

## REVIEW ARTICLE

# Tumour-Derived Glutamate: Linking Aberrant Cancer Cell Metabolism to Peripheral Sensory Pain Pathways

Jennifer Fazzari, Katja Linher-Melville and Gurmit Singh\*

Department of Pathology and Molecular Medicine; Michael G. DeGroot Institute for Pain Research and Care, McMaster University, Hamilton, ON Canada

**Abstract: Background:** Chronic pain is a major symptom that develops in cancer patients, most commonly emerging during advanced stages of the disease. The nature of cancer-induced pain is complex, and the efficacy of current therapeutic interventions is restricted by the dose-limiting side-effects that accompany common centrally targeted analgesics.

**Methods:** This review focuses on how up-regulated glutamate production and export by the tumour converge at peripheral afferent nerve terminals to transmit nociceptive signals through the transient receptor cation channel, TRPV1, thereby initiating central sensitization in response to peripheral disease-mediated stimuli.

**Results:** Cancer cells undergo numerous metabolic changes that include increased glutamine catabolism and over-expression of enzymes involved in glutaminolysis, including glutaminase. This mitochondrial enzyme mediates glutaminolysis, producing large pools of intracellular glutamate. Up-regulation of the plasma membrane cystine/glutamate antiporter, system  $x_c^-$ , promotes aberrant glutamate release from cancer cells. Increased levels of extracellular glutamate have been associated with the progression of cancer-induced pain and we discuss how this can be mediated by activation of TRPV1.

**Conclusion:** With a growing population of patients receiving inadequate treatment for intractable pain, new targets need to be considered to better address this largely unmet clinical need for improving their quality of life. A better understanding of the mechanisms that underlie the unique qualities of cancer pain will help to identify novel targets that are able to limit the initiation of pain from a peripheral source—the tumour.

**Keywords:** Cancer pain, glutamate, glutaminase, system  $x_c^-$ , TRPV1.

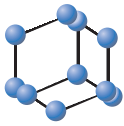
## INTRODUCTION

The central nervous system (CNS) senses diverse endogenous and environmental stimuli, transmitting responding signals to the brain for processing. Particularly intense stimuli have the potential to elicit acute pain, and recurring injury or tissue damage enhance both peripheral and central components that contribute to the transmission of pain signals, leading to hypersensitivity. Physiological initiation of protective responses, although beneficial, may lead to chronic pain when these changes persist. Within the peripheral nervous system, the dorsal root ganglia (DRG) are comprised of somatic sensory neurons that act as mechanoreceptors, nociceptors, pruriceptors, and thermoreceptors [1, 2]. The majority of these DRG neurons are excitatory and glutamatergic, releasing glutamate, one of the most abundant neurotransmitters, onto postsynaptic neurons in the dorsal horn [3-5]. A subset of DRG neurons also release neuropeptides

[6] such as substance P and calcitonin gene-related peptide (CGRP) [1, 4], among others. Glutamate also acts as a peripheral signalling molecule, with its receptors present in the spleen, pancreas, lung, heart, liver, and other organs of the digestive and reproductive systems (reviewed in [7]), as well as the bone microenvironment, where both osteoblasts and osteoclasts release glutamate [8, 9] and in turn respond to extracellular glutamate [10].

Aberrant glutamatergic signalling has been associated with various peripheral diseases, including cancer. As an example, breast cancer cells secrete significant levels of glutamate through the heterodimeric amino acid transporter, system  $x_c^-$  [11, 12], as a consequence of altered glutamine metabolism and changes in cellular redox balance. These cells frequently metastasize to bone [13], where excess glutamate can contribute to bone pathologies [14]. In the restricted bone microenvironment, glutamate acts as a paracrine mediator to coordinate intracellular communication, with even small changes in its levels significantly impacting the skeleton [15]. In addition, the periosteum, bone marrow, and, to a lesser extent, mineralized bone, are innervated by sensory and sympathetic nerve fibres [16]. Notably, these

\*Address correspondence to this author at the Department of Pathology and Molecular Medicine; Michael G. DeGroot Institute for Pain Research and Care, McMaster University, Hamilton, ON Canada; Tel: (905) 525-9140 x28144; E-mail: singhg@mcmaster.ca



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peripheral fibres express functional glutamate receptors and therefore actively respond to this ligand outside of the CNS [17-22].

The majority of breast cancer patients present with bone metastases, which are associated with severe, chronic, and often untreatable bone pain that significantly diminishes a patient's quality of life [23]. Due to increased early detection and an expanding repertoire of clinically available treatment options, cancer deaths have decreased by 42% since peaking in 1986, although research is ongoing to identify tailored small molecules that target the growth and survival of specific cancer subtypes. Overall improvements in cancer management strategies have contributed to a significant proportion of patients living with cancer-induced morbidities including chronic pain, which has remained largely unaddressed. Available interventions such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids provide only limited analgesic relief, and are accompanied by significant side-effects that further affect patients' overall quality of life [24].

Research is therefore focused on developing new strategies to better manage cancer-induced pain. Our laboratory recently conducted a high-throughput screen, identifying potential small molecule inhibitors of glutamate release from triple-negative breast cancer cells [25]. Efforts are underway to characterize the mode of action of a set of promising candidate molecules that demonstrate optimum inhibition of increased levels of extracellular glutamate derived from these cells. While potentially targeting the system  $x_c^-$  cystine/glutamate antiporter, the compounds that inhibit glutamate release from cancer cells do not definitively implicate this transporter, and may instead act through other mechanisms related to glutamine metabolism and calcium ( $Ca^{2+}$ ) signalling. Alternate targets include the potential inhibition of glutaminase (GA) activity or the transient receptor potential cation channel, subfamily V, member 1 (TRPV1). The benefit of blocking glutamate release from cancer cells, irrespective of the underlying mechanism(s), is to alleviate cancer-induced bone pain, potentially expanding the clinical application of "anti-cancer" small molecule inhibitors as analgesics. Furthermore, investigating these targets may reveal how tumour-derived glutamate propagates stimuli that elicit pain. The following review discusses 1. how dysregulated peripheral glutamate release from cancer cells may contribute to the processing of sensory information related to pain, and 2. methods of blocking peripheral glutamate release and signalling to alleviate pain symptoms.

## GLUTAMATE PRODUCTION IN THE TUMOUR: THE ROLE OF GLUTAMINASE (GA)

GA, also referred to as phosphate-activated GA, L-glutaminase, and glutamine aminohydrolase, is a mitochondrial enzyme that catalyzes the hydrolytic conversion of glutamine into glutamate, with the formation of ammonia ( $NH_3$ ) [26] (Fig. 1A). Glutamate dehydrogenase subsequently converts glutamate into  $\alpha$ -ketoglutarate, which is further metabolized in the tricarboxylic acid (TCA) cycle to produce adenosine triphosphate (ATP) and essential cellular building blocks. Glutamate also serves as one of the

precursors for glutathione (GSH) synthesis. It is thought that  $NH_3$  diffuses from the mitochondria out of the cell, or is utilized to produce carbamoyl phosphate [27]. The enzymatic activity of GA serves to maintain normal tissue homeostasis, also contributing to the Warburg effect [28] by facilitating the "addiction" of cancer cells to glutamine as an alternative energy source [29]. The action of GA in a cancer cell is outlined in Fig. (1B).

