ-glutamylcysteine synthase (Wu et al., 2004). This dipeptide is further altered by glutathione synthase which adds the final amino acid, glycine, to make GSH (Wu et al., 2004). GSH is a tripeptide of glutamate, glycine, and a cystine and can be found intracellular at concentrations in the millimolar range, making it one of the most significant antioxidants in somatic cells (Lewerenz et al., 2011). In most cells, the rate limiting step of GSH synthesis is the import of free cysteine. Aside from system x_c^-, there are several other transporters of free cysteine such as system alanine-serine-cysteine (system ASC) which also has been shown to transport threonine, asparagine and glutamine (Christensen, 1990). System A is another efficient transporter of glycine, proline, and alanine which has also been shown to directly import cysteine at low affinity (Christensen, 1990). While there are many importers of cysteine, the extracellular concentration of this amino acid is relatively low in comparison to cystine, and system x_c^- is thought to be the primary method of cystine import for cancer cells (Narang et al., 2003).

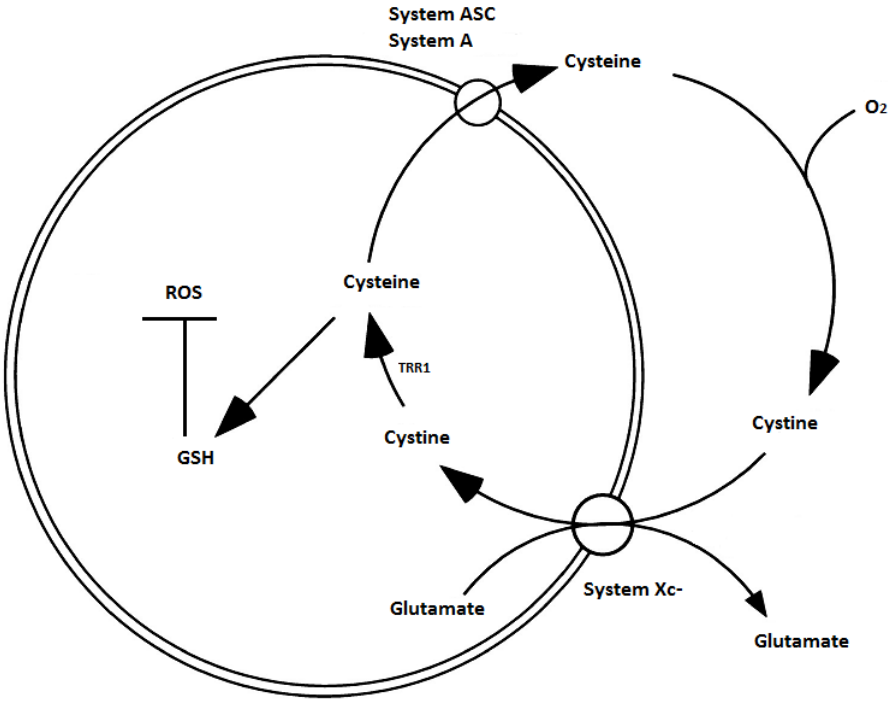


Figure 1.3: The Pathway of Glutathione (GSH) production using primarily system x_c^- for cystine import where it then is reduced to cysteine via TRR1. Glutamate and glycine are then added (via γ-glutamylcysteine synthase and glutathione synthase respectively) to make the tripeptide GSH. (Adapted from Sato et al., 2002).

Glutathione Production

The need for cystine uptake is crucial for GSH production in cancer cells, which experience a constantly elevated level of oxidative stress due to their accelerated metabolic rates (Nogueira and Hay, 2013). Oxidative stress is best defined as an imbalance between ROS production and elimination though various protective systems, GSH being one of the most abundant antioxidants in cancer cells. This need for increased GSH stems from the rapid tumour

growth causing the cancer cells to experience a shift from aerobic to anaerobic energy production, relying on glycolysis over the oxidation of pyruvate (as occurs in most cells during normal metabolism) (Alfarouk et al., 2011). This distinguishing feature of cancer cells is known as aerobic glycolysis, more commonly called the Warburg Effect, postulated in the early twentieth century by Otto Heinrich Warburg (Warburg et al., 1927). Although the reason for this metabolic shift is uncertain, there are host of consequences which can arise from relying on glycolysis. This method of energy production allows for glucose to be converted into metabolically active precursors which aid in cancer cell proliferation and anabolic reactions such as acetyl-CoA for fatty acids, glycolytic intermediates for nonessential amino acids, and ribose for nucleotides. Presence of these precursor molecules may confer an advantage to cancer cells, although this remains controversial (Vander Heiden et al., 2009). It has been shown that increased glycolysis can actually prevent ROS-mediated death, as anti-oxidant function of some scavengers (such as GSH) is closely coupled to the NADPH/NADP balance of the cell. Most of the NADPH/NADP is produced through the pentose phosphate pathway (PPP), a branching metabolic pathway found in glycolysis. Enhanced glycolysis might activate PPP, increasing the level of NADPH as by-products which act to increase GSH`s anti-oxidant ability (Kondoh et al., 2005; Kondoh, 2008; Haigis et al., 2012). Increased proliferation is also accompanied by a vast increase in ROS, suggesting that the Warburg Effect occurs to prevent ROS-mediated cellular death (Cairns et al., 2012).

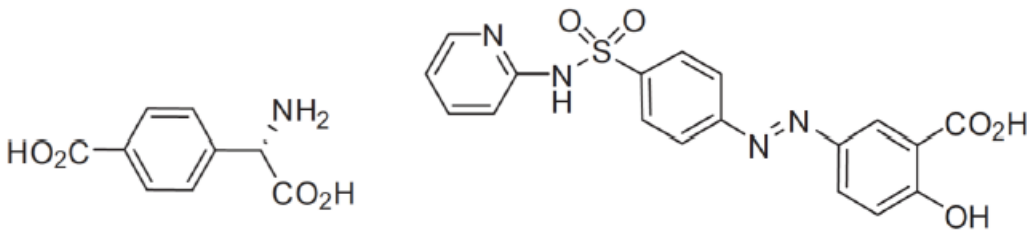
Agonists of System x_c^-

The significance of system x_C^- in many pathologies such as Parkinson's disease (Massie et al., 2008), glioma (Takeuchi et al., 2013), and breast cancer (Ungard et al., 2013) highlights this transporter as an interesting target for pharmacological inhibition. The first inhibitors of system x_C^- were L-cystine and L-glutamate, its substrates. When exposed to high enough concentrations of L-cystine and L-glutamate the transporter stops its import activity. This was evidence that this transporter is gradient dependant. Recently, studies have uncovered a vast number of potential system x_C^- inhibitors including sulfasalazine and (s)-4-carboxyphenylglycine, two antagonists which are used widely in our lab (Figure 1.4).

Sulfasalazine (SSZ), a combination drug developed in the 1950s to treat rheumatoid arthritis was discovered, when intact, to have an inhibitory action on system x_C^-. SSZ is a combination drug composed of sulfapyridine, an antibiotic, and 5-aminosalicylic acid, an anti-inflammatory, bound by a sulphur-containing azo-bridge. This bridge is broken down by azo-reductase, an enzyme largely produced by the gut microflora; where about 70% of orally taken SSZ will be broken apart, leaving only 30% SSZ function to reach other sites (Chung et al., 1992). When SSZ is intact, it can inhibit system x_C^- with an IC_{50} of 30 μM (Shukla et al., 2011). SSZ does not permeate the blood brain barrier (BBB), which is advantageous, as many neurons in the CNS highly express system x_C^-. For its ability to inhibit system x_C^-, SSZ has been used in our lab as a gold standard for reducing cancer-released glutamate (Sharma et al., 2010) as well a novel treatment for cancer induced bone pain, significantly increasing time until the onset of pain

(Ungard et al., 2013). Along with its well documented ability to inhibit system x_C^-, SSZ also has been shown to inhibit the activation of NFκB, a core inflammatory mediator of the immune system, further complicating sulfasalazine's use as a selective inhibitor (Sontheimer & Bridges, 2012). SSZ was recently used in our lab following the same methodologies as this thesis in an attempt to attenuate cancer pain through glutamate reduction. Only limited reduction in pain was observed and onset of pain was only slightly delayed (Ungard et al., 2013).

(s)-4-carboxyphenylglycine ((S)-4-CPG), is a cyclic glutamate analogue and competitive group I metabotropic glutamate receptor antagonist, with increasing selectivity against mGluR 1 over mGluR 5 (Sung et al., 2001). It was also reported to have a non-specific effect on system x_C^- (Chung et al., 2005 and Ye et al., 1999). Our lab has found (S)-4-CPG to be among the most potent inhibitors of system x_C^- (Ungard et al., 2013) with an IC_{50} value of 15 μM, half that of SSZ. (S)-4-CPG has been used successfully as an inhibitor of system x_C^- *in vitro* in many cell cultures including cancer cells (Chung et al., 2005). (S)-4-CPG has also been used *in vivo* following the same methods as this thesis in mice with metastatic breast cancer, but no significant results in pain attenuation were seen (Ungard et al., 2013). It can be said however that the concentration of (S)-4-CPG used for this experiment was small when compared to SSZ and future testing of this compound may prove more fruitful.



(S)-4-CPG Sulfasalazine

Figure 1.4: The chemical structures of (S)-4-carboxyphenylglycine, a cyclic glutamate analogue (left) and the multi-component anti-inflammatory drug sulfasalazine (right).

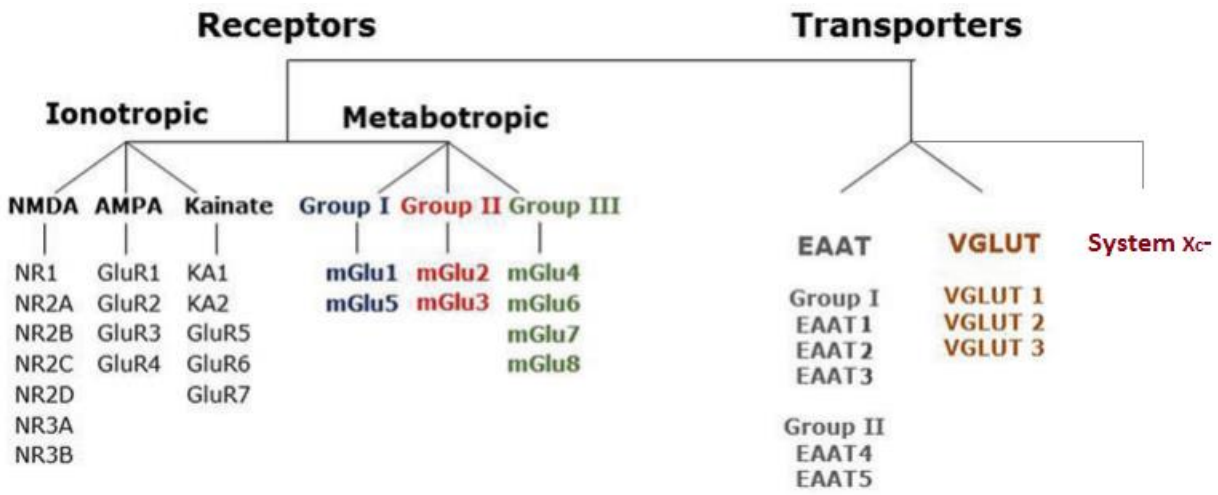


Figure 1.5: Various Receptors and Transporters of Glutamate.

(Adapted from Osikowicz et al., 2013)

TRP-Family Receptors

The transient receptor potential (TRP) family of cation channels is named after the visual defects observed in *Drosophilia* mutants (Moiseenkova-Bell et al., 2007) and consists of seven major branches: TRP-vanilloid (TRPV), TRP-Canonical (TRPC), TRP-ankyrin (TRPA), TRP-melastatin (TRPM), TRP-mucolipin (TRPML), TRP-polycystin (TRPP), and TRP-no mechanoreceptor potential-C (TRPN) (Moiseenkova-Bell et al., 2007). These channels are labeled according to their main agonist type and respond to a diverse range of physical stimuli such as heat, cold and osmotic pressures, as well as being pH gated (Voets et al., 2005; Dhaka et al., 2006). All TRP family receptors are characterized by six complete transmembrane domains and one partial transmembrane domain, which are thought to be associated with the ion channel (Szallasi & Blumberg, 1999). The main focus of this thesis will revolve around TRPV receptors, specifically TRPV1.

TRPV1

TRPV1 is an 838 amino acid long plasma membrane receptor with a molecular weight of approximately 95kDa which was the first isolated TRP receptor (Catarina et al., 1997). TRPV1 consists of four identical subunits each consisting of a cytoplasmic N-terminal region containing 3 ankyrin domains, 6 transmembrane domains (S1-S6), an intracellular pore forming loop between S5 and S6 and a cytoplasmic C-terminus region that includes a calmodulin and a

phosphatydylinositol-4,5-biphosphate binding site (Lee and Gu, 2009; Liao et al., 2013) (Figure 1.5). TRPV1 acts as an ion channel with non-selective permeability to calcium, magnesium, sodium, potassium and caesium (Niemeyer, 2005), with a notable preference for calcium (Caterina et al., 1997). The channel opening is activated by a host of factors including protons, endovanilloids, lipooxgenase metabolites, and temperatures greater than 43°C (Lee and Gu, 2009) and further modulated by prostaglandin E_2 (PGE_2), bradykinins (BK), nerve growth factor (NGF), and various proteases (Jia and Lee, 2007). Since its discovery in 1997, this receptor has been widely recognized as the molecular gateway to pain and nociception in somatic and visceral tissues. Many TRPV1 antagonists have already proceeded to Phase I/II clinical trials for the treatment of pain with the aim of improving the quality of life of people with migraine, chronic intractable pain secondary to cancer, as well as neuropathic pain associated with AIDS and diabetes (Szallasi et al., 2007). TRPV1 also serves a multitude of purposes aside from pain perception. Bladder contractions in mice have been found to be TRPV1 dependant (Birder et al., 2002) as well as body temperature regulation in humans (Gavva et al., 2007). For this thesis, the examination of TRPV1 inhibitions role in pain attenuation will be the focus of this project. For this purpose, the antagonist capsazepine, a synthetic analogue of the naturally occurring capsaicin vanilloid, will be used.

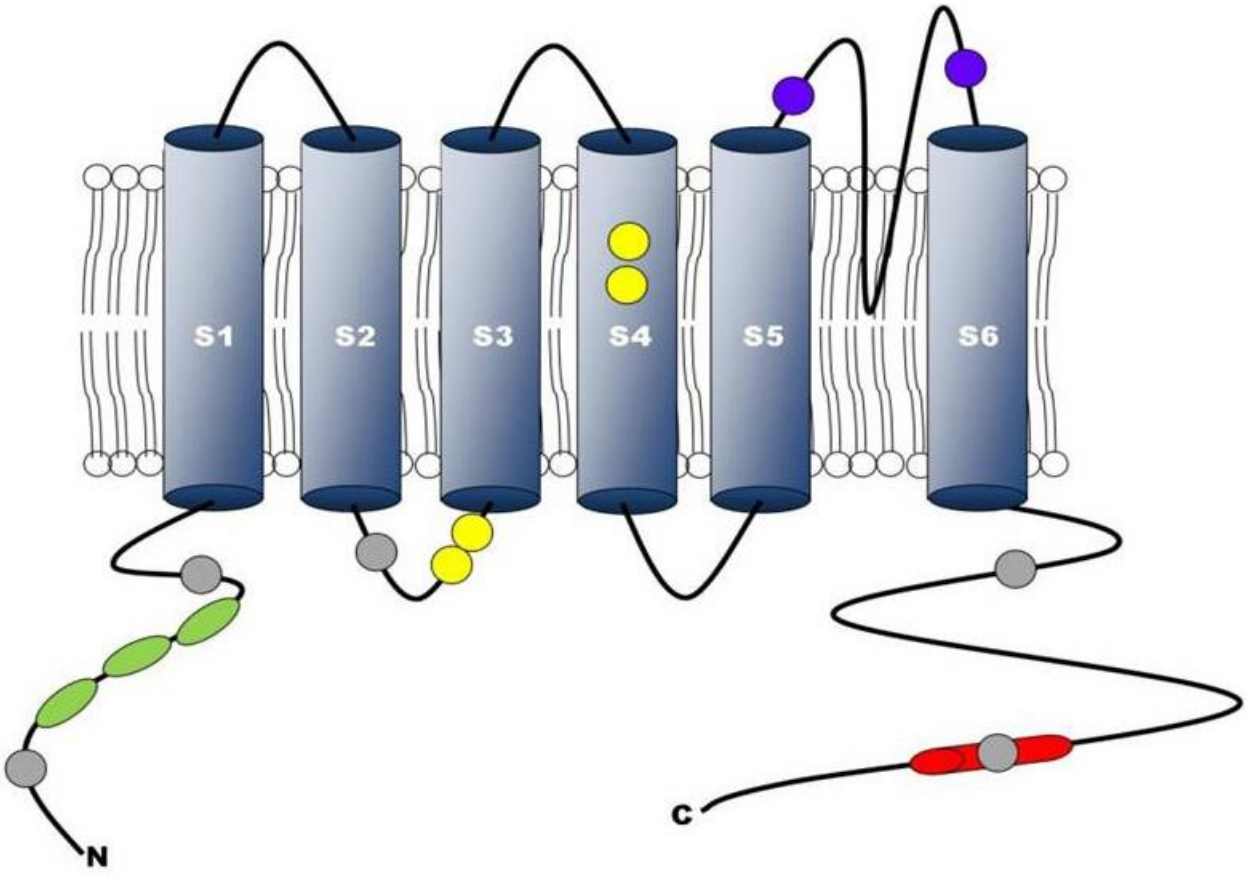


Figure 1.6: Structure of TRPV1 vanilloid receptor. This diagram shows the structure, including the three ankyrin domains (Green) as well as the multiple binding sites of the TRPV1 calcium channel. Binding sites are coloured to indicate specific substrates such as H^+ (Blue), vanilloids (Yellow), calmodulin and phosphatydylinositol-4,5-biphosphate (Red), and protein kinases (gray). (Romac and Liddle, 2012)

TRPV1 Localization

TRPV1 receptors are expressed along the entire length of nociceptive sensory neurons. They primarily are localized to thin, unmyelinated C-fibers (Holzer, 1991), TRPV1 has also been shown to be located on large diameter A**δ**-fibers in tissues such as tooth pulp, where this fiber type predominates over C-fibers. In the tooth pulp, A**δ**-fibers function as mechano-nociceptors where they respond mainly to mechanical stimuli and heat (Ikeda et al., 1997). Vanilloid sensitive neurons, those containing TRPV1, are heterogeneous in morphology, neurochemistry, and functionally, as well as encompassing several subclasses of dorsal root ganglion (DRG) neurons (Holzer, 1988). These neurons all respond similarly to vanilloids and because this is the only common trait that these neurons typically share, they are collectively known as vanilloid sensitive neurons ((Szallasi & Blumberg, 1999). Aside from the nervous system TRPV1 has been discovered in a variety of cell types such as cells of the bladder (Ács et al., 1996), colon (Goso et al., 1993), trachea (Szallasi & Blumberg, 1993), bronchi (Szallasi & Blumberg, 1993), urethra (Parlani et al., 1993), muscle cells (Cavuoto et al., 2007), osteoclasts (Rossi et al., 2008), and osteoblasts (Abed et al., 2008).

Agonists and Antagonists

Capsaicin is the main ingredient of chili peppers and is the primary substance involved in the burning sensation when consuming spicy foods. Capsaicin is a TRPV1 agonist which selectively binds TRPV1 receptors on the membrane of sensory neurons to activate noxious signaling which is then perceived by the brain (Tominaga & Tominaga, 2005). Capsaicin is an

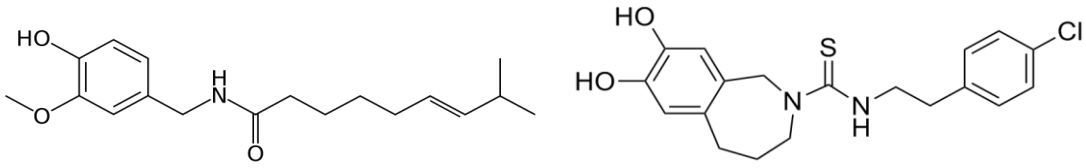
extremely versatile compound, involved in over 900 patents to date (Szallasi & Blumberg, 1999). These uses of capsaicin range from flavourings, to self-defensive aids such as pepper spray, to medical ointments for neuropathic pain relief. Pain relief involving TRPV1 has been documented since the dawn of recorded history, using cactus-like plant, *Euphorbia resinifera* as a vesicant (skin irritation) and sternutative (nose irritation) agent (Figure 1.7), though the mechanisms of pain relief were not known at this time.