## Structure and Expression Profile of GA

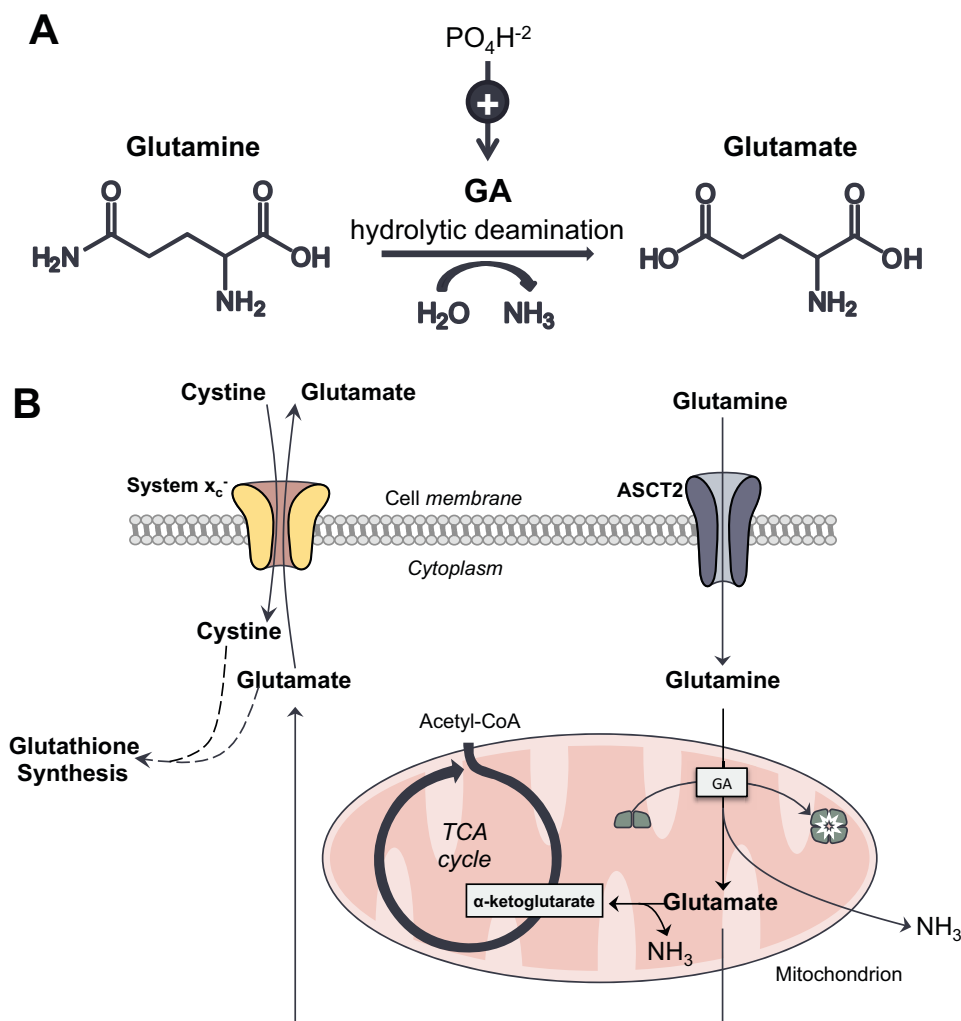
There are currently four structurally unique human isoforms of GA. The glutaminase 1 gene (*GLS1*) encodes two differentially spliced variants of "kidney-type", with *GLS2* encoding two variants of "liver-type" [29, 30] that arise due to alternative transcription initiation and the use of an alternate promoter [31]. The "kidney-type" GAs differ primarily in their C-terminal regions, with the longer isoform referred to as KGA and the shorter as glutaminase C (GAC) [32], collectively called GLS [33]. The two isoforms of "liver-type" GA include a long form, glutaminase B (GAB) [34], and short form, LGA, with the latter containing a domain in its C-terminus that mediates its association with proteins containing a PDZ domain [35]. The GA isoforms have unique kinetic properties and are expressed in distinct tissues [36]. Table 1 provides a summary of the various GA isoenzymes.

A tissue distribution profile of human GA expression revealed that *GLS2* is primarily present in the liver, also being detected in the brain, pancreas, and breast cancer cells [37]. Both *GLS1* transcripts (KGA and GAC) are expressed in the kidney, brain, heart, lung, pancreas, placenta, and breast cancer cells [32, 38]. GA has also been shown to localize to surface granules in human polymorphonuclear neutrophils [39], and both LGA and KGA proteins are expressed in human myeloid leukemia cells and medullar blood isolated from patients with acute lymphoblastic leukemia [40]. KGA is up-regulated in brain, breast, B cell, cervical, and lung cancers, with its inhibition slowing the proliferation of representative cancer cell lines *in vitro* [41-45], and GAC is also expressed in numerous cancer cell lines [41, 46]. Two or more GA isoforms may be coexpressed in one cell type (reviewed in [29]), suggesting that the mechanisms underlying this enzyme's actions are likely complex. Given that the most significant differences between the GA isoforms map to domains that are important for protein-protein interactions and cellular localization, it is likely that each mediates distinct functions and undergoes differential regulation in a cell type-dependent manner [47].

## The Functions of GA in Normal and Tissues and Disease

### The Kidneys and Liver

In the kidneys, KGA plays a pivotal role in maintaining acid-base balance. As the major circulating amino acid in mammals, glutamine functions as a carrier of non-ionizable ammonia, which, unlike free  $NH_3$ , does not induce alkalosis or neurotoxicity. Ammonia is thereby "safely" carried from peripheral tissues to the kidneys, where KGA hydrolyzes the nitrogen within glutamine, generating glutamate and  $NH_3$ . The latter is secreted as free ammonium ion ( $NH_4^+$ ) in the



**Fig. (1).** A. Glutamine, the major circulating amino acid, undergoes hydrolytic deamidation through the enzymatic action of glutaminase (GA), producing glutamate and ammonia (NH<sub>3</sub>). GA is referred to as phosphate-activated, as the presence of phosphate can up-regulate its activity. B. In cancer cells, glutamine enters the cell through its membrane transporter, ASCT2. It is then metabolized in the mitochondria into glutamate through glutaminolysis, a process mediated by GA, which is converted from an inactive dimer into an active tetramer. Glutamate is subsequently transformed into α-ketoglutarate, which is further metabolized through the TCA cycle to produce pyruvate and NADPH, key cellular energy sources. The high rate of glutamine metabolism leads to excess levels of intracellular glutamate. At the plasma membrane, system x<sub>c</sub><sup>-</sup> transports glutamate out of the cell while importing cystine, which is required for glutathione synthesis to maintain redox balance. NH<sub>3</sub>, a significant by-product of glutaminolysis, diffuses from the cell.

**Table 1. Glutaminase isoenzymes.**

GA							
“Kidney-Type”				“Liver-Type”			
Short Form		Long Form		Short Form Gene		Long Form	
Gene	Protein	Gene	Protein	Gene	Protein	Gene	Protein
<i>GLS1</i>	GAC	<i>GLS1</i>	KGA	<i>GLS2</i>	LGA	<i>GLS2</i>	GAB

urine, thereby maintaining normal pH by reducing hydrogen ion (H<sup>+</sup>) concentrations. The liver scavenges NH<sub>3</sub>, incorporating it into urea as a means of clearing nitrogen waste. LGA localizes to distinct subpopulations of hepatocytes [30] and contributes to the urea cycle. During the onset of acidosis,

the body diverts glutamine from the liver to the kidneys, where KGA catalyzes the generation of glutamate and NH<sub>3</sub>, with glutamate catabolism releasing additional NH<sub>3</sub> during the formation of α-ketoglutarate. These pools of NH<sub>3</sub> are then ionized to NH<sub>4</sub><sup>+</sup> for excretion.

### The Central Nervous System (CNS)

In the CNS, the metabolism of glutamine, glutamate, and  $\text{NH}_3$  is closely regulated by the interaction between neurons, surrounding protective glial cells (astrocytes), and cerebral blood flow. This controlled metabolism, referred to as the glutamate-glutamine cycle, is essential for maintaining proper glutamate levels in the brain, with GA driving its synthesis [35]. The localization of GA to spinal and sensory neurons indicates that it also serves as a marker for glutamate neurotransmission in the CNS [48]. GA is active in the presynaptic terminals of CNS neurons, where it functions to convert astrocyte-derived glutamine into glutamate, which is then loaded into synaptic vesicles and released into the synapse. Glutamate subsequently undergoes rapid re-uptake by local astrocytes, which recycle it into glutamine, restarting the cycle. As a major neurotoxin,  $\text{NH}_3$  also factors into this process. Disorders resulting from elevated levels of circulating  $\text{NH}_3$ , such as urea cycle disorders and liver dysfunction, can adversely affect the CNS and, in severe instances, cause death. The primary negative effects of hyperammonemia within the CNS are disruptions in astrocyte metabolism and neurotoxicity. Circulating  $\text{NH}_3$  that enters the brain reacts with glutamate through the activity of glutamine synthetase to form glutamine, and changes in this process can significantly alter glutamate levels in synaptic neurons, leading to pain and disease [49].

### Cancer

The main functions of glutamine are storing nitrogen in the muscle and trafficking it through the circulation to different tissues [50, 51]. While mammals are able to synthesize glutamine, its supply may be surpassed by cellular demand during the onset and progression of disease, or in rapidly proliferating cells. Glutamine is utilized in metabolic reactions that require either its  $\gamma$ -nitrogen (for nucleotide and hexosamine synthesis) or its  $\alpha$ -nitrogen/carbon skeleton, with glutamate acting as its intermediary metabolite. Although cancer cells generally have considerable intracellular glutamate reserves, adequate maintenance of these pools requires continuous metabolism of glutamine into glutamate. The GA-mediated conversion of glutamine into glutamate has been correlated with tumour growth rates *in vivo* [52, 53]. By limiting GA activity, the proliferation of cancer cells decreases, and growth rates of xenografts have been shown to be reduced [54, 55]. Human melanomas exhibit significantly higher GA activity compared to surrounding non-cancerous patient-matched skin [56]. In addition, the expression and activity of GA are up-regulated in various tumour types and cancer cell lines. While glutamine may contribute to cellular metabolism through other mechanisms, the activity of GA is essential for altered metabolic processes that support the rapid proliferation characteristic of cancer cells. Several cellular pathways related to amino acid synthesis, the TCA cycle, and redox balance are supported by glutamine-based metabolism through its intermediary, glutamate (Fig. 1B), and metabolites derived from glutamate are directly relevant to tumour growth. These include nucleotide and hexosamine biosynthesis, glycosylation reactions, synthesis of nonessential amino acids, antioxidant synthesis (*via* GSH), production of respiratory substrates and

reducing equivalents, and ammoniogenesis (reviewed in [57]).

### Relevance of GA in Other Diseases

In addition to the up-regulation of KGA and GAC in various cancers, which contributes to an altered metabolic state associated with a more aggressive cancer phenotype, GA also contributes to other diseases, some of which are associated with pain. During chronic acidosis, *GLS1* expression is up-regulated in the kidneys, and it has been observed that in cultured renal epithelial cells, KGA mRNA levels increase significantly as a means to counter pH changes [58]. Active lesions in multiple sclerosis (MS) express higher than normal levels of GA in macrophages and microglia that closely localize to dystrophic axons [59]. Hyperammonemia within the brain, a typical secondary complication of primary liver disease known as hepatic encephalopathy, affects glutamate/glutamine cycling [60]. Intestinal GA may play a possible role in the pathogenesis of hepatic encephalopathy and has been suggested as a target for novel therapeutic interventions [61]. In hippocampal samples collected from patients with Alzheimer's disease (AD), the number of pyramidal glutamate- and GA-positive neurons are reduced, with remaining neurons displaying shortened, irregular dendritic fields that are consistent with neurofibrillary tangles typically associated with AD [62]. Post-mortem studies of AD patients have indicated loss of GA activity coupled with reduced glutamate levels and a lower number of pyramidal cell perikarya, which are generally correlated with the severity of dementia [63]. Cortical GA has also been linked with AD [64]. In addition, the activity of GA is lower in other neurologically-linked pathological conditions, including Huntington's disease [65].