Pain relief from capsaicin can be achieved using two different methods: a continuous low dose application (Derry and Moore, 2012) or a single high dose (Irving et al., 2012). Low doses of capsaicin are thought to continuously activate TRPV1 channels in a non-noxious situation. This method of pain reduction promotes desensitization of the TRPV1 receptors on unmyelinated C-fibers responsible for pain (Derry and Moore, 2012). The other method, high dose of capsaicin, is used to flood the resident nociceptive neuron with calcium, causing defunctionalisation of nociceptor fibres, through the temporary loss of membrane potential, inability to transport neurotrophic factors, and a reversible retraction of epidermal and dermal nerve fibre terminals (Anand and Bley, 2011).

Figure 1.7: Historical documentation of TRPV1 use as an analgesic. Taken from the Arabic version of *De Materia Medica* (Dioscorides), an encyclopaedia and pharmacopoeia of herbs and the medicines written between 50-70AD. Depicted here is cactus-like plant, *Euphorbia resinifera* which contains an extremely potent TRPV1 agonist, resiniferatoxin being used as a general remedy for various snakebites and poisons during antiquity (Szallasi and Blumberg, 1999).

Capsazepine is a synthetic analogue of capsaicin (Figure 1.7) which acts as a non-functional competitor for vanilloid binding sites on TRPV1. Once bound, capsazepine stops the activation of the TRPV1 ion channel acting as a primary antagonist to TRPV1. Capsazepine, discovered in 1994, was the first competitive antagonist identified against TRPV1 (Walpole et al., 1994). This compound was first used to block the sensory effects of capsaicin and resiniferatoxin (on TRPV1) three years prior to the receptors discovery (Walpole et al., 1994). Since its discovery, capsazepine has been used in many laboratories for its antagonistic properties in a wide variety of studies from pain relief to restoring normal micturition frequency in cyclophosphamide-treated rats (Dinis et al., 2004). When delivered systemically, capsazepine has been linked to many restorative properties including: decreases in nociceptor signalling in an L-arginine-induced pancreatitis model (Wick et al., 2006), decreases in physiological responses to colorectal or jejunal distension in mice (Sugiura et al., 2007), blocking induced cough responses in guinea pigs (Lalloo et al., 1995), and increasing systolic blood pressure in Dahl salt-resistant rats that were maintained on a high-salt diets (Wang and Wang, 2006). In the context of nociception, capsazepine is one of the most widely explored TRPV1 antagonists. It has been shown in a mice model of osteosarcoma that capsazepine can attenuate thermal hyperalgesia, as seen by an increase in paw withdrawal latencies using a hotplate test (Menéndez et al., 2006) as well as thermal hyperalgesia induced by CFA in rats (Menéndez et al., 2004). Mechanical hyperalgesia has also been shown to respond positively to capsazepine in mouse models of inflammatory and neuropathic nociception (Walker et al., 2002). It has also been found that capsazepine, when given in conjunction with morphine, strengthened morphine anti-nociception as well as preventing the development of tolerance and physical dependence in mice (Nguyen et

al., 2010). In our lab, capsazepine was found using a high-throughput screen searching for compounds that attenuate cancer-released glutamate. Capsazepine was one of the more potent inhibitors of glutamate release that did not cause cytotoxicity in the concentrations used. This relationship between capsazepine, TRPV1 and glutamate is not new. Capsaicin, the receptor agonist, has been shown to cause spontaneous releases of glutamate in spinal cord neurons, an effect which was significantly reduced by capsazepine (Köfalvi et al., 2003; Ueda et al., 1993). The proven therapeutic profile and low cytotoxicity of this compound suggest great analgesic potential for capsazepine in our model of metastatic breast cancer and for these reasons it was chosen as the primary analgesic for this thesis.



Capsaicin Capsazepine

Figure 1.8: The chemical structures of naturally occurring capsaicin (left) and capsazepine (right), a synthetic analogue of capsaicin.

Bone Physiology

In healthy adults, there are 213 bones in the human body. Originally thought to be static structures, bones undergo constant turnover in a process called bone remodeling. The skeletal system serves a variety of functions necessary for life. The first is providing the body with a

structural framework: permitting locomotion, movement and attachment sites for tendons, levers for muscles and protection of vital internal organs (Bart Clarke, 2008). The second major function of bone is to act as a mineral reserve, maintaining calcium homeostasis and acid-base balance within the body. Finally, bone is also the main site of blood cell production which occurs in the marrow of the bone matrix. This is the birthplace of the immune system and where production of oxygen-carrying red blood cells (RBCs) occurs. The structure of a bone is composed of a membranous periosteum and two bone types: cortical and trabecular bone. While both bone types are composed of osteoclasts, osteoblasts and osteocytes, they differ in function and location. Superficially, the outside of the bone, with the exception of the joints, is cloaked in the periosteum, a sheath of dense irregular connective tissue which contains many nerve endings and is highly vascularized, supplying blood to the bone. The underlying bone is called compact or cortical bone. This hard outer layer, is far more dense and accounts for 80% of the total bone mass. This bone type is more stable undergoing less frequent remodeling and is typically less metabolically active than trabecular bone. Cortical bone contains the haversian canals, named after British physician Clopton Havers, which are a series of narrow tubes formed by lamellae which house blood vessels and nerve cells as they extend from the periosteum and penetrate through the cortical bone. These canals can also intersect with one another via perforating holes in the cortical bone called volkmann's canals. These canals often carry small arterioles throughout the bone, supplying blood and nutrients to the interior of the bone. Trabecular bone, also known as spongy bone, is extremely porous and is located in the interior of bones and at the epiphysis of long bones (Hadjidakis & Androulakis, 2006).

Bone protein is almost entirely composed of type I collagen with trace amounts of type III and V collagen which are synthesized and deposited by osteoclasts during the remodeling process (Bart Clarke, 2008). These collagen proteins are mineralized by hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], containing up to 99% of the body's calcium stores with trace amounts of magnesium, carbonate and acid phosphate (Bart Clarke, 2008). Bone mineralization provides rigidity and compressive strength required for load-bearing and accounts for 50-70% of overall bone strength (Bart Clarke, 2008). There are two different kinds of bone matrix, woven and lamellar. Woven bone is weaker, but is constructed faster than lamellar bone. During periods of injury or load-bearing tissue, engineering is skewed towards the production of woven bone and osteoblasts rapidly produce and lay down collagen fibers in random orientation. The newly constructed bone slowly becomes replaced by lamellar bone which is far more organized and ordered into concentric sheets reducing the ratio of osteocytes to surround tissues, strengthening the bone structure (McKenzie & Silva, 2010). This process of replacing woven bone with lamellar bone is known as bone substitution. Osteocytes are terminally differentiated osteoblasts which incorporate themselves into the bone matrix, lying within the lacunae in mineralized bone. Although they make up over 90% of all bone cells, the exact function and mechanisms of these cells are not yet known. Osteocytes have however, been shown to participate in processes such as orchestrating bone remodeling, regulating osteoblast and osteoclast activity; they also function as endocrine cells for signalling cells and even organs, such as the kidneys (Lynda Bonewald, 2011). Osteocytes have also been shown to release matrix metalloproteinases (MMPs) which function to degrade the bone matrix and aid in bone turnover and normal matrix maintenance (Holmbeck et al., 2005). Furthermore, these cells seem to be crucial targets of parathyroid

hormone (PTH) where PTH inhibits sclerostin expression on osteocytes, a molecule that inhibits bone formation and indirectly activates osteoclasts (Bellido et al,. 2013).

Bone Remodeling

Bone remodeling is a process involving the removal of old bone for newly synthesized proteinaceous matrix and the eventual mineralization of this matrix to form new bone. The specific sites of bone remodeling occur randomly and sporadically, but are also concentrated at areas in need of repair. This process is necessary to maintain adequate bone strength and mineral homeostasis and is largely carried out by osteoclasts and osteoblasts.

Osteoclasts are the only known cell that can resorb bone. They are large multi-nucleated cells derived from monocyte-macrophage precursor cells. Osteoclastogenesis occurs in the presence of receptor activation of NFκB ligand (RANKL) and the presence of macrophage colony-stimulating factor (M-CSF) is required for proliferation and cell survival (Lin et al., 2008). Osteoclasts are found bound to collagen, fibronectin and laminin fibers in the bone with the main receptor, $\alpha_v\beta_3$ integrin, articulating with osteopontin which allows for tight binding to the boney matrix. Once bound, osteoclasts secrete hydrogen ions using H^+-ATPase hydrogen pumps. Hydrogen ions greatly decrease the surrounding pH in the area between the osteoclast and the bone matrix, known as the resorption pit, causing bone demineralization for bone reabsorption. Following the loss of hydroxyapatite, the resorption pit becomes contaminated with

calcium and phosphate ions from the demineralization process. To correct for this, transcytosis of these minerals occurs by VGLUT1, where it is secreted from the apical end of the cell, or through the periphery of the resorption pits (Morimoto et al., 2006). The bone matrix becomes active with the exocytosis of cathpsin K, contained in acidic vesicles, this process follows closely after bone demineralization. Cathpsin K is used to digest the proteinaceous matrix, consisting mainly of collagen type I and further activates tartrate-resistant acid phosphatase (TRAP), an ROS generating phosphatase which is essential for bone resorption (Sasaki et al., 1994). This process of bone degradation has been targeted using a class of pharmaceuticals known as bisphosphonates which adhere to calcium in the bone. Upon resorption by osteoclasts, the ingested calcium (along with the bisphosphonate) activates inside the cell, causing apoptosis. While these drugs are known to reduce bone pain acutely, they eventually fail to maintain a pain-free state in bone cancer patients and are often accompanied by long term side effect such as gastrointestinal discomfort hypocalcaemia, and bone fractures resulting from the suppressed bone remodeling (Heidenreich et al., 2001).

Osteoblasts are single nuclei cells involved in the creation of bone. Osteoblasts arise through a process known as osteoblastogenesis and are derived from mesenchymal stem cells which are in abundance in the periosteum. Differential commitment of mesenchymal stem cells requires careful orchestration between transforming growth factor-β (TGF- β) and bone morphogenic protein (BMP) signaling, which drives runt-related transcription factor-2 (Runx2) activity, a key regulator of osteoblast differentiation (Chen et al., 2012). Osteoblasts, in their

organized groups, are found along the bone matrix and synthesize type I collagen, osteocalcin, and osteopontin, vital components of the bone matrix. Following protein secretion, osteoblasts release hydroxyapatite to initiate bone mineralization, a process that accounts for about 50% of the total bone mass (Yilmaz et al., 2013). While a single osteoblast cell cannot directly synthesize bone, they work in tightly organized groups. (Ascenzi & Roe, 2012). The newly synthesized bone matrix will eventually encapsulate the osteoblast, burying it and creating an osteocyte. These osteocytes will group together with other cells and the newly mineralized bone, forming the osteon, the fundamental functional unit of most compact bone (Ascenzi & Roe, 2012).

In order to maintain proper bone structure, the balance of osteoclasts and osteoblasts are tightly controlled. Homeostasis of these bone cells are influenced by various hormones, including PTH, calcitonin, and estrogen. PTH is secreted by chief cells in the parathyroid glands and acts to increase the amount of calcium in the blood, through degradation of bone. Osteoblasts, but not osteoclasts, contain parathyroid hormone type-1 receptor (PTH1R) and are the binding sites for PTH. The overall effect of PTH on bone homeostasis through it action on osteoblasts to activate Runx2 transcription, as well as promote anti-apoptotic pathways in the cell, increasing osteoblast numbers and indirectly sequestering osteoclasts for bone turnover (Bellido et al., 2013). This action is achieved when osteoblasts overpopulate, expressing high concentrations of RANKL and M-CSF. Typically osteoblasts also secrete osteoprotegerin (OPG), a decoy receptor for RANKL, inhibiting osteoclast formation, but PTH binding has been

shown to decrease OPG release, allowing for RANKL binding and osteoclast sequestering (Silva et al., 2011). Calcitonin is a polypeptide hormone produced by the parafollicular cells of the thyroid. It acts to counteract the effects of PTH and reduce blood calcium by inhibiting osteoclasts and promoting the creation of new bone. Estrogen is another hormone which has ties to bone homeostasis. It has been shown to reduce osteoclast differentiation leading to an overall loss of bone resorption and opposing the effects of PTH, similarly to calcitonin (Khosla et al., 2012).

The mechanism behind bone remodeling is composed of four phases: activation, resorption, reversal and formation (Raggatt & Partridge, 2010). Activation, the first stage in bone remodeling, is thought to be initiated though osteocyte apoptosis and osteoclast sequestering. Under basal conditions, osteocytes secrete TGF- β, a cytokine which inhibits osteoclastogenesis, damage to bone, and limb immobilization, TGF- β results in an increase in osteocyte apoptosis allowing osteoclastogenesis to occur (Raggatt & Partridge, 2010). Resorption phase is when osteoblasts respond to the apoptotic osteocytes and release (monocyte chemoattractant protein-1) MCP-1, which in turn recruits osteoclast precursor cells to the area. MMPs are released by osteoblasts and act to degrade the unmineralized bone and expose specific binding sites for osteoclast binding. These exposed binding sites flank mineralized bone which is then demineralized when bound osteoclasts secrete hydrogen ions to create an acid microenvironment. The reversal stage occurs when an undetermined cell type (called reversal cells) thought to be of osteoblastic lineage, removes these osteoclasts. Reversal is quickly

followed by the formation stage, where osteoblasts lay the foundations for new bone development (Raggatt & Partridge, 2010).

Glutamate Signaling in Bone

In order to maintain functional homeostasis and normal structure, bone must rely on multiple extracellular signaling pathways, a major one being the glutamatergic pathway. Although no complete picture of the role of glutamate in the bone has been identified, glutamate has been found to be a fundamental extracellular messenger molecule in the bone environment (Cowan et al., 2012). Functional components from each stage of the glutamate signaling pathway have been identified in cells of the bone (Brakspear & Mason, 2012; Figure 1.9). Osteocytes have been shown to contain NMDARs (Itzstein et al., 2001), GluR1 AMPAR (Szczesniak et al., 2005), and EAAT1 (Hugget et al., 2002) at the protein level *in vivo*. Osteoblasts have been shown to express NMDARs (Hinoi et al., 2003; Ho et al., 2005), GluR1 and 2 AMPARs (Hinoi et al., 2002), EAAT1 (Mason et al., 1997), and EAAT3 (Takarada et al., 2004) at the protein level with Kainate receptors (Taylor, 2002), group I mGluRs (mGluR1) (Gu and Publicover, 2000), and group III mGluRs (mGluRs 4 and 8) at the mRNA level (Hinoi etal., 2001). Osteoclasts have been shown to express NMDARs (Merle et al., 2003), GluR1-4 AMPARs (Hinoi etal., 2001), group I mGluRs (mGluR 5) (Lin et al., 2008), group III mGluRs (mGluRs 4, 6, 7, and 8), and EAAT2 and 4 (Hinoi et al., 2007; Takarada & Yoneda, 2008).

Osteoblasts have been shown *in* vivo to express the functional components required for glutamate release which has been shown to regulate osteoblast differentiation (Olkku & Mahonen, 2008) During osteoblastogenesis, glutamate concentrations in the local environemt

spike due to a decrease of glutamine synthase, an enzyme which converts glutamate into glutamine (Olkku & Mahonen, 2008). Glutamate release has also been shown in mature osteoclasts, but not pre-osteoclasts (Morimoto et al., 2006). Although the function of this excess glutamate is not well understood, glutamate appears to be a potent signaling molecule in bone. One possible outcome of excess glutamate is the differentiation of osteoclasts, as NMDA receptor activation has been linked to osteoclastogenesis, with inhibition studies resulting in reduced osteoclast numbers and a reduction in resorption pits in bone (Peet et al., 1999) a result opposing that of our labs findings using MK 801, a known NMDA inhibitor (Seidlitz et al., 2010b).

The system x_C^- transport has also been found to play a role in bone homeostasis. The cystine/glutamate antiporter is expressed and functionally required for the differentiation of pre-osteoblasts, pre-osteoclastic cells, and primary osteoclasts from bone marrow precursor cells, where known inhibitors such as SSZ and glutamate concentrations in excess of 500 µM inhibited differentiation of bone marrow precursor cells (Hinoi et al., 2007; Takarada-Iemata et al., 2010).

Our lab has recently studied the effects of glutamate in the bone environment suggesting even modest increases in extracellular glutamate alters the differentiation of osteoblasts and bone formation (Seidlitz et al., 2010b). It has been postulated by Seidlitz et al. that glutamate secreted from tumour cells may aid in the development of metastasis to bone as well as cancer-induced bone pain, the primary focus of this thesis (Seidlitz et al., 2010a)

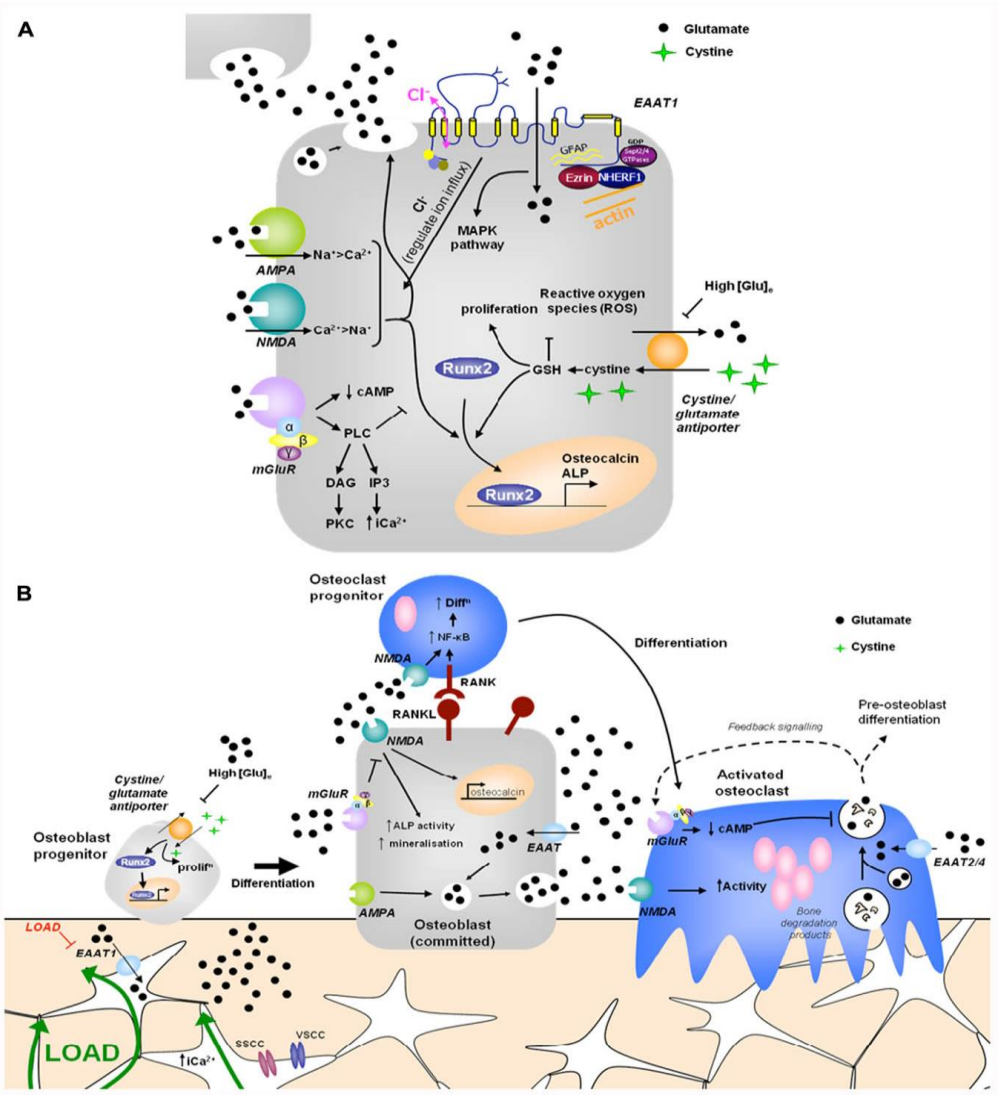


Figure 1.9: Glutamate Signaling in the Bone. (A) Glutamate Signaling in Osteoblasts. Osteoblasts release glutamate to activate glutamate receptors in an autocrine and paracrine manner and express functional ionotropic and metabotropic glutamate receptors. (B) Glutamate signaling during bone remodeling. Osteoclasts express EAATs 2 and 4 and NMDA receptor activation promotes NF-κB stimulated osteoclast

differentiation and increases mature osteoclast activity. Mature osteoclasts also release glutamate in conjunction with bone degradation products. (Brakspear & Mason, 2012).