### GA and Pain

Upon injection into human skin or muscle, glutamate causes acute pain, and painful conditions such as arthritis, myalgia, and tendonitis (reviewed in [66]), as well as MS, are associated with increased glutamate levels in affected tissues. Human chronic pain has been studied using animal models and through the injection of inflammatory agents such as complete Freund's adjuvant [67]. During inflammation, various neurotransmitters, including glutamate, as well as stimuli such as ATP, cations such as hydrogen ions ( $\text{H}^+$ ), and prostaglandins, sensitize afferent primary neurons by lowering their activation threshold, increasing spontaneous neural activity, and increasing and/or prolonging neural firing [66]. One mechanism by which sensory neurons alter their responses to inflammation, noxious stimulation, or tissue damage is to increase the expression and availability of neurotransmitters. Indeed, the levels of glutamate are higher in inflamed tissues, and during inflammation, glutamate sensitizes the axons of primary afferent neurons by decreasing their firing threshold and inducing a hyperexcitable state [68]. The primary afferent neuron may act as a significant possible source of glutamate, and in both humans and animal models, antagonism of glutamate receptors that are expressed on axons of primary afferent neurons during inflammation lessens pain [66]. It has been shown that the peripheral inhibition of GA using 6-diazo-5-oxo-L-norleucine (DON) relieves inflammatory pain, which

is supported by work in rats demonstrating that GA itself may act as a peripheral inflammatory mediator [69]. Inflammation also up-regulates the expression of substance P and CGRP in the DRG [70, 71] and the spinal dorsal horn [72], as well as in the joints and skin [73, 74], with these changes providing a marker of pain-sensing neurons. Neurons that release substance P and CGRP are also glutamatergic [75, 76] and produce glutamate through enhanced GA activity [66, 77]. However, how chronic glutamate production is regulated in pain models remains understudied.

It is known that in response to noxious stimuli, acute glutamate release from primary afferent terminals [78-81], occurring concomitant with the release of substance P and CGRP, drives spinal neuron sensitization, which has been associated with chronic changes [82]. Induced inflammation in the simian knee joint increases fibers in the spinal cord that are immunoreactive for glutamate by approximately 30% at 4 hours and 40% at 8 hours, consistent with a sustained effect [83]. Indeed, in rat spinal cords, extracellular glutamate levels are 150% higher than controls at 24 hours [80], further supporting that glutamate release from central primary afferent neurons is prolonged and activity-dependent during inflammation. These findings indicate that the production and release of glutamate are altered in response to pain, most likely due to modified flux control and local changes in the GA-mediated glutamate-glutamine cycle [84]. In support of this latter notion, persistent inflammation, which was experimentally induced by complete Freund's adjuvant in a rat model of arthritis, was shown to increase GA expression and enzymatic activity in DRG neurons [85]. It was hypothesized that elevated GA in primary sensory neurons could increase the production of glutamate in spinal primary afferent terminals, thereby either directly contributing to central or peripheral sensitization [85]. In an animal model of MS, GA was found to be highly expressed and correlated with axonal damage in macrophages and microglial cells associated with active lesions [59]. A comparison of white matter from various inflammatory neurologic diseases, including MS, with non-inflammatory conditions revealed high GA reactivity only during inflammation [59]. It is likely that dysregulated glutamate homeostasis contributes to axonal dystrophy in MS, and that manipulating the imbalanced glutamate-glutamine cycle may be of therapeutic relevance. GA, as an important regulator of glutamate production, could therefore be targeted for the development of novel therapeutics aimed at treating pain, including cancer-induced pain.

### The Regulation of GA

GA activity is regulated through several mechanisms. *In vitro*, the enzyme may be stimulated by adding inorganic phosphate, and it is therefore often referred to as phosphate-activated (Fig. 1A). While exposure to low phosphate levels activates LGA, a response that is not inhibited by glutamate, KGA activity is dependent on high levels of phosphate and can be inhibited by glutamate [36]. In particular, GAC transitions from a dimer to an active tetramer *in vitro* following the addition of 50 to 100 mM of inorganic phosphate [36, 86].

The conditions above suggest that LGA and KGA are differentially regulated. One activator of GLS2/LGA is

adenosine diphosphate (ADP), which lowers the enzymatic  $K_m$ , with the opposite effect occurring in the presence of ATP, and both effects dependent on mitochondrial integrity [87]. GLS2 is linked with increased metabolism, decreased levels of intracellular reactive oxygen species (ROS), and decreased DNA oxidation in both normal and stressed cells. It has been suggested that the control of ROS levels by GLS2 is mediated by p53 as a means of protecting cells from DNA damage, also supporting cell survival in response to genotoxic stress [27]. Depending on the cell type, as well as the level and type of stress, the extent of GLS2 transcriptional up-regulation by p53 differs in normal and cancer cells [27].

### Positive Regulators

Relative to healthy tissue, the levels of GLS protein are increased in breast tumours [41]. In particular, increased GAC levels have been associated with a higher grade of invasive ductal breast carcinoma [33]. The oncogene c-Myc positively affects glutamine metabolism, as its up-regulation is sufficient to drive mitochondrial glutaminolysis [88, 89]. Of the two GLS isoforms, mitochondrial GAC is stimulated by c-Myc in transformed fibroblasts and breast cancer cells [41]. c-Myc also indirectly influences GLS expression through its action on microRNA (miR) 23a and 23b [54]. Under normal conditions, miR23a and b bind to the 3' untranslated region of GLS transcripts, thereby preventing translation. c-Myc transcriptionally suppresses miR-23a/b expression, de-repressing the block on GLS translation and thereby facilitating glutamine metabolism [54]. Interestingly, acting through its p65 subunit, NF- $\kappa$ B also positively regulates GLS expression by inhibiting miR-23a [90]. NF- $\kappa$ B is the common intermediary that modulates GA activation downstream of Rho GTPase signalling [2]. Another protein regulating glutamine metabolism is signal transducer and activator of transcription (STAT) 1, the phosphorylated/activated form of which binds within the GLS1 promoter region, with interferon alpha (IFN $\alpha$ ) -stimulated STAT1 activation up-regulating GLS1 expression [91]. Mitogen-activated protein kinase (MAPK) signaling and changes in GA expression are also linked based on a report demonstrating that KGA binds directly to MEK-ERK [92]. Activation of the MEK-ERK pathway in response to epidermal growth factor (EGF) treatment, or pathway inactivation by the selective MEK1/2 inhibitor U0126, activates or represses KGA activity, respectively, suggesting a phosphorylation-dependent mode of regulation [92]. This latter point is in line with alkaline phosphatase exposure completely blocking basal GAC activity [41].

### Negative Regulators

There are several mechanisms by which GA is negatively regulated. Anaphase-promoting complex/cyclosome (APC/C) associates with cadherin 1 (CDH1), acting as a ubiquitin ligase to down-regulate GA [93]. The APC/C-CDH1 complex targets proteins with either a destruction box (D box; [RH]xxLxx[LIVM]) or KEN box (Lys-Glu-Asn) for ubiquitination, followed by targeted proteosomal degradation. Of the two GLS1 splice variants, only KGA has both boxes in its C terminus [93], making the APC/C-CDH1 pathway a potential target for down-regulating KGA in cancer cells. Another

negative GA regulator is Lon protease, which localizes to the mitochondrial matrix and preferentially targets misfolded or unassembled proteins [94]. Diphenylarsinic acid (DPAAV) rapidly promotes Lon protease-mediated GAC tetramer dissociation and subsequent proteosomal degradation in a human hepatocarcinoma cell line without affecting GAC mRNA levels or translation [94].

### GLUTAMATE RELEASE FROM THE TUMOUR: SYSTEM $X_c^-$

Glutamate release from cancer cells has been associated with over-expression of the system  $x_c^-$  cystine/glutamate antiporter [95, 96], which is up-regulated as an antioxidant defense mechanism to counter high levels of ROS associated with altered glutamine metabolism. The primary role of system  $x_c^-$  in the tumour is to acquire cystine for the intracellular synthesis of GSH [97]. In addition to GSH synthesis within the cell, cystine reduction to cysteine across the plasma membrane also confers antioxidant potential by mitigating extracellular levels of ROS [98]. As an obligatory antiporter, import of cystine through system  $x_c^-$  must be coupled to the release of glutamate. Increased levels of glutamate are ultimately a by-product of the dysregulated, malignancy-associated metabolic changes that promote the rapid growth and continuous survival of cancer cells. This phenomenon has been well documented [99, 100]. System  $x_c^-$  activity may be regulated through several mechanisms, including by glutamate itself [101], as well feedback from changes in cellular redox balance. Its expression at the mRNA level is affected by ROS in MCF-7 human breast cancer cells *via* the KEAP-1/NRF2 pathway [102], nutrient sensing as mediated by ATF4 in human T24 bladder carcinoma cells [103], STAT3 and/or STAT5-mediated signalling in human breast cancer cells [104], and in response to the RNA-binding protein huR in primary mouse astrocytes [105]. We have shown that system  $x_c^-$  contributes to cancer-induced bone pain, as inhibition of glutamate release with sulfasalazine [13] attenuates mechanical allodynia in an animal model [11]. Importantly, glutamate transport through system  $x_c^-$  represents an intermediate mechanism linking the dysregulated production of glutamate at the tumour site with its detrimental extracellular effects (reviewed by [106]), including the glutamate-promoted migration and invasion potential of aggressive cancer cells [107] and increased cancer-induced pain. Having implicated this particular transporter in *in vivo* pain models, the focus of this review is to discuss the possible mechanisms by which excess glutamate initiates nociceptive responses in cancer.

### PERCEPTION OF EXTRACELLULAR GLUTAMATE IN THE PERIPHERY: TRPV1 AND ITS INTERACTION WITH GLUTAMATE RECEPTORS

TRPV1 was first identified based on its response to heat and vanilloids such as capsaicin [108]. It is a gated, non-selective cation channel of the transient receptor potential family composed of identical tetramers comprised of six transmembrane domains [109]. TRPV1 is permeable to  $Ca^{2+}$  and localizes to both spinal nociceptive afferent fibres [110-112] and supraspinal structures where they can also play a role in central sensitization [113, 114], enabling it to

modulate membrane potential and to transduce sensory signals along excitable cells. Cation permeability of TRPV1 is not static and can vary its ionic selectivity based on both the type and concentration of agonist [115]. Therefore, this channel plays a major role in integrating a variety of noxious stimuli [112] with pain perception by initiating and propagating nociceptive signalling cascades along small, unmyelinated primary afferent fibres [108].

### Regulation of TRPV1

TRPV1 is subject to sensitization and desensitization by a diverse range of factors that can both directly and indirectly activate channel activity through recognition and/or phosphorylation sites on TRPV1.

#### Positive Regulators of TRPV1

Commonly described as a thermoreceptor, TRPV1 is physiologically activated at temperatures greater than 43°C. It is also directly gated by protons that initiate signaling at a non-physiological change in pH below 5.9. Endogenous TRPV1 ligands include the fatty acid-like molecule anandamide, as well as N-arachidonoyl dopamine (NADA) and N-oleoyldopamine (OLDA), which are both metabolites of arachidonic acid [116]. Interestingly, diacylglycerol (DAG) can directly activate TRPV1, linking it to G-protein coupled receptor (GPCR) signalling [117]. In this manner, TRPV1 is sensitized by downstream signalling mediators that include phospholipase C (PLC), protein kinase A (PKA), and protein kinase C (PKC). This channel can also be activated by exogenous vanilloids such as capsaicin, the pungent component of chilli peppers, and resiniferatoxin (RTX), a naturally occurring capsaicin analog found in the *Euphorbia* plant [112]. TRPV1 agonists constitute a diverse population of small molecule ligands that have been extensively reviewed [118].

In response to tissue injury and inflammation, endogenous factors are modulated in order to increase the response to pain, whereby pain-transducing factors are up-regulated in sensory nerve endings, heightening their ability to perceive noxious stimuli associated with pathological changes. Translocation of TRPV1 to the cell membrane is essential for its activity and is mediated by a variety of factors, including bradykinin, insulin-like growth factor (IGF-1) [119], and nerve growth factor (NGF) [120]. Ultimately, TRPV1 activation is voltage dependent, relying on membrane depolarization. The specific factors that initiate channel activation also, in part, shift the membrane potential to a voltage that sensitizes the channel to temperature [121]. Therefore, persistent depolarization of neurons would be expected to reduce the threshold for temperature-mediated activation of TRPV1, allowing it to propagate allodynia and hyperalgesia in response to physiological changes in temperature [121].

#### Negative Regulators of TRPV1

Due to its role in pain signalling, TRPV1 is an attractive pharmacological target for the development of analgesics. Capsazepine was the first competitive antagonist developed against TRPV1 [122]. A more potent antagonist was created by modifying the agonist, Resiniferatoxin (RTX), generating

5-iodo-RTX (IRTX), which has a forty times higher affinity for TRPV1 compared to capsaizepine [123]. Interestingly, TRPV1 is susceptible to desensitization by agonists such as capsaicin, where prolonged exposure decreases the receptor's ligand-mediated response, thereby providing long-lasting but reversible analgesia in a complex process reviewed by Touska *et al.* [124]. A heterogeneous population of TRPV1 antagonists and their therapeutic potential have also been comprehensively reviewed [125]. Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) has also been shown to tonically inhibit TRPV1 at the membrane in lieu of PLC activity [126].