TRPV-1 Signaling in Bone

Recently there has been an emergence of literature showing the role of TRPV family receptors in maintaining normal bone structure and turnover. TRPV5 is localized in the ruffled membrane of osteoclasts, but is absent from osteoblasts. Importantly, TRPV5($^{-/-}$) mice exhibit significant disturbances in bone structure, with a reduced trabecular network and diminished cortical bone thickness (Nilius et al., 2007). TRPV4 is also expressed on osteoblasts, with TRPV4-deficient mice demonstrating deformed chondrocytic columns and irregularly shaped bone trabeculae. Thus, TRPV4 has been implicated in the regulation of chondrocyte polarity, differentiation, and ossification (Mizuno et al., 2006).

TRPV1 has also been revealed to be associated with bone recovery, as newly fractured bone was found to contain significantly higher levels of TRPV1 than control, although this could be due to sensory distress from the damaged bone rather than aiding in the remodeling process (Kawarai et al., 2014). TRPV1 receptors have also been shown to be located on osteoblasts and osteoclasts, where the TRPV1 antagonist capsazepine inhibits osteoclast and osteoblast differentiation, *in vitro* and ovariectomy induced bone loss *in vivo* in mice (Pan et al., 2013; Idris et al., 2010). This effect was reversed, increasing RANKL and causing osteoblast differentiation with the application of capsaicin, a TRPV1 agonist (Idris et al., 2010). TRPV1 has been

implicated in RANKL signaling, aiding to previous evidence that TRPV1 mediates osteoclast differentiation (Rossi et al., 2009).

Bone Metastasis

Metastatic bone disease occurs when cancers from a primary site spread to the bone. Prior to the development of bone metastasis, the primary cancer must acquire certain traits which allow it to thrive under these new physiological conditions. These traits, or "hallmarks" of cancer, as defined by Hanahan and Weinberg (2011), comprise six biological qualities that are essential in the development of human tumours. They include sustaining proliferation, evading growth suppressors, resisting apoptosis, maintaining cellular immortality, inducing angiogenesis, and activating metastasis by the primary tumour (Hanahan & Weinberg, 2011). The bone microenvironment is a common site of metastasis for many cancers and has been found to aid in the pathogenesis of metastasis under the influence of primary cancers (Hanahan & Weinberg, 2011). Immune cells, most notably macrophages, neutrophils and mast cells are made in the bone marrow and act to increase metastasis by triggering angiogenesis, enhancing tumor migration and invasion while suppressing existing antitumor immunity (Condeelis & Pollard, 2006; Qian & Pollard, 2010; Ferrara, 2010). Vascular endothelial growth factor (VEGF), an essential signal for angiogenesis, can be produced by macrophages in addition to large quantities of MMP9, which releases matrix-bound VEGF (Qian & Pollard, 2010). VEGF has been shown to stimulate angiogenesis required for tumour cell invasion (Du et al., 2008).

Not all primary cancers metastasize with the same site distribution. Cancers of the lung, breast, and prostate have a high avidity for bone. This may be in part to the increased glutamate release associated with aggressive cancers of these sites (Seidlitz et al., 2009). In light of these cancer's strong predispositions for bone, breast cancer will be the sole target for the duration of this thesis. Breast cancers have many factors that have been linked to an increase in bone preference. In addition to increases in extracellular glutamate and VEGF expression, TGF- β signaling mediator Mothers-against-decapentaplegic-homolog 3 (Smad3) has also been implicated in the progression of breast cancers to the bone (Seidlitz et al., 2009; Sethi et al., 2011; Petersen et al., 2010). Knockdown of Smad3 in MDA-MB-231 cells has been correlated with prolonged latency and a delayed growth of bone metastasis in mice (Petersen et al., 2010). Conversely, knockdown of Smad2, another signaling mediator for TGF-β, was found to increase aggressive phenotypes when compared to control cells (Petersen et al., 2010). The mechanism behind these changes in aggressiveness is thought to be linked to specific members of the activator protein 1 (AP-1) transcription factor family: c-Jun, JunB, c-Fos and Fra1 which were found to be essential factors for TGF-β-induced tumour invasion (Sundqvist et al., 2013).

Secondary breast cancers in the bone are usually osteolytic in nature, resulting in pathological activation of osteoclasts leading to extensive demineralization and resorption of bone (Yoneda et al., 1994; Cook et al., 1998; Sethi et al., 2011). The pathological activation of osteoclasts can arise from many tumour related factors such as Jagged1 (Sethi et al., 2011), parathyroid hormone–related protein (PTHrP) (Johnson et al., 2011), and Gli2 (Johnson et al.,

2011). Jagged1 is a Notch signaling ligand found in breast cancers to promote tumour growth and bone destruction through osteolysis. Jagged1 has been implemented in the release of IL-6 from nearby osteoblasts and the direct activation of osteoclasts (Sethi et al., 2011). PTHrP is thought to be one of the primary factors affecting osteoclast activation and differentiation in breast cancer-induced osteolytic lesions (Tsai et al., 2013). PTHrP is structurally homologous with PTH and acts to stimulate osteoclast proliferation in a similar manner to that previously described, decreasing OPG release and allowing for RANKL mediated sequestering of osteoclasts (Silva et al., 2011). PTHrP inhibition using microRNA has been shown to decrease bone metastasis in lung (Tsai et al., 2013) and breast cancers *in vitro* and *in vivo* (Zheng et al., 2013). Gli2, a hedgehog signaling molecule, has also been found in many cancer cell lines, including MDA-MB-231 cells, to be responsible for osteolytic bone metastasis. Gli2 has been shown to alter TGF-β dependant PTHrP production, where its inhibition reduced endogenous PTHrP mRNA levels and visible osteolytic lesions in a xenograft mouse model using MDA-MB-231 cells (Johnson et al., 2011).

Another mechanism tumours can utilize to break down existing bone structure is through the use of MMPs, specifically MMP2 and MMP9 (Johnson et al., 1998; Ehrenfield et al., 2011). MMPs are a family of zinc-dependent endopeptidases that play a crucial role in various physiological processes including tissue remodeling, organ development, and tumour metastasis (Kassenbrock et al., 2010). The general structure of MMPs contains three domains that are common to almost all MMPs: the pro-peptide, the catalytic domain, and the hemopexin-like C-

terminal domain that is linked to the catalytic domain via a flexible hinge region (Kassenbrock et al., 2010). These enzymes are initially synthesized in an enzymatically inactive state via the interaction of a cysteine residue of the pro-domain with the zinc ion of the catalytic site. Activation occurs after cleavage of the pro-domain or chemical modification of the cysteine residue via other MMPs or various convertases (Kassenbrock et al., 2010). In the case of metastatic cancers, MMPs degrade the extracellular matrix (ECM) and, therefore, prepare the path for tumor cells to migrate, invade and spread to distant secondary sites, such as bone (Bourboulia & Stetler-Stevenson, 2010). The detection of active MMP-2 alone is considered a very sensitive indicator of metastatic breast cancer (Jezierska & Motyl, 2009) with lower levels of either MMP2 or MMP9 indicating a favorable prognosis to cancer patients (Hagemann et al., 2004), whereas increasing expression of either MMP correlated significantly with tumor size and neoangiogenesis, with overall survival significantly shortened in patients with MMP-2 or MMP-2/MMP-9 positive tumor cells (Ranogajec et al., 2013). MMPs may be produced by the invading cancer cells through immune cell interactions with the primary tumour, adding the evidence that the immune system can also contribute to tumour invasiveness (Hagemann et al., 2004).

Pain

Pain is a common term used to describe an unpleasant sensory experience associated with actual or potential tissue damage. Pain can be broadly divided into three classes: nociceptive pain, inflammatory pain and pathological pain (Figure 1.10). Nociceptive pain represents the

sensation associated with the detection of potentially tissue-damaging noxious stimuli and is protective in nature, warning the host of potential damage. Inflammatory pain is a pain state associated with direct tissue damage and the infiltration of immune cells, whereas pathological pain is a disease state caused by damage to the nervous system (neuropathic) or by abnormal functions of other dysfunctional systems due to pathology (Woolf, 2010). Pain is a complex state which can be hard to quantify, as it is subjective and no objective test can measure pain intensity. This elusive nature of pain can make it difficult to accurately diagnose and treat. The process of nociception is the encoding and processing in the nervous system of signals from specialized free nerve endings, called nociceptors, in response to stimuli such as temperature, chemicals, or pressure (Loeser & Treede, 2008). Nociception is not necessary for the pain sensation, but pain is an essential consequence of nociception, as it warns the body of impending damage. Under pathological conditions, nociception can also arise from non-noxious stimuli. This condition is called allodynia, and is different from hyperalgesia, which is a pathologically exaggerated reaction to a normally painful stimulus (Merskey & Boguk, 1994).

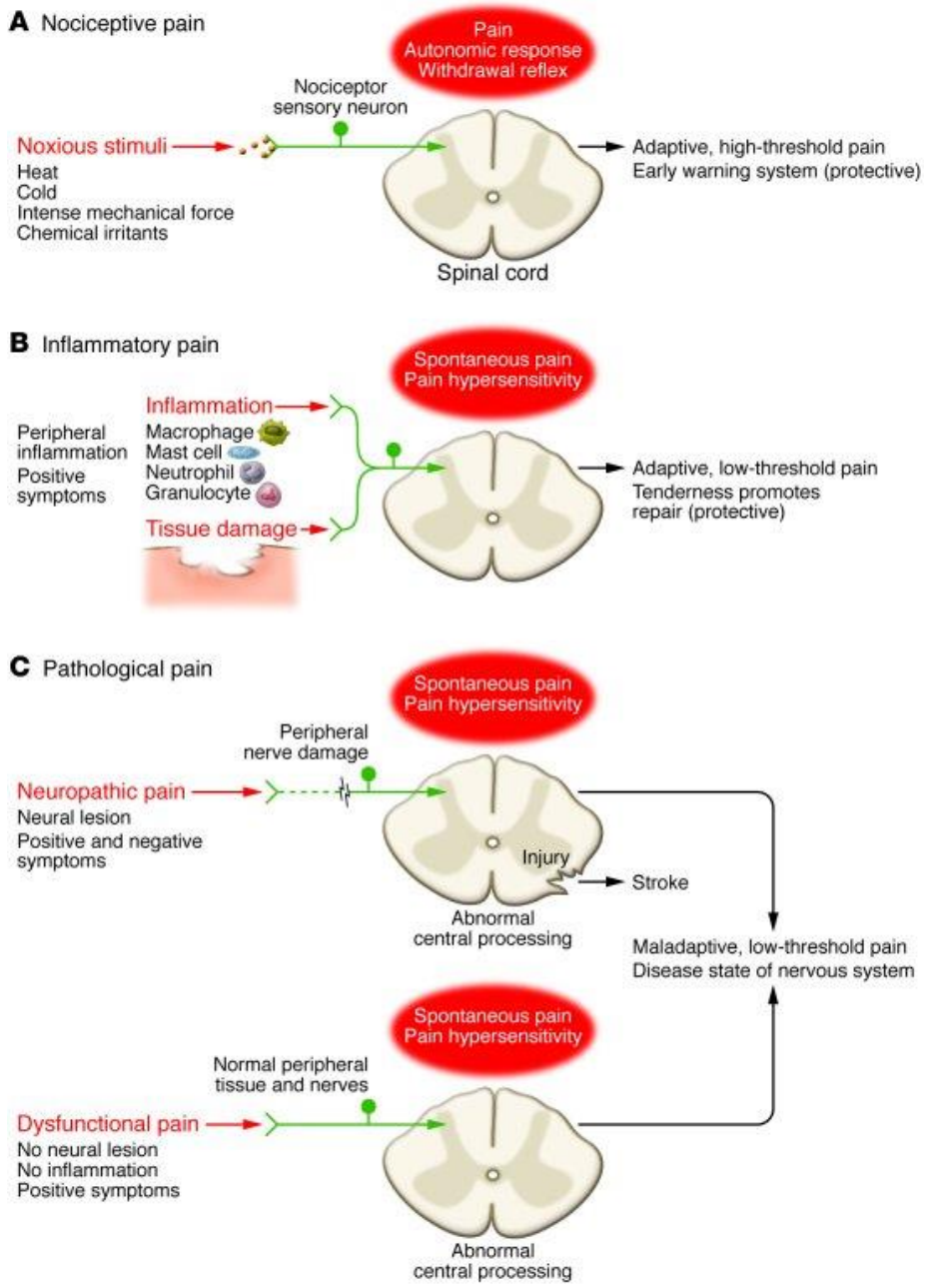


Figure 1.10: The multiple pain pathways in humans. Pain can be divided broadly into three classes .The three primary pain states are Nociceptive, Inflammatory and Pathological Pain (Adapted from Woolf, 2010).

Glutamate and Pain

Glutamate is the principal excitatory neurotransmitter of the CNS, but an accumulating body of evidence is emerging which suggests that glutamate has an expanded functional repertoire, which includes the modulation of pain (Bleakman et al., 2006). Glutamate receptors are located along nociceptive pathways at synapses in the periphery, spinal cord, and brain where they modulate pain transmission both in inflammatory and neuropathic pain models (Chiechio & Nicoletti, 2012). Nociceptive primary afferents release glutamate, activating postsynaptic glutamate receptors on spinal cord dorsal horn neurons with both ionotropic and metabotropic glutamate receptors having been implemented in the onset and attenuation of pain (Chappell et al., 2014; Chiechio & Nicoletti, 2012; Nashed et al., 2014).

Both large and small DRGs have been found to express NMDAR mRNA (Sato et al., 1993; Bardoni, 2013). While most NMDA receptors are localized to A fibers, C fibers can also express additional NMDARs (Hummel et al., 2008). Interestingly, the average current density of NMDA is 2.8-fold larger in DRG neurons from female rats when compared with male rats. Furthermore, addition of 17-β-estradiol potentiates NMDA currents, particularly in female rats (McRoberts et al., 2007). NMDA has also been found to cause nociception. Injection of NMDA into the cerebrospinal fluid of the rat spinal cord mimicked the changes that occur with persistent injury, producing not only pain, but significant internalization of substance P, a known pain signal (Aanonsen & Wilcox, 1987; Liu et al., 1997). Finally, zinc, an abundant ion in the CNS, has been found to regulate pain through NMDAR inhibition (Nozaki et al., 2011). These results

are further supported by pain attenuation through the administration of either magnesium, a natural blocker of NMDAR activation, or MK801, an NMDAR antagonist (Rondón et al., 2010).

AMPARs have been demonstrated to be directly involved in nociceptive signalling from noxious stimuli, with AMPAR antagonists alleviating inflammation, nociception and pathology in a rat antigen-induced arthritis model (Bleakman et al., 2006; Bonnet et al., 2011). In addition to noxious stimuli, ROS have also been shown to modulate AMPAR phosphorylation, leading to pain-related behaviours in mice (Lee et al., 2012). It was found that intrathecal administration of NMDA, a substance known to cause nociception, induced pain-related behavior in mice as well as causing an increase in AMPAR phosphorylation in the spinal dorsal horn in a ROS-dependent manner (Lee et al., 2012).

Kainate receptors have also been implicated in nociception. Kainate receptor, iGluR5, is the predominant kainate receptor subunit expressed by DRG neurons and is mostly expressed by C fiber nerves which act as nociceptors, relaying pain signals to the CNS (Partin et al., 1993). iGluR5 activation has been associated with pain, a response which was attenuated with administration of LY545694, a selective iGluR5 antagonist (Simmons et al., 1998). Recently, iGluR5 has been linked to two chronic pain conditions: osteoarthritis and diabetic peripheral neuropathic pain (Chappell et al., 2014). LY545694 administration was recently shown to cause a significant decrease in secondary hyperalgesia in thermally-induced pain models, but failed to reduce the primary pain at the source (Peterson et al., 2014).

Metabotropic glutamate receptors are expressed abundantly in the spinal cord and have been shown to play an important role in the modulation of pain and nociceptive plasticity (Karim et al., 2001). Group I metabotropic glutamate receptor (mGluR1 and mGluR5) agonists cyclopropan[b] chromen-1a-carboxylate (CPCCOEt) and 2-methyl-6-(phenylethynyl) pyridine (MPEP) were found to significantly attenuate the enhanced nociception and noxious stimulus-induced glutamate release in the spinal cord dorsal horn of rats in vivo (Kumar et al., 2010). Recent evidence has also pointed to group III mGluRs, such as mGlu4 and mGlu6–8, being responsible for the onset of pain and as potential targets for analgesic drugs (Goudet et al. 2008) showing that mGluRs play distinct and sometimes opposing roles in nociception depending on the site of activation and pain modality (Osikowicz et al., 2013).

Furthermore, glutamate injected subcutaneously into the masseter and temporalis muscles created significantly higher visual analogue scale (VAS) scores, an indicator for pain. The same experiment also found a difference in VAS scores based on sex, with women showing statistically higher pain scores than men (Castrillon et al., 2012), although this may be caused by the fact that women are known to have a higher nociceptor density (Mogil, 2012).

TRPV-1 Pain

The TRPV1 receptor is a common acid sensing ion channel localized on small diameter C and A**δ** sensory fibers of the DRG which are responsible for the perception of noxious stimuli

(Palazzo et al., 2012). Interestingly, TRPV1 has been found to be co-expressed with group II mGluRs on many DRG cells (Carlton et al., 2009). Along with acidic conditions, TRPV1 hosts a wide array of natural agonists such as capsaicin, resiniferatoxin, endovanilloids, lipooxygenase metabolites, and temperatures greater than 43°C, as discussed earlier. This wide array of stimulants allows TRPV1 to become susceptible to a plethora of environmental changes, resulting in pain.

Early work by on TRPV1, by Bodnar et al. demonstrated that intracerebroventricular (ICV) capsaicin injections decreased nociceptive thresholds and reduced morphine induced analgesia in mice (Bodnar et al., 1982, Bodnar et al., 1983) Recent studies have further elaborated upon this observation that TRPV1 activation leads to pain perception in humans (Ro et al., 2009; Gibson et al., 2009; Nilsson et al., 2014).

In the bone, the process of remodeling is a continuous process where osteoblasts build new material, while the old bone is resorbed by osteoclasts. During osteoclast resorption of bone, protons are released to degrade the unmineralized bone. If the balance of bone degradation is skewed, as in the case of osteolytic bone tumours (Yoneda et al., 2011), the number of osteoclasts increases, causing more bone destruction and higher proton levels in the local environment, lowering the pH past the threshold of TRPV1 (Nagae et al., 2007; Yoneda et al., 2011). TRPV1 is also found to be up-regulated by bone-derived growth factors, which may be a reason why bone pain is progressive, sustained, and intolerable in some cancer patients (Yoneda

et al., 2011). Human tumours cells have also been shown to express V-ATPase in their plasma membrane (Martinez-Zaguilan et al., 1993), causing the local microenvironment to become acidic. This tumour derived acidity is also thought to be a potential activator of cancer-induced pain (Nagae et al., 2007).