### The Function of TRPV1 in Cancer

TRPV1 expression has been documented in colon [127], pancreatic [128], and prostate [129] cancers. Interestingly, the effects of capsaicin vary between cancer cell types, possibly due to off-target effects or the level of channel expression. Also, the role of TRPV1 in cell proliferation varies, which could be due to the degree of Ca<sup>2+</sup> signalling induced by channel activation. For example, it has been shown that capsaicin does not affect the proliferation of TRPV1-expressing MCF-7 breast cancer cells, but does induce apoptosis [130]. The latter effect has recently been associated with a rise in intracellular free Ca<sup>2+</sup> concentrations upon TRPV1 activation [131]. The same anti-tumour activity has been observed in gliomas, in which TRPV1 gene expression is inversely correlated to tumour grade [132]. However, due to the heterogeneity of responses elicited by TRPV1 activation in cancer cells, therapeutically targeting this channel may present a risky strategy, as its inhibition has been reported to promote proliferation in some cancers [133]. Expression levels of TRP family proteins, including TRPV1, can be used as a marker of cancer progression [134]. In addition, TRPV1 expression levels in peripheral cancers have been correlated to pain scores [128], suggesting that channels not directly localizing to afferent nerve terminals may initiate a pain response, possibly by inducing the release of mediators such as glutamate from these terminals [135]. In an osteosarcoma model of bone cancer-induced pain, TRPV1 expression increased in the DRG [136], and TRPV1 antagonists inhibit both central [113] and peripheral [137] nociceptive transmission.

### TRPV1 Activation in Response to Inflammation

TRPV1 levels in DRG and spinal neurons increase in response to inflammation [120] and the presence of tumour-secreted factors [138] through signal transduction pathways that overlap with those engaged by lipopolysaccharide (LPS) [139, 140]. Peripheral inflammation induces the MAPK signalling cascade in nociceptive neurons, which increases both TRPV1 levels in the DRG and the subsequent transfer of these channels to peripheral terminals of nociceptive neurons, thereby promoting hypersensitivity [120]. Initiation of the MAPK cascade lies downstream of Toll-like receptor 4 (TLR4) activation in trigeminal sensory neurons [141]. Cancer cells secrete damage associated molecular patterns (DAMPs) [142-144] which can activate TLR4 receptors on peripheral sensory neurons proximal to tumour. Therefore, the role of TLR4 extends beyond that of the innate immune response and plays a role in non-infectious excitation of

primary sensory neurons (Reviewed in [145]), including sensitization of TRPV1 on sensory nociceptive fibres (Fig. 2) [139]. Furthermore, TLR4/MAPK signalling also induces the release of pro-inflammatory cytokines such as interleukin 1-beta (IL-1 $\beta$ ) and tumour necrosis factor-alpha (TNF- $\alpha$ ) from tumour-infiltrating immune cells, and by cancer cells themselves [146], which can in turn activate/sensitize TRPV1 channels [147, 148]. MAPKs also influence PKA and PKC activity in modulating neuronal excitability [149], which are both known regulators of TRPV1 activity [150-152].

### Role for TRPV1 in Cancer-Induced Pain

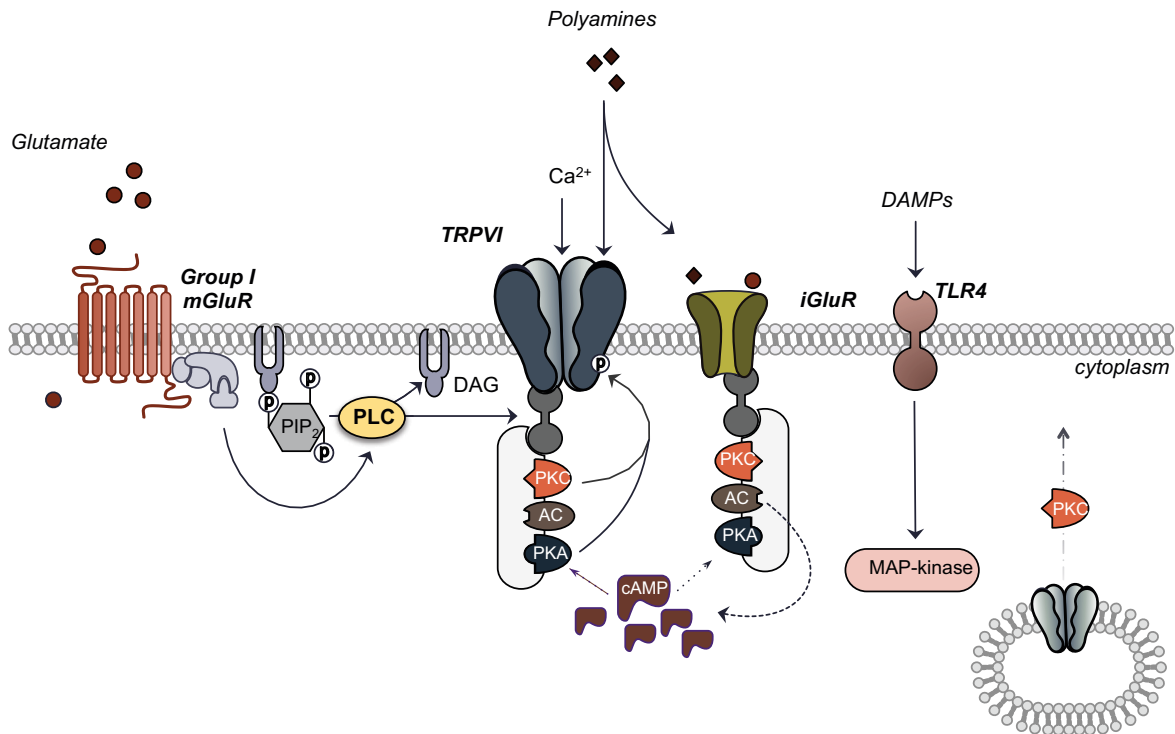
Numerous studies have documented the role of TRPV1 in nociception in diverse tissues, including those composed of non-excitabile cells. The potential role of TRPV1 in the propagation of cancer-induced pain will therefore be discussed with a focus on its peripheral effects and how the channel functions in conjunction with glutamatergic signalling to evoke a nociceptive response from peripheral (tumour-secreted) mediators.

In the periphery, glutamate, a mediator of inflammation and tissue injury, plays a role in physiological nociceptive transmission [153] through both ionotropic [154-156] and metabotropic [157, 158] glutamate receptor activation. Several studies have shown that in both humans [159, 160] and animal models [19, 161, 162], glutamate is released from peripheral terminals of C-fiber neurons, increasing its local concentration. This excitatory amino acid is then able to stimulate neighboring glutamate receptors in an autocrine fashion, promoting not only the development, but also the maintenance and propagation, of pain. Many of these nociceptive responses can be blocked by local, peripheral administration of ionotropic glutamate receptor antagonists [20, 154, 156].

The transmission of sensory information by glutamate and glutamate receptor activation is potentiated by TRPV1 phosphorylation. TRPV1 contains phosphorylation sites on its cytoplasmic N- and C-termini, and its phosphorylation status underlies its ability to respond to noxious stimuli [163]. Extracellular glutamate in the periphery promotes phosphorylation of TRPV1 on the terminals of primary afferents, resulting in channel sensitization. Group I metabotropic glutamate receptors (mGluRs; R1 and R5) are also expressed on the peripheral termini of unmyelinated nociceptive afferents, propagating glutamate-induced hyper- and thermal sensitivity [17]. Activation of group I mGluRs by peripheral glutamate induces DAG production *via* PLC. DAG can then activate TRPV1 directly [117] or through downstream activation of protein kinases [150]. In addition, PKC [151, 164, 165] and PKA [166] have both been shown to phosphorylate and activate TRPV1 activity downstream of glutamate receptor activation. In this manner, increases in local extracellular glutamate levels can initiate a nociceptive response. This nociceptive processing can be amplified by increasing the number of TRPV1 receptors that are available on peripheral afferents.

Interestingly, PKC signalling also initiates TRPV1 translocation from vesicular pools to the plasma membrane of sensory neurons (Fig. 2) [119, 165], enhancing neural





**Fig. (2).** TRPV1 located on peripheral afferent terminals of sensory neurons indirectly responds to increased local levels of extracellular glutamate secreted from the tumour. Glutamate-mediated activation of TRPV1 occurs *via* metabotropic glutamate receptors of the group I class as well as ionotropic glutamate receptors that integrate downstream signalling kinase-mediated signalling cascades. Protein Kinase C (PKC) and Protein Kinase A (PKA) phosphorylate and therefore sensitize and/or activate TRPV1. Activation of these kinases lies downstream of mGluR-coupled phospholipase C (PLC) activation which promotes the association of anchoring kinase association protein 79/150 (AKAP 79/150) to TRPV1 where it localizes kinase activity proximal to the channel. AKAP 79/150 also tethers to iGluRs which can mediate activity of anchored kinases. This scaffold system also includes adenylyl cyclase (AC) which promotes cAMP production aiding in activation of PKA. PKC also mediates the translocation of cytoplasmic TRPV1 to the plasma membrane in response to stimuli. In addition to glutamate, exogenous, tumour-secreted factors initiate TRPV1 activation through iGluR polyamine recognition sites and danger associated molecular pattern-induced toll-like receptor 4 (TLR4) activation.

transmission in response to noxious stimuli, as well as the maintenance of hyperalgesia. Transport of TRPV1 from the dorsal root ganglion to peripheral nerve terminals has also been observed in response to peripheral inflammation *via* retrograde transport of NGF from a peripheral site of inflammation to the DRG. In the DRG, NGF induces sustained MAPK activation, increasing TRPV1 translation and its transport to peripheral terminals [120]. In addition to its signalling in the DRG, NGF also plays a role in sensitizing the peripheral TRPV1 channels, again through a PKC-mediated mechanism [167, 168]. Together, these observations illustrate a mechanism by which peripheral glutamate engages TRPV1 in a nociceptive response and promotes ongoing nociceptive signalling.

Pro-inflammatory agents are also able to activate the TRPV1 channel through second messenger signalling cascades [112] that lead to the development of inflammatory hyperalgesia through PLC activation [169]. Extracellular agonists of TRPV1 increase during inflammation and in response to cancer [170, 171]. In particular, polyamines are often produced during inflammation, and increased pools of these organic cations have also been observed in tumour cells. As by-products of amino acid metabolism, the synthesis and catabolism of polyamines may contribute to

tumourigenesis (reviewed by [172]). Therefore, TRPV1 activation by tumour-derived polyamines provides another potential mechanism that propagates cancer-induced pain signals. Polyamines are able to directly sensitize and activate TRPV1 channels and to induce pain behaviours [170, 173, 174]. The pain responses induced by polyamines can also be mediated indirectly by glutamatergic input independent of substance P [174]. In this case, glutamate mediates polyamine-induced activation of TRPV1 through N-methyl-D-aspartate (NMDA) ionotropic glutamate receptors (iGluR). NMDA receptors are responsible for increased synaptic strength and long-term potentiation of C-fiber synapses [175, 176]. They modulate TRPV1 activity through protein kinase-directed phosphorylation mechanisms (Fig. 2) [177-180]. Similar to mGluR expression, NMDA receptors localize along the length of DRG neurons, including their peripheral processes [18], where they would be proximal to TRPV1 channels. The functional localization of these glutamate receptors on peripheral afferent terminals has been further confirmed by the induction of allodynia and hyperalgesia following peripheral administration of agonists against this class of ionotropic receptor [21].

Scaffolding proteins mediate the interactions between protein kinases and TRPV1 to promote ion channel



phosphorylation. The A kinase anchoring protein 79 (human)/150 (rat) (AKAP 79/150) facilitates the targeting of PKC and PKA to TRPV1, and underlies the NMDA-induced activation of TRPV1 *via* PKC in sensory neurons [177]. AKAP150 (rat) mediates the formation of a signalling complex that initiates nociceptor sensitization [177]. AKAP79/150 also facilitates cyclic AMP (cAMP)-dependent PKA phosphorylation of TRPV1 by coupling this signalling complex to adenylyl cyclase. This increases the local concentration of cAMP around TRPV1 and the co-anchored PKA, enhancing phosphorylation and sensitization of TRPV1 (Fig. 2) [181]. Similarly, peripheral mGluR-induced TRPV1 activation is dependent on AKAP150 [182, 183]. Therefore, formation of organized signalling complexes on AKAP ensures rapid phosphorylation of TRPV1 in response to local signals, including cancer-secreted glutamate, revealing another mechanism by which this neurotransmitter can induce sensitization of peripheral sensory neurons and induce pain behaviours. Interestingly, NMDA receptors contain polyamine recognition sites, which potentiate NMDA activation only in the presence of glutamate and glycine [184]. Taken together, it can be hypothesized that excess tumour-derived glutamate initiates a pain response *via* paracrine activation of glutamate receptors on peripheral afferent terminals of nociceptive fibres, propagating a central pain response *via* subsequent activation of proximal TRPV1 receptors.

When applying these interconnected processes to the bone microenvironment, it becomes evident how these mechanisms are able to play a role in propagating cancer-induced bone pain signals. TRPV1 is expressed on sensory fibres that innervate mineralized bone [185]. This channel can therefore respond to local changes in the bone microenvironment that arise in response to dysregulated glutamate signalling, which is known to occur in the presence of cancer [186-189]. Given the multiple chemical mediators, including those involved in inflammation and bone degradation (acid), as well as tumour-secreted factors (glutamate), TRPV1 may become sensitized and activated under physiological conditions by its endogenous agonists. Indeed, subcutaneous injection of capsaicin has been shown to induce peripheral glutamate release, which could be inhibited by pre-administration of capsazepine, a TRPV1 antagonist [149, 190].

## DEVELOPMENT OF THERAPEUTIC INTERVENTIONS

The role of glutamate-induced nociception in cancer-induced pain implicates several targets that, when blocked, may inhibit the production and release of glutamate from the tumour, also potentially affecting the perception of glutamate as a noxious stimulus by peripheral primary afferent nerve endings.