To illustrate the dynamics of glutamate and TRPV1 in pain perception, capsaicin, the TRPV1 agonist, and glutamate were directly injected into human tendon. The results showed that capsaicin was able to elicit a significantly higher pain score than glutamate; however, injected glutamate resulted in pain which was significantly longer in duration then that of capsaicin (Gibson et al., 2009). These results suggest that capsaicin may play a role in quick onset, acute pain whereas glutamate may be responsible for long duration, chronic pain (Gibson et al., 2009). Combination studies were also implemented in this experiment. It was found that VAS scores, an measure of pain, were reported as being significantly higher when glutamate was given before a subsequent injection of capsaicin when compared to the reverse where capsaicin is the initial injection. These results indicate that glutamate may help in the sensitization process of TRPV1 nociceptive channels, due to their acidic nature (Gibson et al., 2009). The acidity of glutamate has also been linked to activation of TRPV1-induced pain. In a glutamate study by Meotti et al. it was found that nociception induced by glutamate in mice is potentiated by protons, as using a pH buffered glutamate solution dramatically decreased nociception (Meotti et al., 2010). The experiment also used capsazepine to inhibit proton mediated nociception, resulting in significantly deceased pain when compared to the unaltered glutamate, but not the buffered

glutamate solution (Meotti et al., 2010). These results show that acid-sensing channel, TRPV1 is vital in both capsaicin and glutamate-induced nociception. Exploring this theme of TRPV1 and glutamate in the tumour environment and their relation to the onset of pain will be the focus for the remainder of this thesis.

Cancer-Induced Bone Pain

Cancer-induced bone pain arises from either primary bone sarcomas or from secondary metastatic lesions, most commonly cancers of the lung, prostate, and breast, with pain being the primary symptom (Urch, 2004). There are two major classes of bone pain; ongoing or breakthrough pain. Ongoing pain is described as dull in character, persistent, and tending to gradually worsen with tumour progression and disease severity (Portenoy & Hagen, 1990; Jimenez-Andrade et al., 2011). Breakthrough pain is named for its ability to "break through" analgesic regimens and can be further characterized as spontaneous pain, a debilitating exacerbation of sharp pain sensations without an apparent trigger, as well as "movement-evoked pain," a pain initiated by movement of the tumor-bearing bone (Portenoy & Lesage, 1999; Lozano-Ondoua et al., 2013). The spontaneity and irregularity of breakthrough pain makes breakthrough pain difficult to control or treat despite otherwise adequately controlled medication regimens, and can severely compromise a patient's quality of life (Mercadante, 2012)

Cancer-induced bone pain elicits neurochemical changes different from other chronic pain states, such as inflammatory or neuropathic pain (Lozano-Ondoua et al., 2013). The bone environment is innervated with sensory and sympathetic neurons throughout the periosteum with the bone itself being heavily innervated by Aβ, A**δ**, and C fibers responsible for nociception (Mach et al., 2002; Kalliomaki et al., 2011). Clinical studies have shown that most cancers of the bone contain no nerve fibers within the tumours or in the adjacent peripheral bone; implying that cancer-induced bone pain is predominately caused by secreted factors or acidic conditions which arise in the tumour microenvironment (O'Connell et al., 1998). Most recent evidence supports this with the observation that anatomical location, size and number of tumor and the extent of bone destruction are not necessarily correlated with the severity of bone pain (Yoneda et al., 2011).

While not all tumours rely on structural damage to relay pain signals, some tumours may still initiate pain though mechanical means. Stretching of periosteum by tumor expansion in turn could initiate the surrounding nociceptive sensory neurons (Mercadante, 1997; Yoneda et al., 2011). Microfractures, as a result of a loss in bone density or disequilibrium in osteoclast and osteoblast differentiation, has also been implicated as a source of bone pain. Osteoclasts degrade bone by lowering pH in the microenvironment through the release of protons into the extracellular space. These protons can also act on TRPV1 receptors typically found on nociceptors which respond to the reduced pH (<6.0) (Yoneda et al., 2011). This mechanism of pain generation is backed by the observation that bisphosphonate therapies, which cause

apoptosis in osteoclasts, have been shown to reduce bone pain in cancer patients (Finter et al., 2007 & Anagha & Sen, 2014), but challenged by the observation that not all cancers in the bone cause pain.

In addition to direct bone damage, tumour cells secrete a host of noxious stimuli including protons, bradykinins, substance P, histamine, NGF, brain-derived neurotrophic factor (BDNF), VEGF, prostaglandins, ATP, and glutamate (Yoneda et al., 2011). However, the observation that not all cancers in the bone are painful and that elimination of any one of these mechanisms thus far has failed to completely eradicate bone pain indicates that cancer-induced bone pain is complex and could be heterogeneous in origin.

Cancer-induced bone pain has been shown to greatly increase patient morbidity, reduce patient performance, and cause an increase in depression and anxiety, leading to an overall loss of quality of life (Portenoy & Hagen, 1990; Bruera et al., 1995; Portenoy et al., 1999). For these reasons it is imperative that we explore alternate methods of treating cancer-induced bone pain, for which we will look at capsazepine. Capsazepine has also been found in our lab to prevent glutamate secretions from tumour cells far exceeding the gold standard of sulfasalazine, as well as antagonizing the nociceptive receptor, TRPV1. With the emerging evidence that these two systems of pain are more linked than originally thought, the use of capsazepine may be an attractive analgesic with a multi-pronged approach at preventing cancer-induced bone pain through the reduction of free glutamate and the inhibition of nociceptive TRPV1 receptors.

Rationale

Glutamate has been linked to neurocommunication, intracellular signaling, bone homeostasis, and pain. Our lab has found that aggressive breast cancers, such as MDA-MB-231 cells, actively release increased amounts of glutamate when compared to control epithelial cells. In a high throughput screen to identify compounds that limit glutamate release from these cells, Capsazepine was identified. Capsazepine is an antagonist of TRPV1, a receptor shown to be necessary for spontaneous glutamate release from nociceptive neurons leading to pain and that has recently been localized on many cancer cells, including the MDA-MB-231 cell line. I have hypothesized that capsazepine will block excess glutamate release by these cancer cells and that the pharmalogical reduction of free glutamate will reduce pain in a mouse model of metastatic breast cancer.

Hypothesis

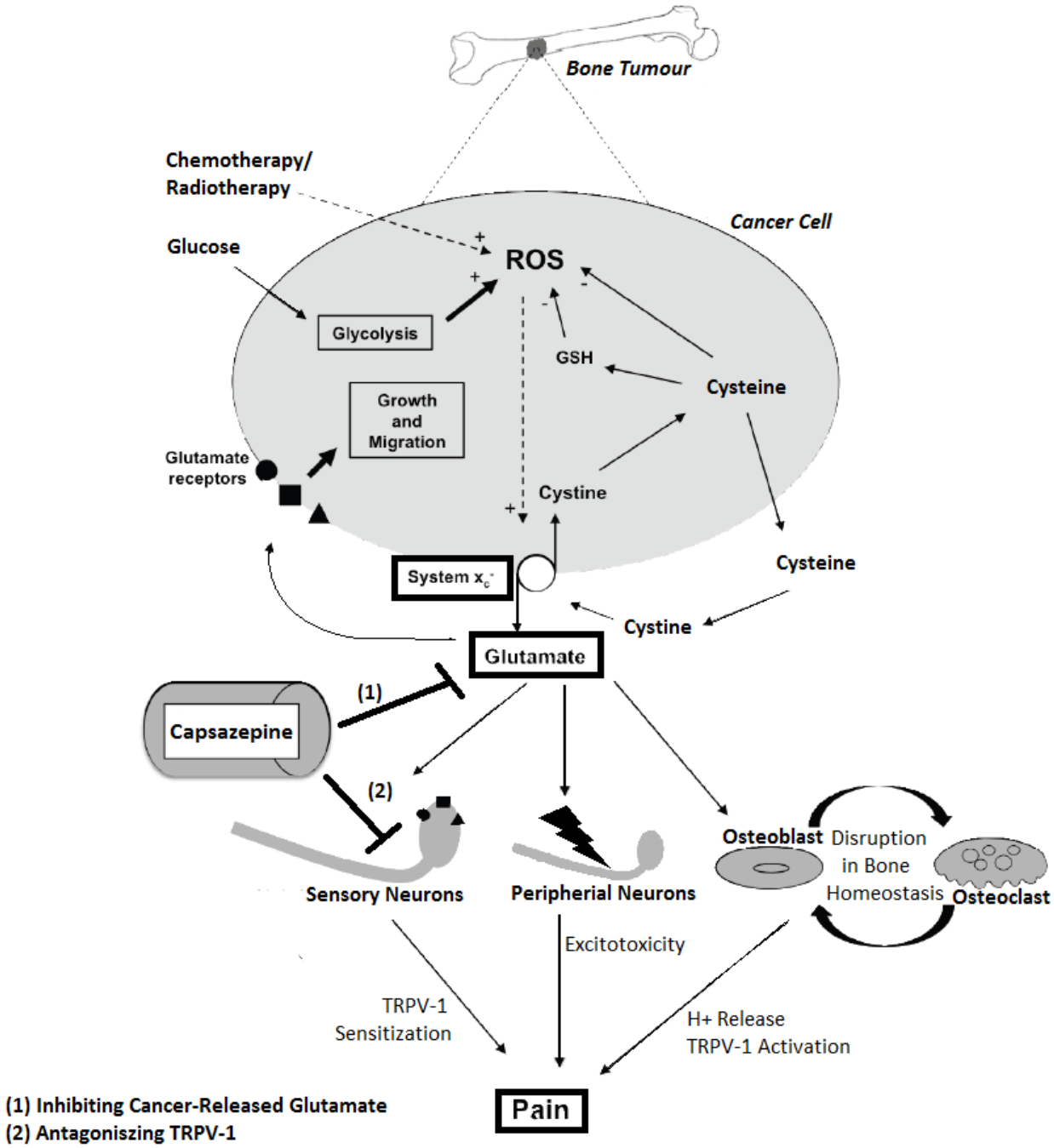
The high throughput screen will be used to identify compounds to inhibit cancer glutamate release. The most suitable of which will be used to limit the release of glutamate from MDA-MB-231 cancer cells injected into the bone, limiting the amount of pain-related behaviours seen in a mouse model of metastatic breast cancer.

Objectives

1) To observe the connection between glutamate release and bone cancer pain.

2) To determine if the discovered compound can cause a reduction of pain *in vivo.*

3) To validate that capsazepine was able to show a reduction of glutamate *in vivo.*



Hypothesis Concept Map (Adapted from Rob Ungard, 2012)

Chapter 2: Materials and Methods

Tissue Culture

For the comparison of cancer-released glutamate to control cells *in vitro* the 184B5 mammary gland epithelium obtained from ATCC (Chicago, IL, USA) were used as the control cells. The highly metastatic MDA-MB-231 human breast adenocarcinoma cell line acquired from American Type Culture Collection (Manassas, VA, USA) was the primary cell line used for all *in vitro* and *in vivo* experiments. These cells were mycoplasma free and tested again before each animal experiment began. Testing of the cells for mycoplasma contamination was done through the PlasmoTest detection assay kit (Invivogen, San Diego, CA, USA) to ensure the cells remained free of contamination and were safe for animal use.

During culture the cells were stored in humidified incubators at 37°C with 5% carbon-dioxide. The media used during culturing was Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Burlington, ON, Canada) for the MDA-MB-231 cell line and Mammary Epithelial Cell Growth Medium (MEGM) (Lonza, Mississauga, ON, Canada) for the 184B5 cell line, unless otherwise stated. The DMEM was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic and antifungal solution (100 U/mL penicillin sodium and 100µg/mL streptomycin sulfate) (Invitrogen).

Cell Assays

For the glutamate release experiments all cells were cultured in DMEM for a 4 hour period to allow for cell adherence. After this time the media was aspirated out and treated media in DMEM without FBS was used for the duration of the experiment. DMEM without FBS was used as it contains no supplemental glutamate and as such would minimize the variability of the results when run through the AMPLEX Red glutamic assay kit (Invitrogen). The mechanism for glutamate release in these cells is also gradient dependant so the excess glutamate in the media may cause transport inhibition of the cancer cell glutamate. Antibiotics were also not added to the media during experiments as β–lactam antibiotics such as penicillin can also alter the expression of many glutamate transports, giving an increased variability of the results (Rothstein et al., 2005).

Cell Viability

Counting cell viability was completed using crystal violet staining as optimized for our lab (Seidlitz et al., 2009). This assay involves fixation of the adhered cells using 10% phosphate buffered formalin (Fisher Scientific), followed by staining with 50 μL of 0.1% crystal violet stain (Sigma-Aldrich, St. Louis, MO, USA) in 25% methanol. After a 30 minute staining period, a gentle rinse in warm water was used to remove excess stain, and a resolubilisation of the crystal

violet in 100 μL of 0.05M sodium dihydrogen phosphate (BDH, Toronto, Ontario, Canada) in 50% ethanol was done. Using a microplate spectrophotometer (BioTek, Winooski, VT, USA) set to λ=570 nm. Crystal violet binds and stains deoxyribonucleic acid (DNA) to give an indicator of cell number. Establishing a standard curve was necessary to quantify the assay results. A gradient of MDA-MB-231 cells were seeded in 96-well plates at densities of 0-40,000 cells/well in increments of 4,000. Following a 4 hour period at 37°C to allow cell adhesion, crystal violet assays were performed to generate a linear relationship of staining at known cell densities. This equation (y =2E-05x + 0.0745) was used to consistently determine cell number from staining value in all further crystal violet assays.

Animal Model

For the animal model of cancer-induced bone pain, a xenograft of human MDA-MB-231 breast adenocarcinoma cells were injected into the rear right femur in order to isolate bone injury or nociception. The MDA-MB-231 cell line was chosen as the primary cell type for its highly metastatic traits, making it a greater analogue of the human condition. MDA-MB-231 cells are also have an exceptionally high rate of glutamate secretion and to be able to retain this ability *in vivo* (Sharma et al., 2010; Siedlitz et al., 2009). Homozygous Balb/c nude mice were used as they lack a thymus, halting production of mature T-cells. This characteristic renders the mice immunodeficient and unable to mount adaptive immune responses against the xenografted cells,

ensuring the highest possible success rate for this experiment. The mice were all female and acquired at 4-6 weeks and given two weeks acclimation period before testing began. These mice were chosen due to laboratory success working with them in a similar model of bone cancer pain (Ungard et al., 2013). During the course of experimentation there was no instance of food or water deprivation. All mice were housed in clean vent-rack facilities with sterilized food and water.

All protocols for animal experimentation was reviewed and approved by the Animal Research Ethics Board (AREB) of McMaster University, Hamilton, ON, Canada, and adhered to the guidelines of the Canadian Council on Animal Care. The Animal Use Protocol number for this project is 11-07-29.

Model Induction

Mice arrived at 4-6 weeks of age and underwent a mandatory two week acclimation period. During the second week the mice were identified using an ear clip. Behavioural experiments began eight days prior to cell injection. Three days before cell injection the mice were anaesthetized using isoflurane and 21 day-release pellets containing 0.25 mg of 17β-estradiol (Innovative Research of America, Sarasota, FL, USA), were implanted in each animal subcutaneously at the base of the neck. The addition of estrogen to the mouse was found to

improve the consistency of the bone graft. Estrogen, as explained previously, is implicated in bone homeostasis and regulation.

On experimental day 0, the mice were set to undergo cell injection. The mice were again anaesthetized using isoflurane as well as injected subcutaneously with 0.05mL of 1:10 buprenorphine solution (Schering-Plough, Welwyn Garden City, Hertfordshire, UK) for an analgesic. The right rear limb was curled around the finger at a 90° angle exposing the patella and the target injection site. Cells used in animal work were supplemented with an additional 10% FBS 24 hours prior to injection to support increased growth rates and increase grafting success in the bone tissue of the mice. Before the cells were injected, the media was spun out three times to ensure safe injection before being suspended in phosphate buffered saline (PBS) solution at a density of $2x10^6$ cells/ 100µL. The entirety of the MDA-MB-231 cell suspension was implanted into the right distal epiphysis of the femur in all cancer mice. Non-tumour injected mice received a PBS solution of similar volume. The mice which received PBS solution were chosen at random using Microsoft Excel random number generators. The solutions were slowly injected as to cause minimal damage to the bone environment and the needle gently removed. No dental plug was required to seal in the tumour as tumour invasion to the periosteum and surrounding superficial layers of the bone closely mimics metastatic breast cancer in humans. This surgical procedure resulted in temporary nociception and minimal damage to the limb which was recoverable during the early stages of the experiment.

On experimental day 14, the animals were again randomized into further treatment groups (See all experimental groups Table 2.1). Groups consisted of a 5 mg/kg capsazepine dose, a 10 mg/kg capsazepine dose, or a vehicle dimethyl sulfoxide (DMSO) solution. The dosages were selected using laboratory tested capsazepine on MDA-MB-231 cells (APPENDIX 3) with a double dose used for to ensure maximal exposure to the drug. Both concentrations of capsazepine used were far less than other animal experiments using capsazepine, as the concentrations required for glutamate reduction was less than that required for TRPV1 inhibition (Asai et al., 2005; Menendez et al., 2006; Venes et al., 2012). After the randomization process, the mice were again anaesthetized using isoflurane and the drug was delivered using Alzet model 1004 mini-osmotic pumps (Durect, Cupertino, CA, USA), inserted intraperitoneally following the steps for surgical implantation (Alzet, 2014). These pumps operate through an osmotic gradient between the pump compartment, called the salt sleeve, and the tissue environment in which the pump is planted. Water is drawn in from the surrounding tissues into the salt sleeve; the resulting expansion compresses an impermeable inner reservoir filled with the desired solution which forces the release of that substance through a flow moderator (Alzet, 2014). The rate of delivery by an ALZET pump is controlled by water permeability. Thus, the delivery profile of the pump is independent of the drug solution used giving a consistent 28 day delivery of all solutions. Day 14 was chosen for this procedure to allow sufficient time for the xenograft cells to establish colonies within the bone as well as gauge the recovery of the mice from the first procedure before the randomization process.

On experimental day 36 the mice reached an endpoint which agreed with our AUP and upon final veterinarian observation, were scheduled for sacrifice. CO_2 was used to cull the mice for organ harvesting. The figure below shows a summary of the animal model induction highlighting the important procedures and dates (Figure 2.1)

NAME	Limb Injection	Pump Solutions
Group 1 (Veh)	$2x10^6$ MDA-MB-231 Cells	DMSO
Group 2 (Capsazepine)	$2x10^6$ MDA-MB-231 Cells	5mg/kg Capsazepine
Group 3 (Capsazepine)	$2x10^6$ MDA-MB-231 Cells	10mg/kg Capsazepine
Group 4 (Control)	PBS Sham Injection	5mg/kg Capsazepine
Group 5 (Control)	PBS Sham Injection	DMSO

Table 2.1: Behavioural experiment groups and controls. Listed here are the various groups used in the animal experiments throughout this thesis. There was no control for 10 mg/kg capsazepine as Group 4 failed to show deviation from Group 5 as well as other studies showing no side effects while using higher doses of capsazepine in mice.

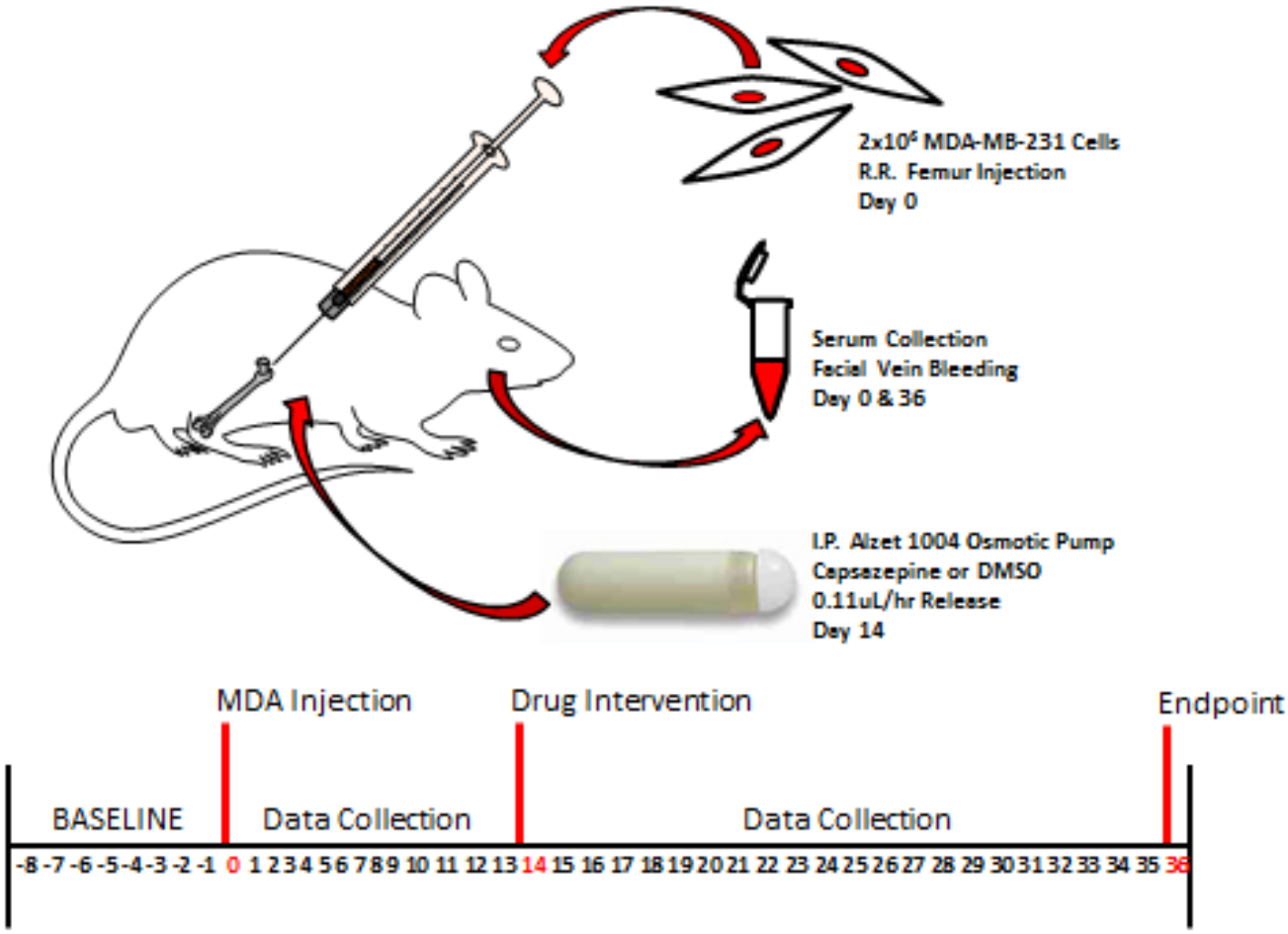


Figure 2.1: Behavioural experiment setup and timeline. The timeline (show at bottom) shows the experimental days in which the various tests and surgeries were performed on the mice with major days highlighted in red. Behavioural tests were run at both BASELINE and Data Collection peroids in which the later was then compared to the former for an accurate look at each individual's pain scores.

Behavioural Testing

The testing period started eight days prior to cancer cell implantation and was performed every other day for a total of four baseline tests. These tests were averaged to get a baseline score for which the rest of the experiment was compared to. After cell implantation the testing was performed three days a week on alternating days until endpoint was reached. There were three tests performed utilizing two behavioural systems, the dynamic plantar aesthesiometer (DPA) (Ugo Basile, Comerio, Italy), and the dynamic weight bearing (DWB) system (BioSeb, Vitrolles, France)

Dynamic Plantar Aesthesiometer

The DPA is a behavioural test using a flattened metal filament attached to an electrical actuator and recording device. The actuator, when triggered, raises the metal filament with adjustable maximum force and speed. The mice are placed individually in holding areas with grated floors through which the filament will come. The device is manually moved under the injected paw of the chosen mouse and the actuator is triggered, raising the filament to the plantar surface of the paw. Once contact is made the amount of force the filament uses increases until the paw is withdrawn and measurements of reaction time and necessary force are gathered. The DPA is an electronic Von Frey instrument designed for mechanical allodynia and hyperalgesia, intending on measuring the force required for withdrawal (in mice experiencing pain). Consistent

force ramping and timing are the advantages of the electronic version of this test. The DPA was done five times per mouse for every testing day with the average result being that day's "pain score" for the mouse. The time of withdrawal was not used in the results section as it was a formula for gathering the force and both graphs are identical in shape and features. Only mice which had visible tumours using the radiograph and histology staining were used for the results section in order to remove interference from unsuccessfully grafted animals. The final group numbers of successful tumour models were: (Vehicle): n=13; (5mg/kg capsazepine): n=11; (10mg/kg capsazepine): n=10; (Sham Vehicle): n=5; (Sham 5mg/kg capsazepine): n=3. No differences were seen from the two sham injected groups and both sham injected groups remained healthy for the duration of the experiment. For these reasons, only the cancer injected mice will be shown in the results in order to have a better comparison of the treatment groups.

Dynamic Weight Bearing System

The DWB system is a way of recording parameters such as weight distribution and time spent on each limb over the course of 3 minutes. Taken collectively, these tests serve as an indirect measure of pain. The DWB consists of a plastic housing with specialized weight sensors covering the floor of the case. These sensors are calibrated to the weight of the mouse with a video camera mounted overhead to analyse the mouse's movements as the experiment progresses. The key advantage of this test is that it requires no incapacitance as it lets the mice roam freely for the duration of the experiment, thus more closely mimicking leg pain without human subjectivity. After the test is completed, the digital recordings from the overhead camera

were manually validated to provide the software with the mouse's orientation on the sensor. The duration was set for 3 minutes per mouse and once per testing day giving ample time for the mouse to settle down from the excitement of the new surrounding as well as experiencing the true posture of the uninhibited mouse. The DWB recorded the mean weight of the mouse for each limb separately as well as the mouse as a whole. For this weight bearing test only the right rear limb was used for the results. The daily mean weight placed on the rear right paw was then compared to the baseline mean weight for a change in weight, shown in the results section. Reduction in mean weight when compared to the baseline was taken as evidence of aversion to limb use due to nociception and scored as an indirect measure of pain for this experiment. Baseline scores were used to account for an animal's predisposition to favour one paw over another as mice have been shown to have a preference between paws (Signore et al., 1991). The other measure the DBW makes is the time spent on each paw. This is a recording where a set threshold must be met in order to acknowledge the presence of the paw on the sensor. This test is very specific for nociception as hovering the rear right limb is not a common stance that mice take. Deviations in time spent on the injected limb were very subtle and as such was considered to be a valuable measurement of severe nociception in mice. Similarly to the DPA test, only mice which had visible tumours using the radiograph and histology staining were used for the results section in order to remove interference from unsuccessful animals that exhibit relevant behaviour. The final group numbers of successful tumour models were: (Vehicle): n=13; (5mg/kg capsazepine): n=11; (10mg/kg capsazepine): n=10; (Sham Vehicle): n=5; (Sham 5mg/kg capsazepine): n=3. No differences were seen from the two sham injected groups and both sham injected groups remained healthy for the duration of the experiment. For these

reasons, only the cancer injected mice will be shown in the results in order to have a better comparison of the treatment groups.

Blood Collection

The collection of blood was used for the comparison of glutamate in the serum before and post-cancer injections. Whole blood was collect via the facial vein at experimental day 0 and again at endpoint using the same method. The mandibular vein was chosen in accordance to the guidelines for survival bleeding of mice and rats (Fernández et al., 2010; Beth Israel Deaconess Medical Center, 2010) for minimal risk to the animal and so that repeated collections could be completed. 100uL of blood was collected after a puncture into the left superficial temporal vein. Blood collection was done using standard 200uL Eppendorf safelock tubes which were then taken to a centrifuge no later than 2 hours post collection. The samples were spun for 15 minutes at 1400 revolutions per minute (RPM) at 4°C. Endpoint samples were taken 4 hours before sacrifice to ensure consistent blood collection methods. Blood serum glutamate was counted using the AMPLEX Red assay kit following a dilution of 10:1 of the serum in 1X reaction buffer.

Histochemistry

For this thesis the primary staining technique used was the haematoxylin and eosin staining to observe the tumour in the bone cavity in addition to the extent of the bone damage in the injected limb. Immediately after sacrifice, the mice were harvested collecting liver, lungs, heart, kidney, spleen, spine and both hind legs. The samples were placed in formalin containers for 48 hours prior to an ethanol wash. Following this step, the harvested samples were placed in an agitated 10% EDTA, 4% formalin buffered solution for two weeks to decalcify, with one set of organs not undergoing decalcification as a control. This step is necessary to deplete the bones of calcium making them malleable for slide production and future staining techniques. After the decalcification process the samples were washed twice in distilled water then placed in paraffin wax. The samples are then ready for slicing and slide production. Slices of 4µm thickness were mounted on glass slides and baked at 65°C for 25 minutes prior to staining. Once cooled, the slides were deparaffinized and rehydrated using multiple xylene, ethanol and dH2O washes before adding the first stain (Haematoxylin (Sigma-Aldrich) diluted 1:2 with dH2O) for 3 minutes. This stain was followed by two dH2O rinses each consisting of ten seconds in alkaline lithium carbonate to induce the haematoxylin stain to change colour. Slides were then stained for 45 seconds in 1% eosin diluted 1:3 in 80% ethanol solution. Following both of these staining processes, slides were then dehydrated in ethanol and xylene before being cover slipped with xylene miscible permount (Fisher Scientific, Pittsburgh, PA, USA). Permount was allowed to dry overnight before visualization under light microscopy was done.

Radiographical Analysis

Immediately following harvest (during the formalin fixation stage for Histochemistry) the leg samples were taken for radiographical analysis. High resolution images were collected using the Faxitron X-ray MX-20 system (Faxitron X-ray Co., Wheeling, IL) on Kodak MIN-R2000 Mammography Film (Eastman Kodak, Rochester, NY, USA). This allowed for a clear view of tumour location and bone destruction in the injected limb when compared to the non-injected limb.

Statistical Analysis

All *in vitro* data was measured using one-way ANOVA followed by a Tukey test to compare all groups. The significance for all experiments was set to $P < 0.05$. All behavioural data was measured using a one-way repeated-measures ANOVA followed by a Tukey test comparing all data sets to one another. Behavioural data was calculated from day 25 until endpoint to limit the effects of the long lag phase from tumour implantation to symptoms. All data presented is in terms of the mean ± the standard error of the mean (SEM). GraphPad Prism software version 5 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) was used for all graphing and statistical analyses.

Chapter 3: Results

Cellular Glutamate Release

The MDA-MB-231 cells were found to release approximately three times more glutamate into the extracellular environment than control 184B5 breast epithelial cells (Figure 3.1). This finding was expected, as MDA-MB-231 cells have been found in our lab to contain higher concentrations of the glutamate transporter, system x_C^-. 10,000 MDA-MB-231 breast adrenocarcinoma cells and 185B5 mammary epithelial cells were seated and glutamate release was tested after a period of 48 hours. Interestingly, the use of SSZ was found to cause a reduction in glutamate in both cell lines, with a larger affect in MDA-MB-231 cells, which is not surprising considering its role in system x_C^- and that these cells possess more xCT than 184B5 cells. The results in Figure 3.1 show a direct comparison of these two cellular glutamate releases at a glutamate in µM/10,000 cells, as to account for faster MDA-MB-231 growth.

The glutamate release experiment was the primary finding and allowed for continuation with dose inhibition experiments. The follow up to this finding was to conduct a high throughput screening for compounds that inhibit cancer-released glutamate from MDA-MB-231 cells. This screening was done by the lab and led to the discovery of capsazepine, the primary drug for this thesis.

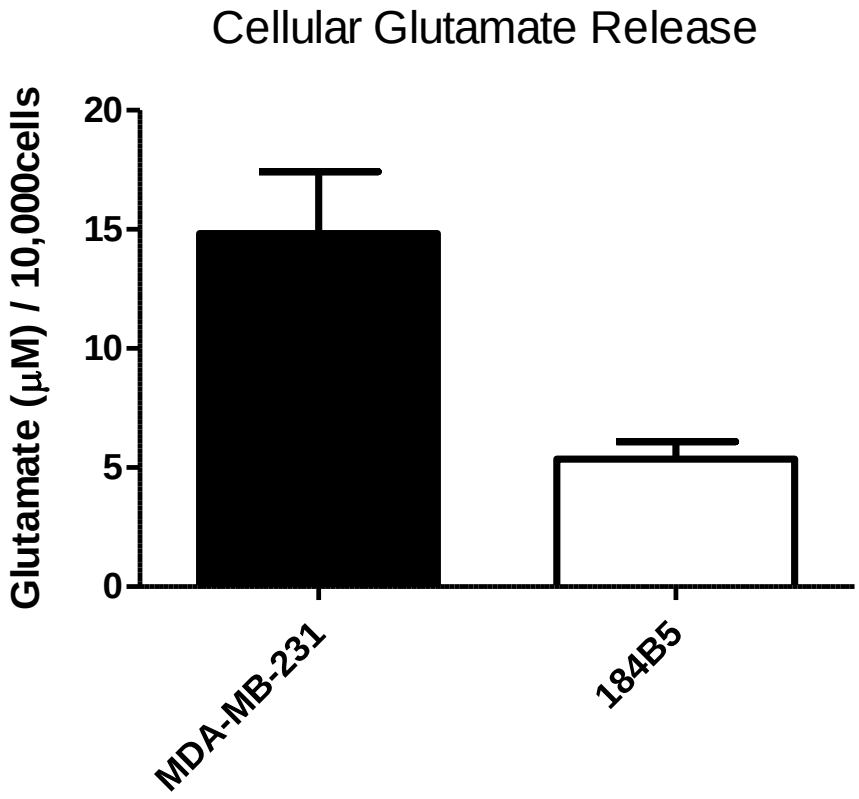


Figure 3.1: Glutamate release assay comparing MDA-MB-231 breast adenocarcinoma cells with 184B5 breast epithelial cells. This graph shows a 3 fold increase in glutamate release from MDA-MB-231 cancer cells when compared to control breast epithelial cells (P=0.0247).

Capsazepine was tested against SSZ, the gold standard in literature, for glutamate inhibition. It was found that capsazepine once again proved to be the more potent inhibitor of cancer-released glutamate (APPENDIX 2). Following up on capsazepine, a dose-dependent drug response curve was completed on 10,000 cells over a 48 hour period. Capsazepine was found to

exhibit an extremely effective response at low doses with a continuing effect until 200 μM, which then became too cytotoxic to be useful for the animal experiments (APPENDIX 3).

Behavioural Analysis

Bulb/C nude mice were injected with $2x10^6$ MDA-MB-231 cells at the distal epiphysis of the right femur. This allowed for the affected limb to be isolated for behavioural experiments, granting consistent and repeatable testing. All mice which did not have successful tumour graft to the bone, as confirmed by radiographical and histological means, were removed from the analysis. The DPA and both DWB tests were consistent, showing a steady increase in nociception with respect to time in the cancer-injected mice. The treatment of these tumours with capsazepine was separated into two doses; a high dose (10 mg/kg) and a low dose (5 mg/kg), with both does showing modulating effects on pain. The expectation was for dose dependant results in all three tests as well as pain attenuation greater than that of SSZ, a treatment which gave moderate levels of success in previous experiments (Ungard et al., 2013). The low dose capsazepine (5 mg/kg) group resulted in a modulation of pain-related behaviours consistent with all three behavioural tests while the high dose capsazepine group was found to be significant in all three tests as well as non-significant from sham-injected mice, suggesting that cancer-induced bone pain was completely attenuated in this group, although the increasing the length of the experiment may have led to these mice eventually experiencing nociception.

DPA

The DPA analysis showed that both capsazepine doses delayed the onset of pain-related behaviours, with the higher dose slightly trending in favor of nociception-free state over the lower dose when observing the pain scores. Nociception was observed by a decrease in force required for paw withdrawal which this behaviour was associated with nociception. The 5 mg/kg dose of capsazepine was statistically significant (P=<0.05) from the vehicle group. The 10 mg/kg group also was statistically significant from the vehicle group (P=<0.05) while not being significant from the non-tumour mice (Figure 3.2). This shows that the larger dose was more closely associated with baseline behaviour and effectively alleviated symptoms of nociception in the weight bearing test, although the experiment was ended too early to tell if symptoms of pain would have eventually arisen in the 10 mg/kg group, due to increasing tumour size and the overall condition of the mice at this time.

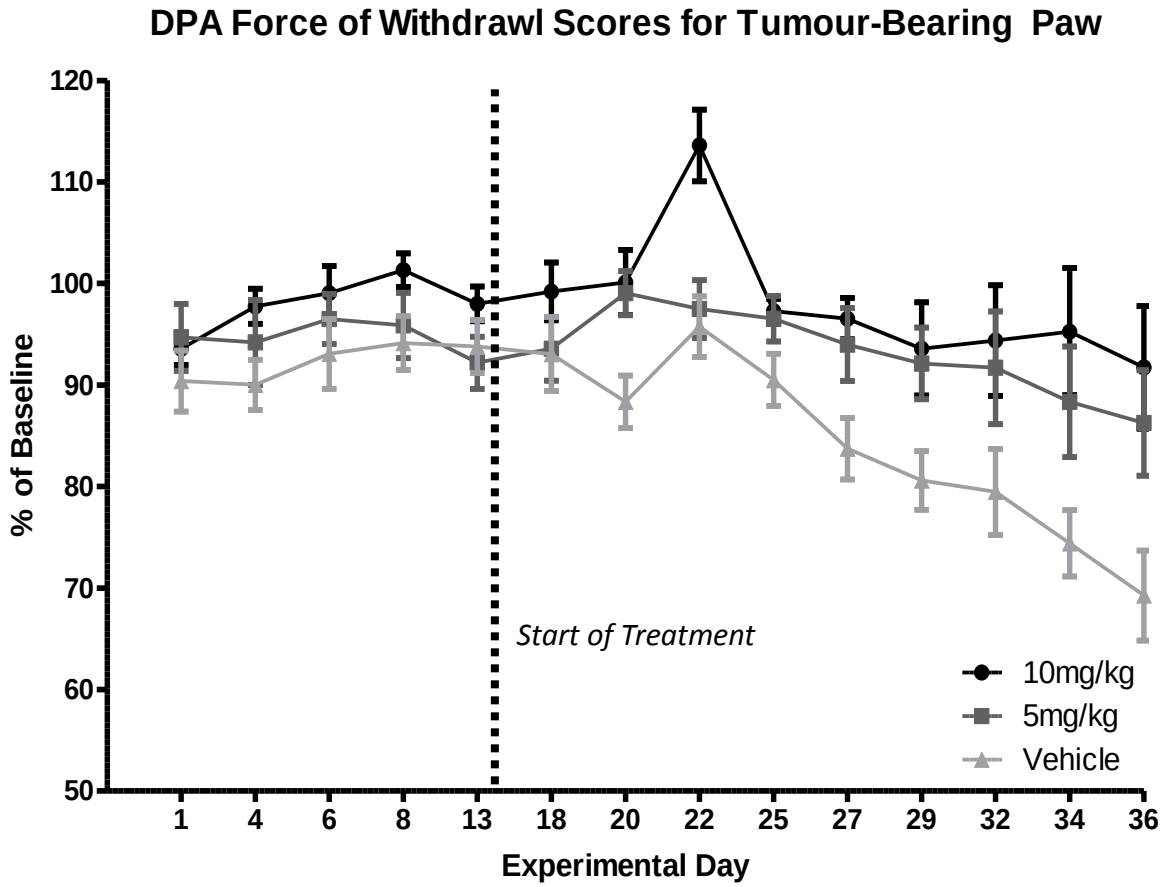


Figure 3.2: DPA measurements of force required for withdrawal of the injected limb compared to the baseline results in capsazepine treated and non-treated mice. 100% on the y-axis is therefore equivalent to the animal's behaviour pre-tumour implantation surgery. This graph shows a significant increase in force required for paw withdrawal in the treated mice (10 mg/kg) n= 10; (5 mg/kg) n= 11; (Vehicle) n= 13. One way repeated measures ANOVA was used on the measurements past Day 25 showing significant differences between groups (P= <0.0001). While both doses were significantly different from vehicle mice (P=<0.05), differences between doses were not significant. Data are expressed as the mean required force as a percentage of the baseline score ± the standard error of the mean (SEM).

DWB

The DWB analysis was done similarly to the DPA and showed consistent results. The sensitivity of the weight percentage test was found to be far greater than that of the time percentage test, with the time-based test being very specific to mice in a high pain state. In the weight-based test, the mice experiencing nociception showed a dramatic decrease over time in the weight exerted on the tumour-injected limb when compared to their baseline weight distribution. Both doses of capsazepine were found to cause significantly less nociception when compared to the pain score of the vehicle treated mice ($P=<0.05$). The time-based test was used to measure limb favouring and it was shown that only mice experiencing severe nociception in the other behavioural tests would lift their rear right limb for any significant period of time. This could be due to pain or an inability to move the damaged limb. Vehicle treated mice showed no hindrance in limb use and also displayed a dramatic drop from their baseline pain scores. During this experiment, only the mice with the most severe pain were shown to rise their hind leg for times significantly different that their baseline preferences. Similarly to the other tests, both doses of capsazepine were found to be significant from the vehicle group when comparing pain scores ($P=<0.05$), but failed to create a dose-dependent response. Interestingly, it was also observed that both doses of capsazepine were not significantly different in pain scores than sham injected mice. For both weight-bearing and time on paw tests a one way ANOVA with repeated measures was used past day 25.

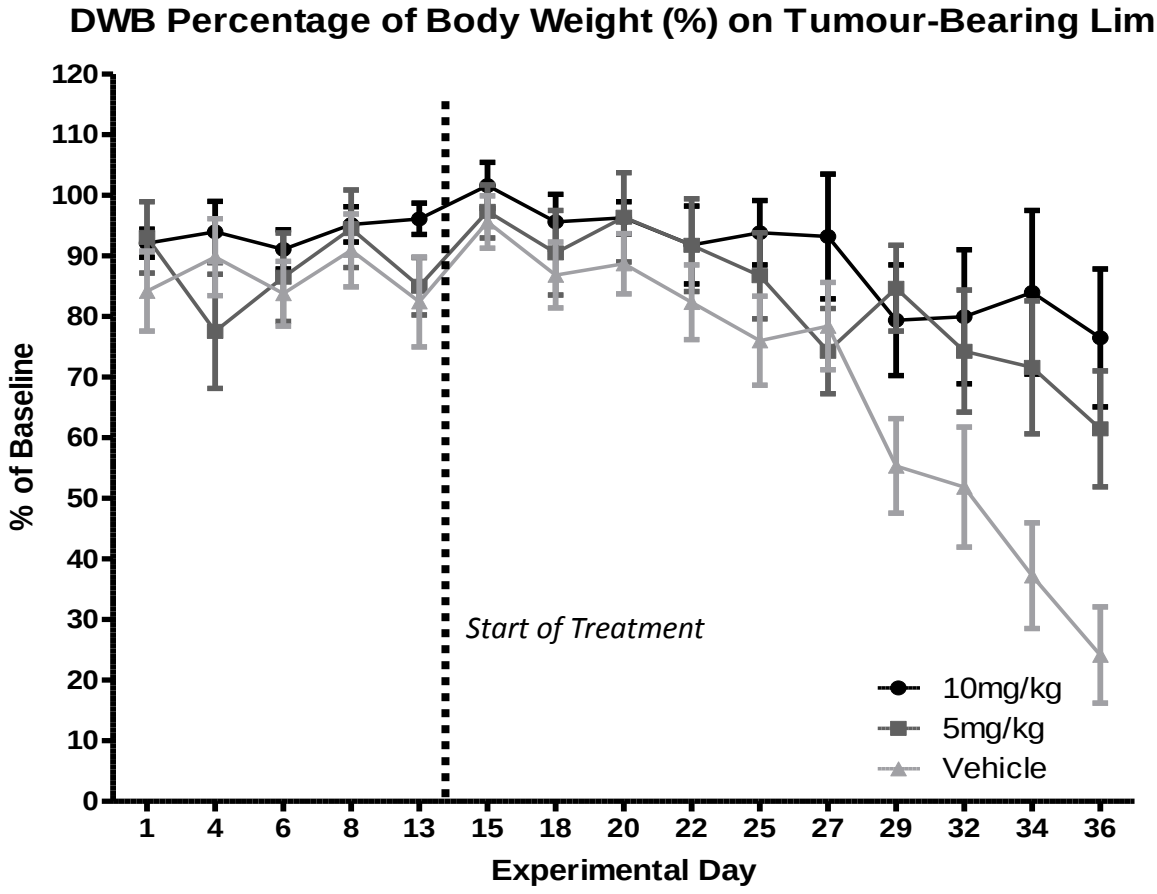


Figure 3.3: DWB measurements of weight distribution compared to the baseline results in capsazepine treated mice and non-treated mice. This graph shows a significant increase in weight distribution in the treated mice (10 mg/kg) n= 10; (5 mg/kg) n= 11; (Vehicle) n= 13. One way repeated measures ANOVA was used on the measurements past Day 25 showing significant differences between groups (P= 0.0008). While both doses were significantly different from vehicle mice (P=<0.05), differences between doses were not significant. Data are expressed as the mean required force as a percentage of the baseline score ± the standard error of the mean (SEM). Data are expressed as the mean weight

bearing in the injected limb as a percentage of the baseline score ± the standard error of the mean (SEM).

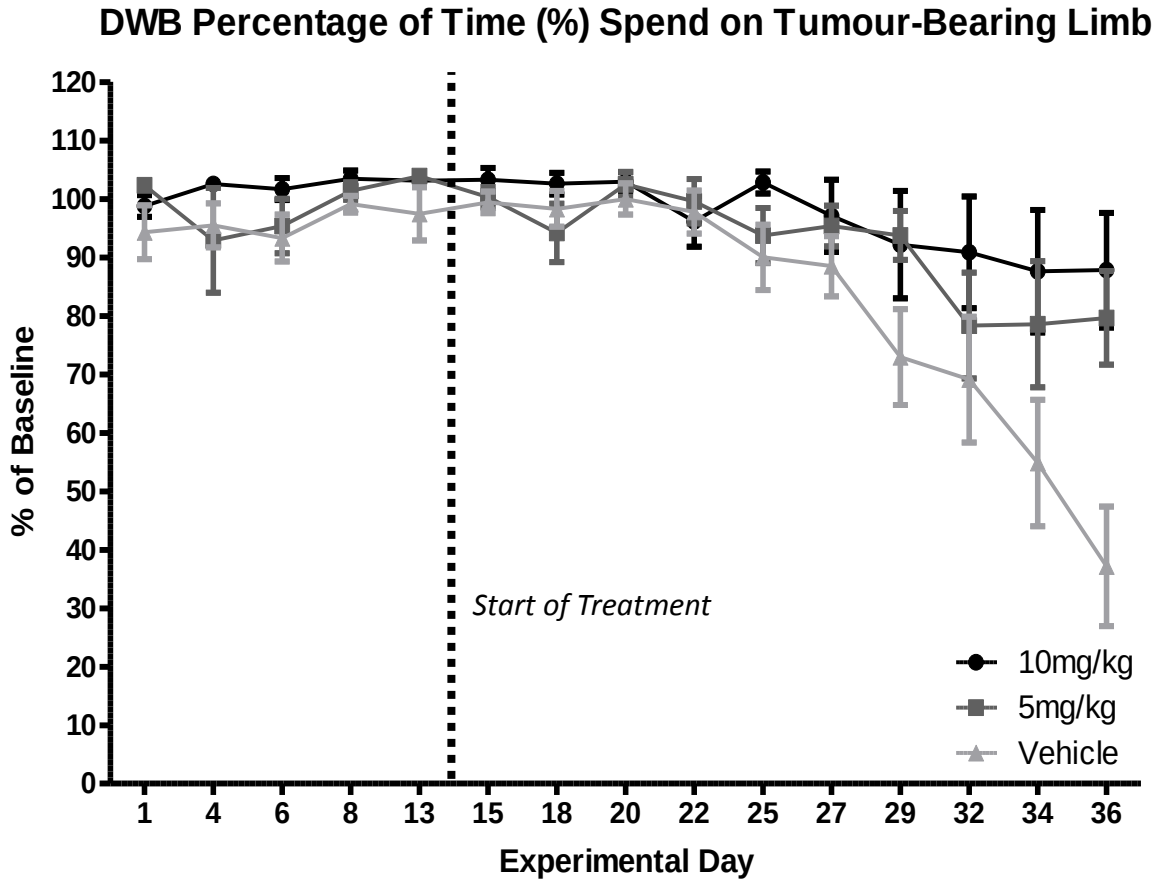


Figure 3.4: DWB measurements of time spent on the injected limb compared to the baseline results in capsazepine treated mice and non-treated mice. This graph shows a significant increase in time spent on injected limb in the treated mice (10 mg/kg) n= 10; (5 mg/kg) n= 11; (Vehicle) n= 13. One way repeated measures ANOVA was used on the measurements past Day 25 showing significant differences between groups (P= <0.0022). While both doses were significantly different from vehicle mice (P=<0.05), differences between doses were not significant. Data are expressed as the mean time (s) on the

injected limb as a percentage of the baseline time ± the standard error of the mean (SEM).

Blood Serum Data

The follow up to the *in vitro* glutamate work, the blood of each mouse was collected to test and see if this effect on glutamate by capsazepine was observable *in vivo* (Figure 3.5). Blood was collected from the facial vein of six SCID mice (three with tumour and 3 control mice) and spun down to collect the serum, where glutamate could then be measured using the AMPLEX red assay kit. Three mice having $1x10^6$ MDA-MB-231 cells intrafemorally injected into the femur and 3 mice with sham injections were used for this experiment. The result is that serum glutamate levels of the cancer-injected SCID mice was significantly increased after a two month period when compared to the sham injected mice (P=0.03). This result was the primary stepping-stone for testing glutamate serum levels in the larger capsazepine experiments, showing promising ground work for the testing of capsazepine in the *in vivo* attenuation of cancer-released glutamate.

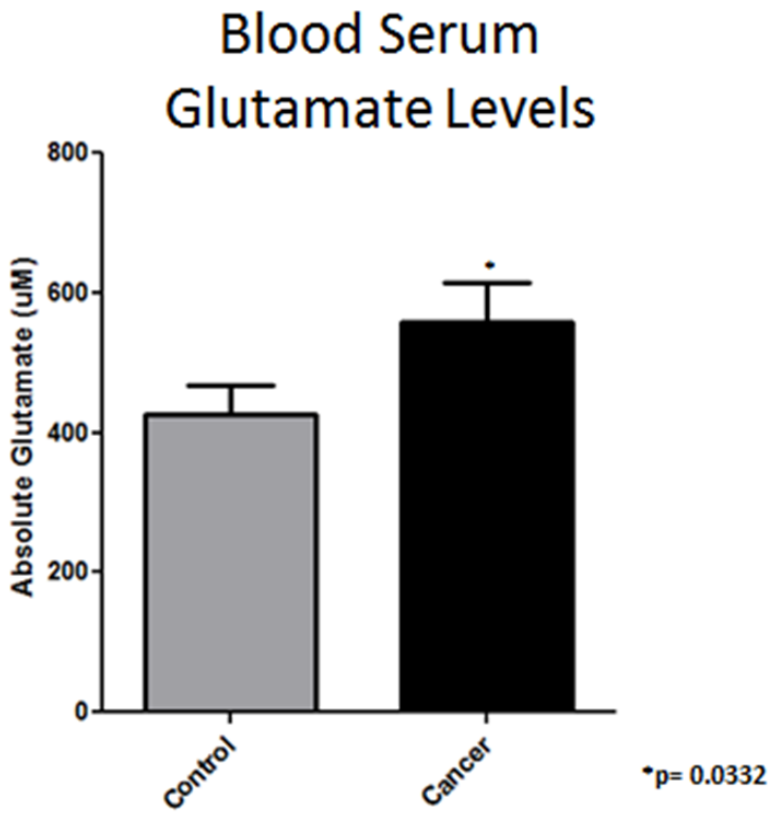


Figure 3.5: Preliminary blood serum study in SCID mice. 3 mice from each group injected with cancer for approximately 2 months were used. Blood was collected using the method described in the methods section, using an AMPLEX Red assay kit for quantification. This result shows the mice with cancer having ~15% greater serum

[glutamate] level. Two tailed T-test was used to measure the statistical significance of $p=0.0332$.

To expand upon these results, blood levels were taken during the much larger capsazepine experiment at both baseline, before the introduction of the tumour cells, and endpoint, where the differences of glutamate levels can be compared to the release of cancer-secreted glutamate. Glutamate testing of each trial using the AMPLEX RED assay kit with the same methods as previously discussed. This was done with the hope that the cancer-injected mice with vehicle treatment would show a similar result to the excess glutamate found in the first study. This effect would then be abolished in the treatment groups in a dose-dependent manner. The results from the capsazepine experiment blood serum analysis shows that non-treated cancer-injected mice indeed, had a significantly higher level of serum glutamate than both treatment groups, but not in a dose-dependent manner (Figure 3.6). Both doses of capsazepine efficiently returned serum glutamate concentrations to baseline levels, exceeding expectations.

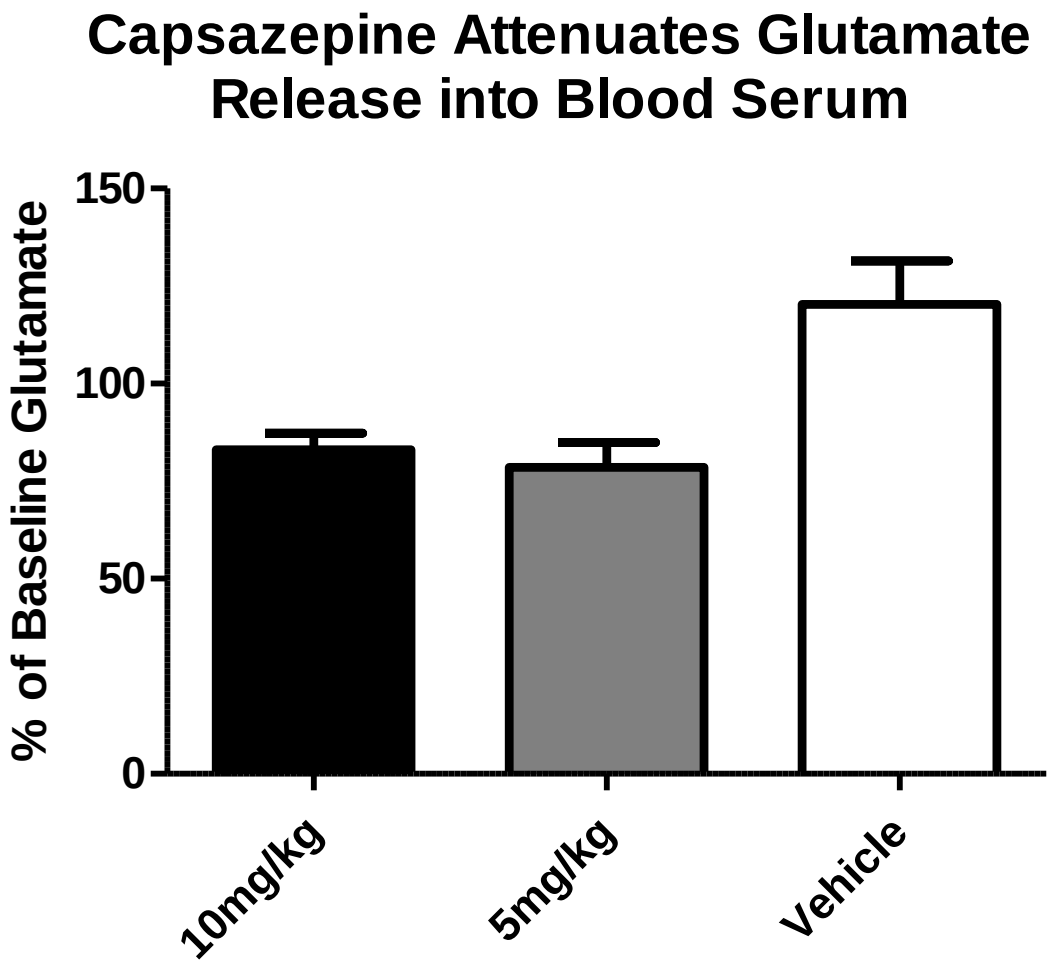


Figure 3.6: Serum glutamate from cancer-injected mice. This graph represents the change of glutamate levels in the blood from baseline after 36 days of tumour growth and intervention with either 5 mg/kg or 10 mg/kg capsazepine. One way ANOVA was used with a TUKEY test to compare each column, which significant results were found (P=0.0029). Similarly to the *in vivo* testing, both doses were significantly lower than the vehicle treated mice (P=<0.05) while not significant from each other.

Radiograph Analysis

For the radiographic analysis, the radiographs were taken using a MX-20 faxitron X-ray system and within 24 hours following sacrifice to ensure tumour graph success (Figures 3.7; 3.8). Mice with no visible osteolytic lesions were immediately removed from the study. Only the right rear limb was examined with the rest being harvested for organ studies or discarded. On the tumour-bearing limb, the focus of the radiograph was on the distal epiphysis where osteoplastic lesions could be easily viewed. Interestingly, secondary muscular lesions were also seen in some mice, but further analysis of the behaviour data did not support the notion that this affected pain more than tumours isolated to the bone. Sham injected mice showed normal phenology with no bone or muscular lesions. The amount of bone destruction or tumour size was not shown to be a significant factor in determining pain.

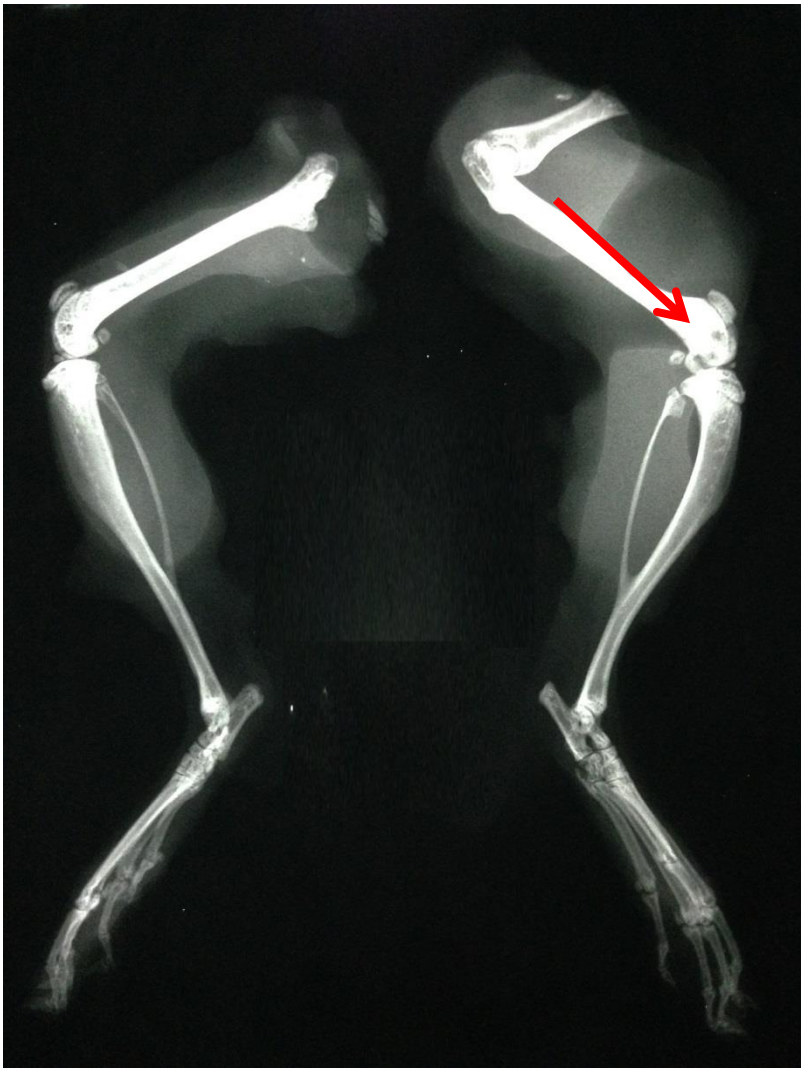


Figure 3.7: X-Ray of a Sham Injected Mouse. Above is a port-mortem radiograph of a sham-injection mouse. The right femur is completely intact with no signs of osteolytic lesions. The site of injection has also fully healed and is no longer distinguishable from the left femur. Arrow indicates the site of injection.

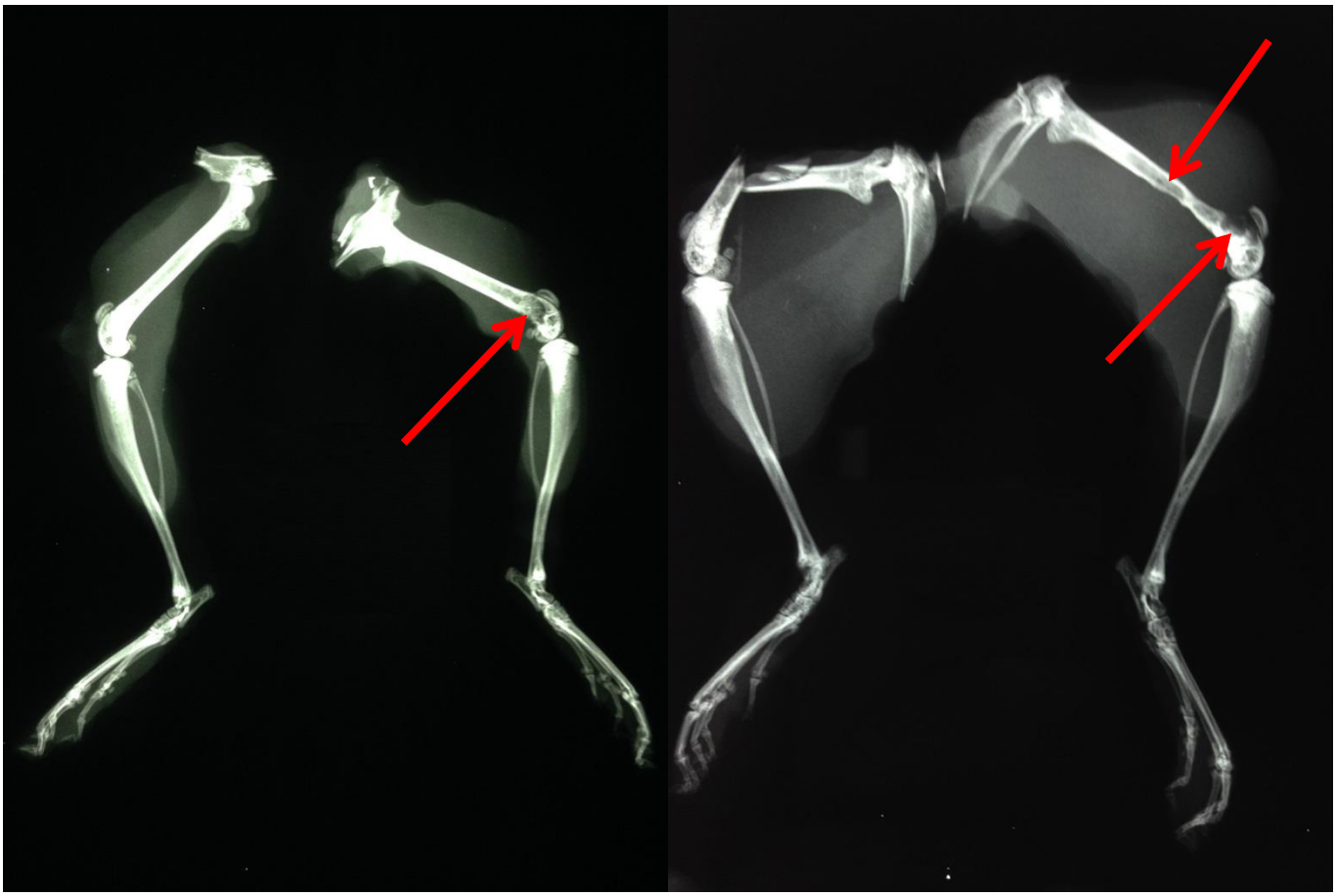


Figure 3.8: X-Rays of tumour injected mice. The image of the left shows osteolytic lesions in the distal epiphysis of the right femur. Dark areas in the bone structure indicate sites of bone loss. The image on the right is another example of tumour-induced osteolysis, but separate in that the lesion has spread to the outside of the bone, affecting the periosteum. This type of injury closely mimics that of the human condition. Arrows indicate osteolytic lesions.

Histology

Histological staining was done using haematoxylin and eosin stains. These stains revealed the extent of the tumours throughout the femur and surrounding tissues. Tumours were mainly isolated to the distal epiphysis and diaphysis of ipsilateral femurs, with some lesions appearing in the muscular tissues, knee join, and surrounding periosteum. While no changes in pain were correlated with tumour damage and location it provided a further control for the experiment. Animals failing to have adequate tumour growth in the right femur were discarded from the study and subsequent analysis. Invading tumour cells were present in the trabecular and cortical bone in all injected mice, as well as in the bone marrow. In the sham injected mice, no evidence of osteolysis was found. Normal bone phenology was observed in all mice used for control purposes.

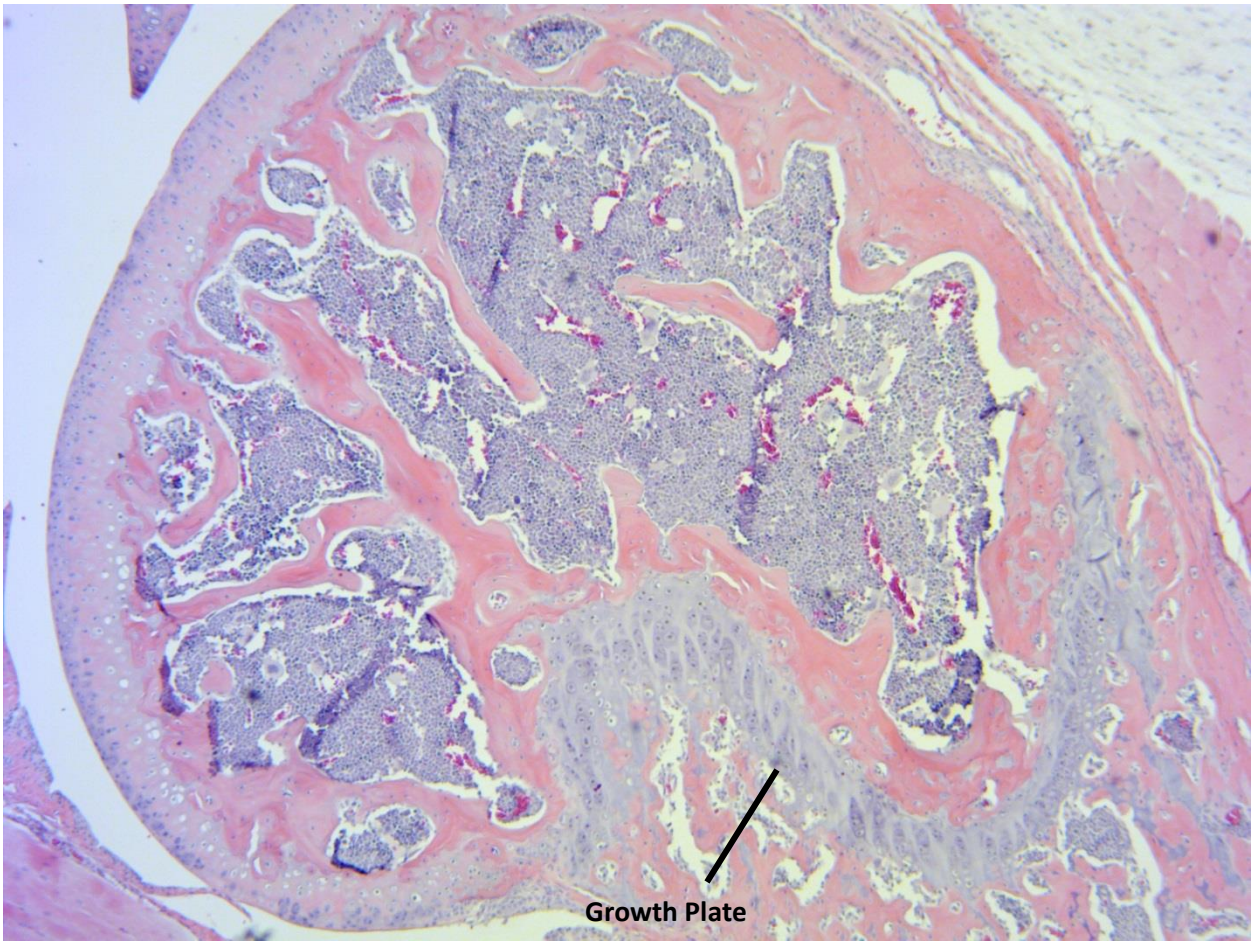


Figure 3.9: H&E stain of a sham-injection mouse femur. Pictured is the H&E stain of a control mouse femur. Note that normal bone histology is present without disruption to the growth plate or bone structure.

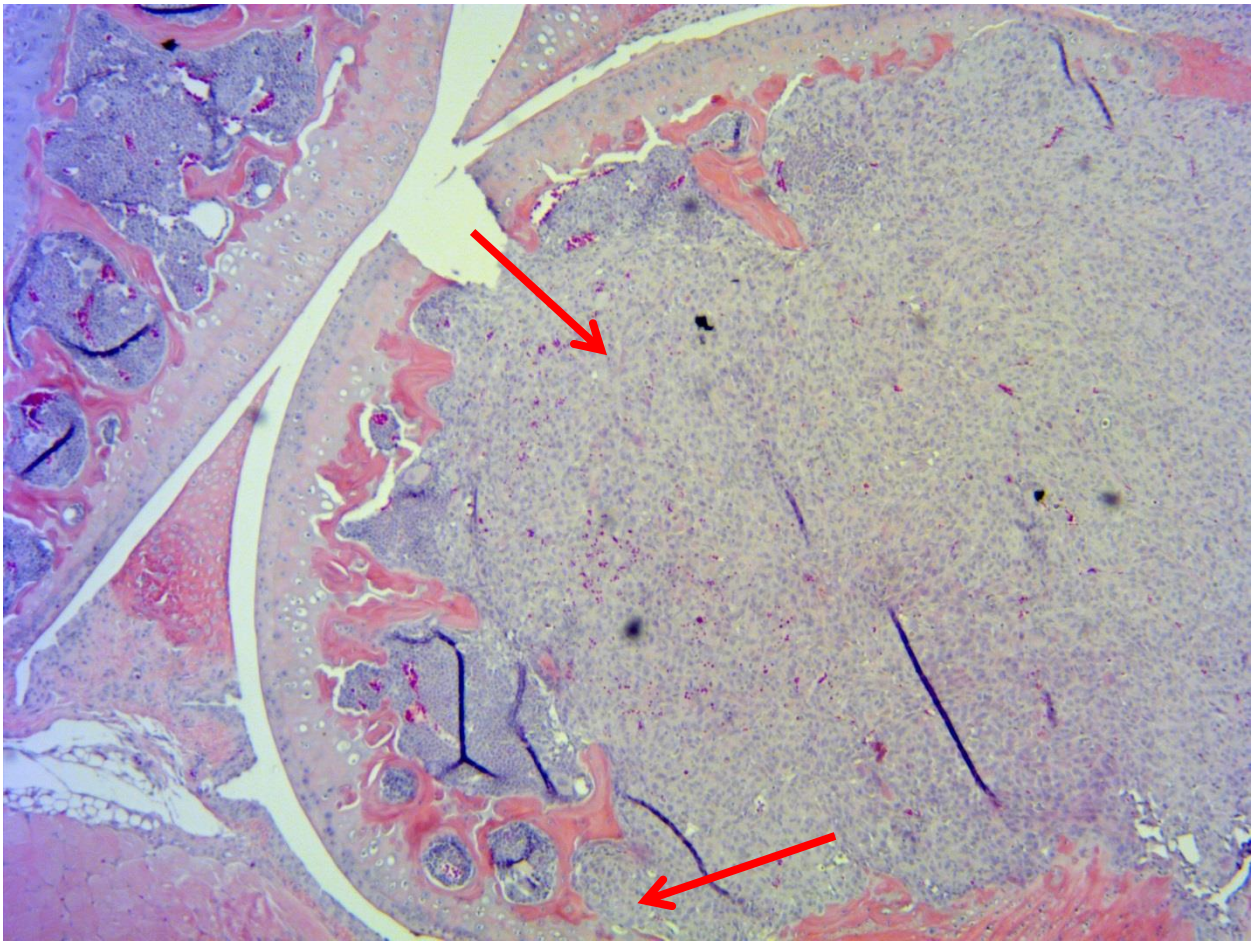


Figure 3.10: H&E stain of a tumour-injected mouse. This slide shows extensive tumour invasion of the femur. No evidence of a functional growth plate is present. Arrows indicate sites of tumour presence.

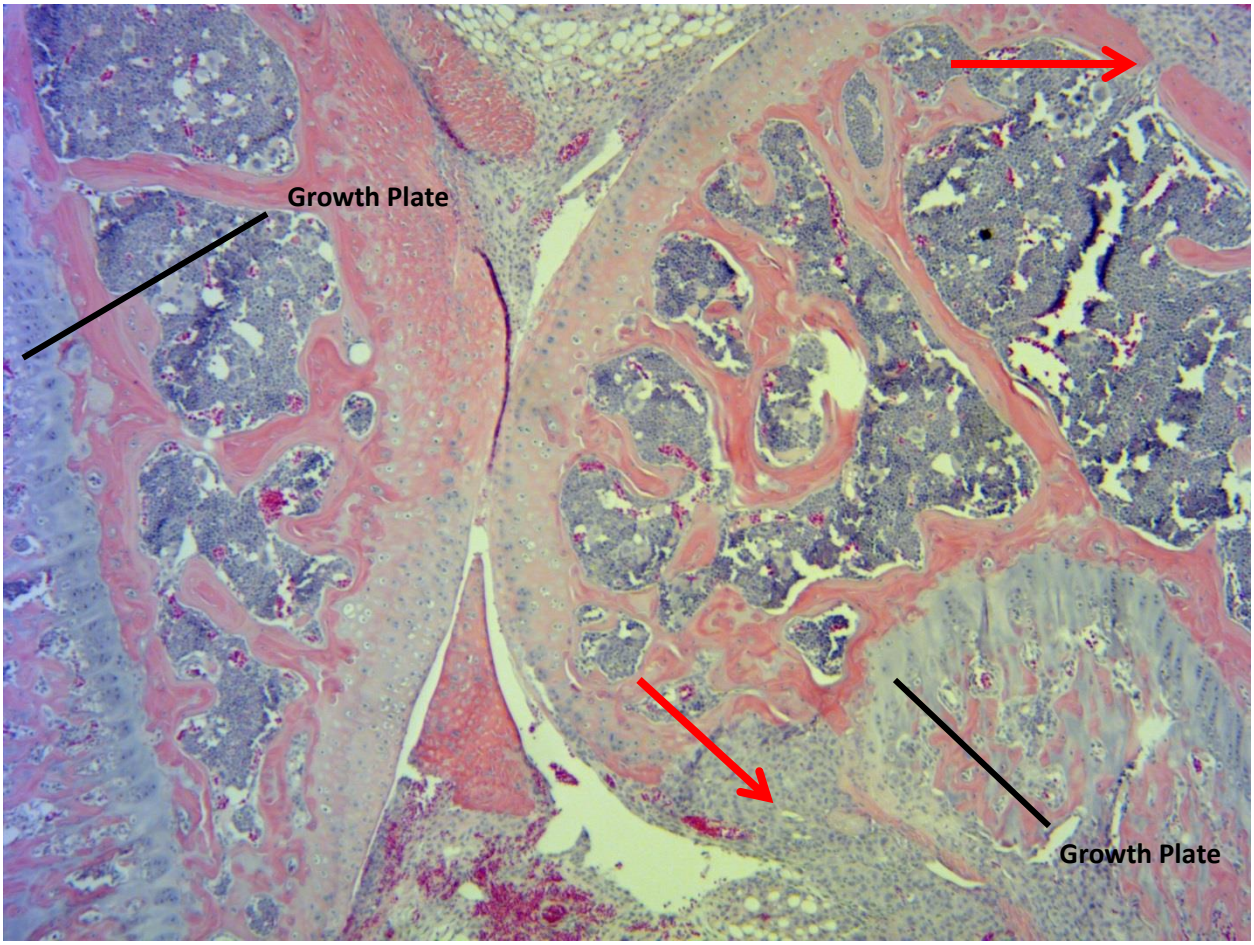


Figure 3.11: H&E stain of tumour breaching periosteum. The H&E stain was able to uncover extensive damage to the growth plate and surrounding periosteum. Normal bone histology is present in the head of the femur. Tumour is also seen breaching in the top of the femur head. Arrows indicate sites of tumour growth and invasion.

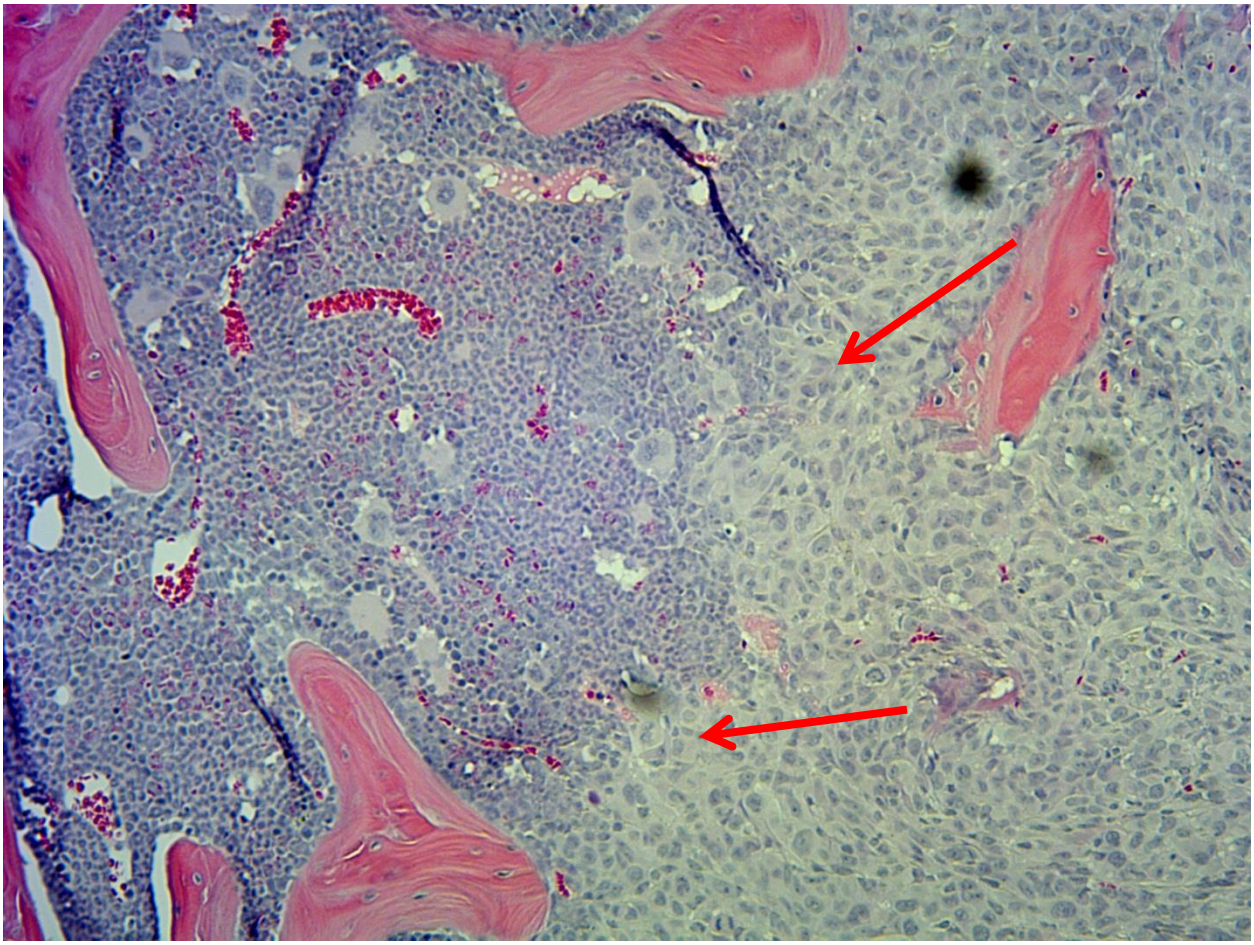


Figure 3.12: Tumour invasion of existing bone. MDA-MB-231 tumour cells on the right are seen invading the existing bone structure in a mouse injected with tumour cells. The degree of osteolysis varied, but was present in all mice injected with tumour cells. Arrows indicate sites of tumour invasion into the existing bone structure.

Chapter 4: Discussion

Advanced cancers of the breast are the most common source of bone metastasis in women. When in the bone, these cancers are followed by a host of pathological consequences, the primary symptom being pain (Jimenez-Andrade, 2010). Cancer in the bone produces a physiologically complex pain state which can arise from a multitude of factors such as altered bone remodeling, damage to surrounding nerves, fracturing of the bone and nociceptive factors released from the surrounding cells or secreted directly secreted from the invading tumour cells. This multiplicity of nociceptive pathways makes the treatment of cancer-induced bone pain resistant to current therapies, with over 50% of patients having persistent pain (Wilson et al., 2014).

The objective of this project was to explore alternative methods of achieving analgesia through the inhibition of cancer mediated glutamate release. Glutamate is the most common neurotransmitter in the CNS, and is known to play a role in pain as well as modulating cellular homeostasis in the bone. It has also been shown to be secreted much more heavily by cancer cells such as the MDA-MB-231 cell line when compared to control breast epithelial cells (Figure 3.1). The greatly increased glutamate in the tumour microenvironment has an effect on a plethora of nociceptive pathways, therefore it was hypothesized that if cancer cell glutamate release could be attenuated then this would result in a reduction of cancer-induced bone pain. Prior to this experiment, a similar trial using SSZ was used and slight decrease in pain-related behaviours were observed, although these were not significant.

During the course of this project a high throughput screen was run examining 30,000 compounds to isolate a potent inhibitor of the MDA-MB-231 cell line's ability to release glutamate which was better than that of SSZ, presently the gold standard for system x_C^- inhibition in cancer cells. Capsazepine was found to have the best result without the effect of cytotoxicity, a common complicating factor with many compounds isolated in the screen. Capsazepine is a synthetic analogue of capsaicin, a naturally occurring TRPV-1 agonist found in many spicy foods. Capsazepine binds the capsaicin binding spots on TRPV-1, but fails to initiate a signal, acting as a competitive inhibitor. TRPV-1 is a newly discovered receptor, with considerable evidence already having been accumulated to support its roles in pain perception for acidic and high temperature conditions (Brandt et al., 2012). A study using capsazepine was done utilizing a similar mouse model then the one used in this thesis project for studying osteosarcoma and heat tolerance. It was found that capsazepine in the dose range of 3-10 mk/kg was successful in abolishing temperature derived behaviours relating to pain, but failed respond following complete Freund's adjuvant (CFA), suggesting it plays little role in inflammatory triggered nociception (Menéndez et al., 2006). Capsazepine has also been shown to reduce mechanical hyperalgesia, carageenan-induced thermal hyperalgesia, and achieve up to 80% reduction in withdrawal behaviour from sciatic nerve ligation in guinea pigs (Walker et al., 2002). Prior to implantation in animals, *in vitro* experiments involving capsazepine were done using doses ranging from 0-200µM and testing cancer glutamate release was established (Appendix 2; Appendix 3). The final concentrations to be used in the animal model were then decided at 5 mg/kg and 10 mg/kg.

Both doses of capsazepine were delivered intraperitoneally via Alzet mini-osmotic pumps. This allowed for stable drug delivery without constant surgical interference which would skew behavioural results. Although these pumps, once inserted into the animal models, cannot be checked for consistent drug release these Alzet mini-osmotic pumps have been used *in vitro* and *in vivo* previously in our lab and were found to be exceptionally reliable in timed drug release studies done over several weeks for animal models of cancer (Foebel & Singh, 2006).

The animal model of cancer-induced bone pain was developed to accurately mimic human metastatic breast cancer to the bone, allowing for limb isolation to permit the use of behavioural observations. Using the DPA we observed a significant increase in mechanical hyperalgesia, as evidenced by a decrease in the force required to trigger paw withdrawal (Figure 3.2). Both doses of capsazepine were associated with a return of this behaviour towards their baseline scores. This test however has limitations, the main one being that it is manually aimed at the plantar surface of the right hind limb, allowing for inconsistent striking locations which may lead to variable data. Another factor that may have played a role is a training effect. This test was repeated several times each experimental day which could help develop changes in withdrawal times and forces, although this would hypothetically affect all groups. The fact that no change was seen in the groups with the sham injection argues against a training or learning effect.

The DWB system also showed great success in differentiating capsazepine treated mice from vehicle treated mice in both measures of weight distribution and time spent on the cancer-

injected limb (Figure 3.3; 3.4). The DWB test has some advantages over the DPA in that there is a reduction of human interaction and a greater standardization of the testing process (which results in a decrease in human error and subjectivity). The manual validation stage however, was done by a single operator which allows for some degree of subjectivity not seen in other behavioural tests. All tests were done by a single operator in order to ensure that all methods and measurements were repeatable. The weight distribution measurement is a method of validating mechanical pain in mice. This test indirectly measures both weight-bearing and spontaneous breakthrough pain providing a behaviour-related to pain in the mice. This test has a high sensitivity, with for large variations in between test results from the same mouse. The value of this measurement is the low human interaction with the mice, allowing for a closer analogue to the natural condition as well as the sensitivity to detect pain-related behaviour earlier then with the DPA. Measurements taken for the time spent on each limb were also found to be a useful parameter when measuring change in behaviour. This test quantified the time during which the mouse raised its right rear limb, a movement uncommon in normal mice, which allowed for a selective measure of nociception with little deviation in the sham injection animals. This test proved to be the most reliable parameter differentiating the vehicle group from both capsazepine doses. The high selectivity and low sensitivity of this test proved to be its main limitation. When early detection of pain-related behaviours were observed other tests, the same mice would be see as healthy if only looking at the time spent on limb.

The serum analysis allowed for the behavioural results to be associated with glutamate concentrations. Blood analysis from previous experiments had linked the increase of glutamate

in the serum to cancer (Figure 3.1), which was followed up in the capsazepine study. Serum analysis shows that capsazepine was able to lower the circulating glutamate. This was the hypothesized result considering that xenograft mice had higher serum glutamate concentrations and that capsazepine inhibited cancer-released glutamate. The surprising result however was that both doses of capsazepine equally abolished raises in serum glutamate, suggesting the differences in pain scores between the two tests may come from another player in cancer-induced bone pain, possibly TRPV1.

The use of TRPV1 inhibitors, such as capsazepine, is one of the fastest growing branches of analgesic study in the last decade, with a widely accepted rationale for the development of TRPV1 antagonists for the treatment of various inflammatory pain conditions (Brandt et al., 2012). However, the role of TRPV1 antagonists for chronic pain states, where conditions of tactile, mechanical and spontaneous pain mainly predominate, is less clear. This thesis aims to support the idea that glutamate inhibition and TRPV1 antagonists can come together to provide a multi-pronged approach to chronic pain.

Conclusion

The glutamate release in cancer cells is thought to be a consequence of their highly metabolic states. Relying on system x_c^- for cystine uptake, some breast cancer cell lines have been known to release increased levels of glutamate when compared to standard mammary epithelial cells. Excess glutamate in the local environment manifests as disruption of normal bone remodeling and activation of surrounding sensory neurons, creating pain. The experiments conducted were to assess the ability of glutamate inhibition, via capsazepine, to attenuate the behavioural responses associated with pain in a mouse model. The treatment of bone pain has been approached using a variety of methods with little success. The hope is that these experiments offer an alternate pathway for pain management, treating the source of pain rather than coving up pain transmission, as most common analgesics do.

The results of the experiments imply that glutamate is a direct cause of pain. Blood levels of glutamate were raised due to glutamate releasing MDAMB-231 cancer cells injected into the bone. This effect was shown to be attenuated with capsazepine in a non-dose dependant fashion. Capsazepine also demonstrated a dose-dependent attenuation of bone pain. This approach to treating cancer pain could be a novel path to relieving pain with limited side effects and increased effectiveness.

Future Directions

Several future directions are suggested: including glutamate, xCT expression, TRPV1, and nerve growth factors. Starting with glutamate, experiments could be done to determine if increasing glutamate concentrations would onset of pain-related behaviours earlier than that of control mice. The behavioural experiments could be continued with varying concentrations of glutamate in the Alzet pumps to see if giving more glutamate would have the opposing effects on pain. Other inhibitors of glutamate such as SSZ and (S)-4-CPG have had little success, but the concentrations of these compounds used were far below that of cytotoxic and increased concentrations may yield greater results.

It would be valuable to measure xCT expression in patient derived tumour samples. Functional system x_C^- has been confirmed in cultured cells immediately derived from patient tumours (Collins et al., 1998), although cultured cells have been known to upregulate xCT expression and testing if this was an artifact of culture should be tested completely.

Further advances in TRPV1 research and pain are another area which, although highly explored, still leaves much to be discovered. Increasingly selective TRPV1 inhibitors have recently been introduced which could provide interesting future directions. Combined studies of (S)-4-CPG and one of these inhibitors may provide a way to obtain stronger analgesic results for both glutamate and TRPV1 inhibition in cancer-induced bone pain.

Furthermore, our lab has been increasingly interested in NGF and BDNF, two nerve growth factors found to be vital to neurogenesis and present in the tumour microenvironment. Staining for these molecules using IHC and other methods of testing cancer cell secretions of these molecules could reveal important information about how tumour cells change their surrounding microenvironment. We are currently seeking information as to the correlation of glutamate with these factors.

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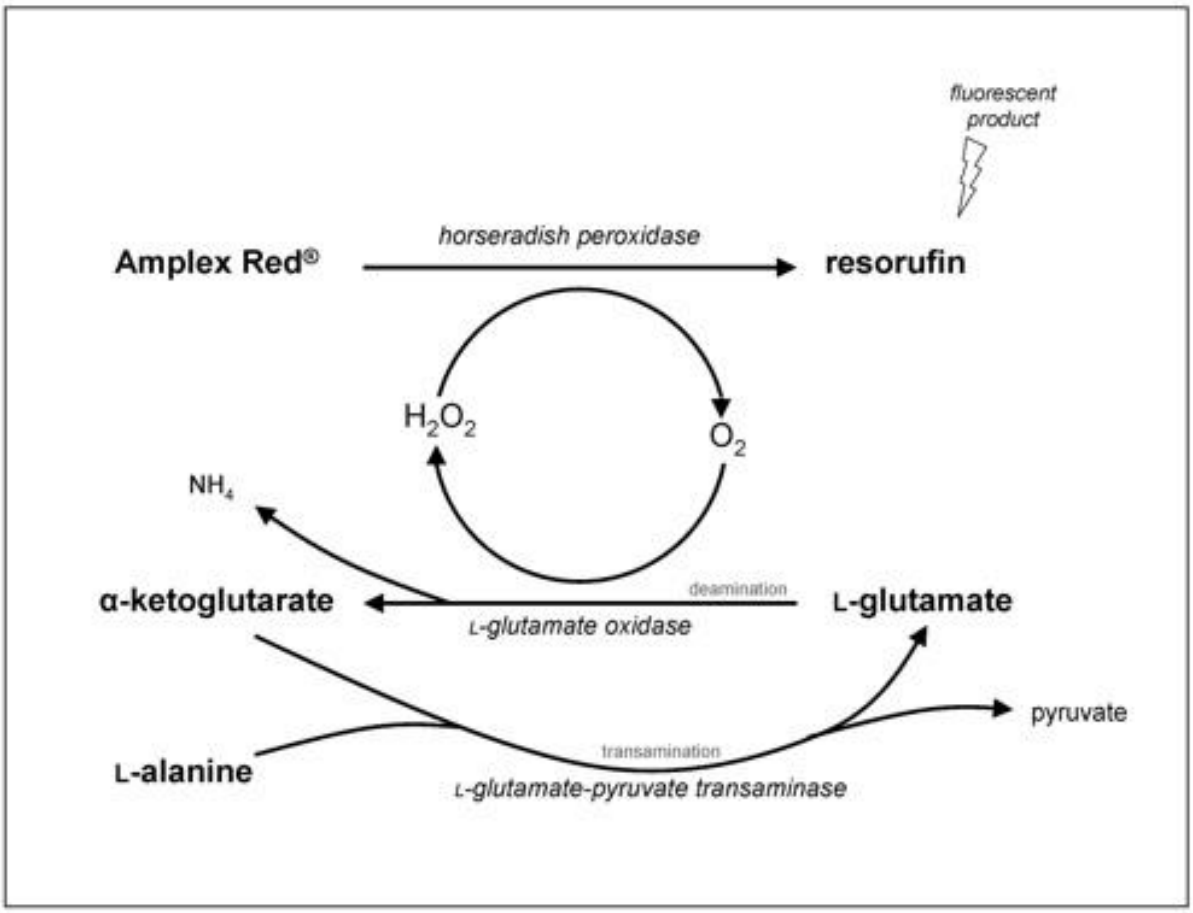
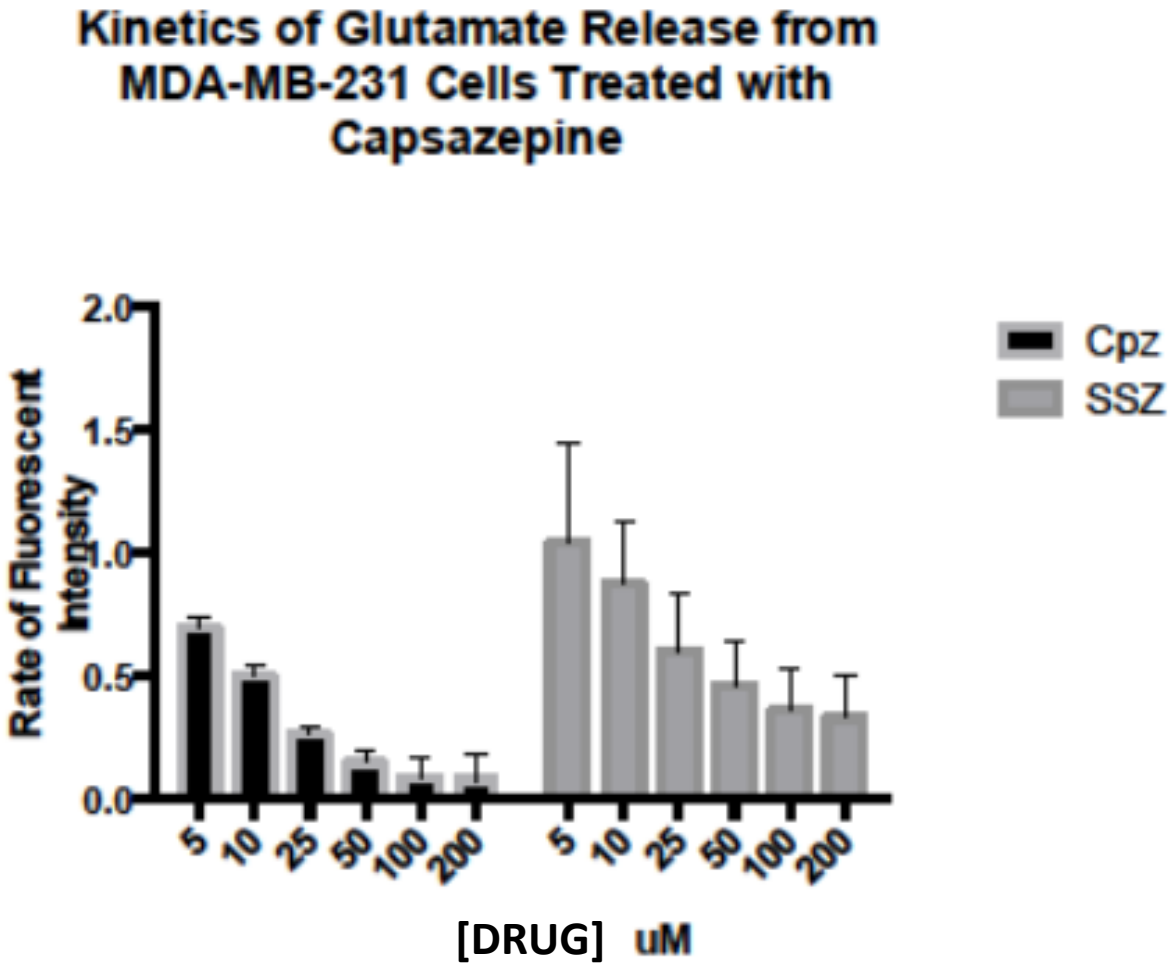
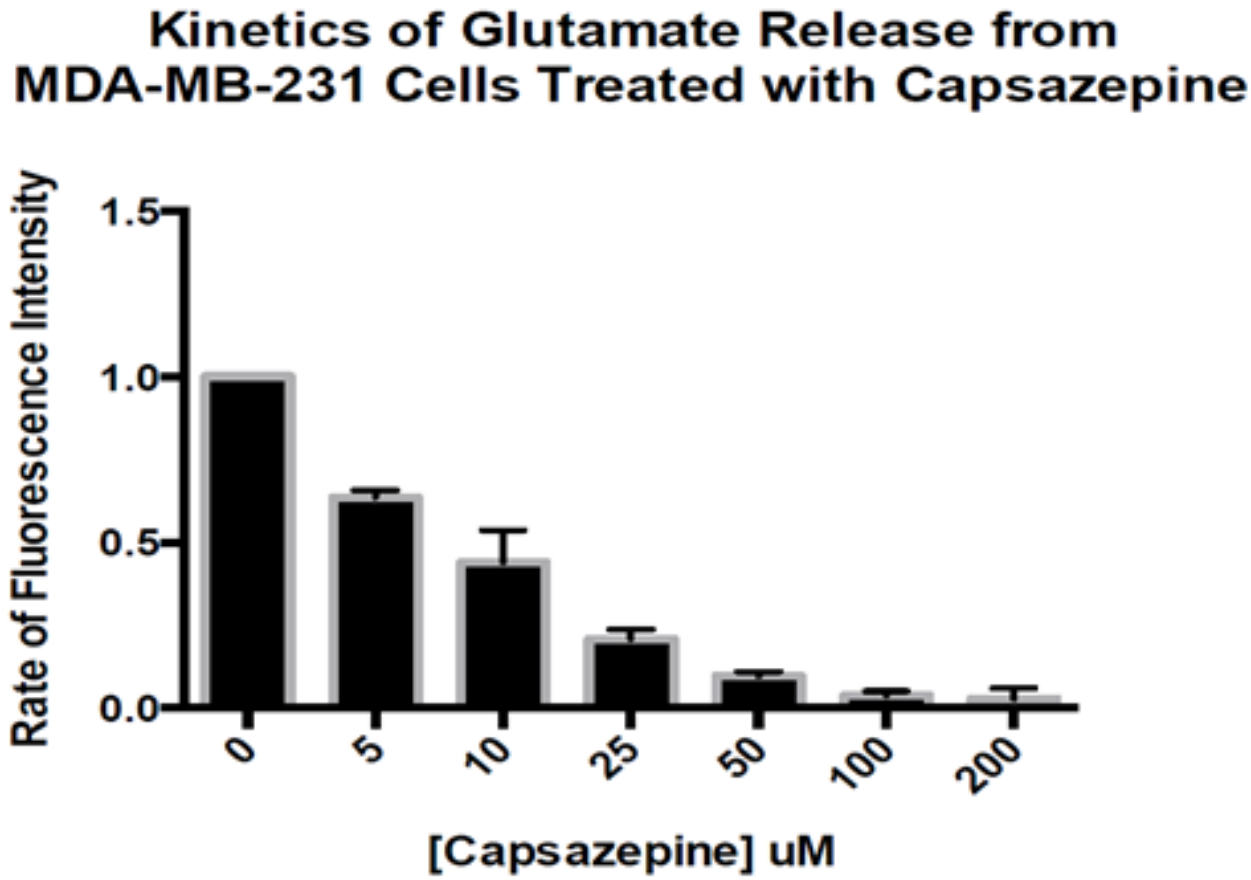
Appendix



APPENDIX 1: AMPLEX Red assay reaction. Reaction concept map outlining the conversion of L-glutamate to resorufin, a fluorescent compound measurable by the microplate reader.



APPENDIX 2: Rate of Reaction for Sulfasalazine & Capsazepine Dose-Response. Used to show that capsazepine, found the in high throughput screen, was better than sulfasalazine *in vitro* at inhibiting cancer released glutamate. This data was collected and graphed by Jennifer Fazzari.



APPENDIX 3: Dose-Dependent attenuation of cancer released glutamate as compared to non-treated cells (0uM). This displays a rapid decline in glutamate production with doses up to 200uM of capsazepine. Dosages used for animal models estimate at 70uM (5 mg/kg) and 140uM (10mg/kg). This data was collected and graphed by Jennifer Fazzari.