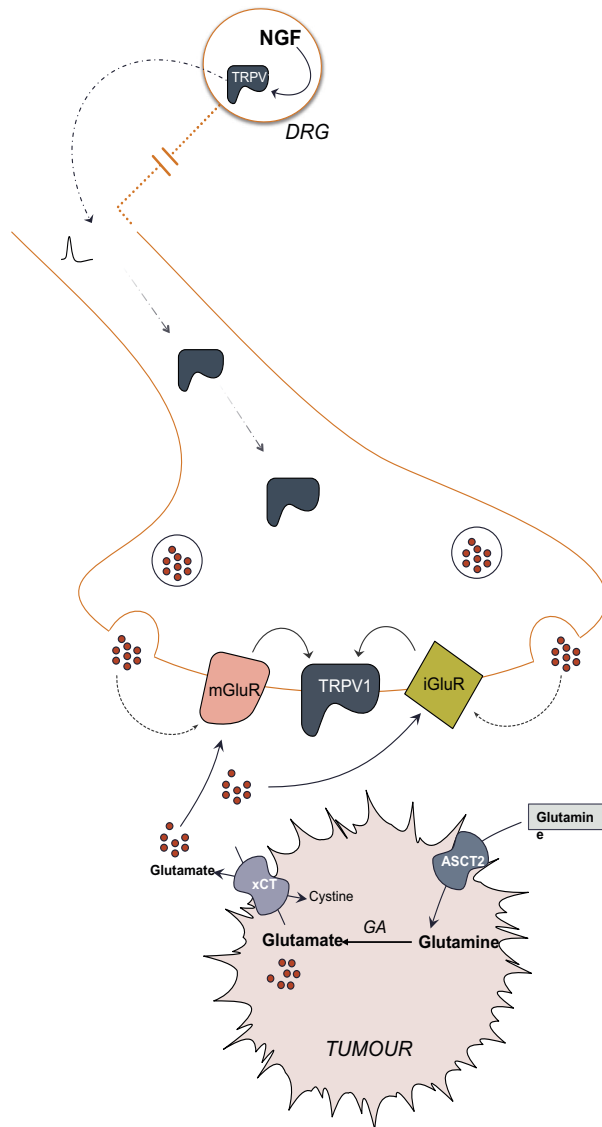
### Inhibition of GA

DON, a non-selective irreversible glutamine-competitive inhibitor with several other targets [191], has been used to effectively block KGA activity [192]. Other glutamine analogs with diazo groups, including azaserine and acivicin, irreversibly bind to the active site of GA, but also affect various other enzymes that utilize glutamine as a substrate,

as well as glutamine transporters [191]. DON and azotomycin showed promising antitumour activity against human cell lines implanted into nude mice, although clinical trials with these small-molecule inhibitors were less efficacious and associated with toxicities [193]. New small molecules have been discovered that inhibit both KGA and its GAC splice variant. Molecule 968, although resisting enzyme co-crystallization, allosterically regulates GAC without competing with glutamine [41, 194]. Its inhibitory potential has been described in various cancer cell lines *in vitro* and in a mouse xenograft model [41], although its hydrophobic nature has made it difficult to apply *in vivo*. The effects of 968 on metabolically sensitive epigenetic markers and their effects on cancer-related genes were also examined. In this context, GA inhibition enhanced histone acetylation at H4 while down-regulating the expression of *AKT* and *ERBB2*, suggesting that 968 could potentially be applied as an effective epigenetic therapeutic agent [195, 196]. In addition, 968 has been used to test whether GA-driven glutamine metabolism has evolved in cancer cells more as a means to control intracellular pH through the release of NH<sub>3</sub> than to provide metabolites to fuel the TCA cycle [43]. Although not in line with established doctrine, this study presents evidence that modulating cellular acidity is an important component of glutamine metabolism. Glutamine withdrawal elicits less drastic effects on the viability of HeLa or MCF-7 cells when their growth media is maintained at a neutral pH 7.3 rather than under acidic conditions (corresponding to pH 6.3), with 968 treatment inhibiting cell proliferation only at the lower pH. However, cell lines resistant to glutamine withdrawal have been shown to regain sensitivity to this amino acid when exposed to glutamine synthetase inhibitors, and glutamine synthetase, through its production of glutamine, consumes NH<sub>3</sub>, thereby potentially acidifying the cellular microenvironment, which were not considered in the study [197]. Nevertheless, these findings present an intriguing secondary consequence of glutamine metabolism in cancer cells, meriting further investigation into acid/base balance.

Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) has emerged as an important allosteric GA inhibitor that specifically targets KGA over LGA. BPTES binds at the interface where two KGA dimers join to form a tetramer, stabilizing a region near its active site and controlling access to its catalytic pocket, thereby inactivating the enzyme [198-201]. Similar to 968, BPTES inhibits KGA activity in various tumour types [44, 45], but, unlike 968, BPTES remains effective even in the presence of inorganic phosphate. BPTES analogs have been designed to improve upon its poor metabolic stability and low aqueous solubility [201]. Other small molecules have been described that inhibit KGA and/or GAC [202], including thiourea molecules designed to function as farnesyl diphosphate mimetics that have been proven to be efficacious against GA activity [203]. However, even the most potent novel compound was less efficacious than 968, BPTES, or DON.

Recently, CB-839, a novel, orally bioavailable inhibitor selective for KGA and GAC, has been developed and characterized, which potently blocks the proliferation of HCC-1806 triple-negative breast cancer cells *in vitro* while also decreasing glutamine catabolism and the levels of



**Fig. (3).** Overview of peripheral nociception induced by tumour-derived glutamate. Dysregulated cancer cell metabolism promotes glutamine uptake by ASCT2 transporter and production of large intracellular glutamate pools that drive the activity of the cystine/glutamate transporter, xCT to accommodate the intracellular demand for cysteine, the limiting reagent in glutathione synthesis. Upregulation of glutaminase (GA) and system x<sub>c</sub><sup>-</sup> increases the extracellular concentration of glutamate that can be perceived by proximal nociceptive terminals that can translate glutamate into a nociceptive signal by integrating the activities of glutamate receptors with TRPV1. These terminals also release glutamate that can act in an autocrine fashion also activating these same glutamate receptors. TRPV1 translocation to these terminals increases in response to peripheral noxious stimuli through the action of NGF in the dorsal root ganglion.

glutamate, GSH, and intermediates of the TCA cycle [204]. Interestingly, in the T47D estrogen receptor-positive breast cancer cell line, no anti-proliferative effect was observed in response to CB-839, although its administration did modestly down-regulate glutamine metabolism and the levels of its

metabolites. A screen of 23 breast cancer cell lines revealed that while expression of LGA, KGA, and GAC could be detected at some level in most cells, GAC protein levels were high, primarily in triple-negative cell lines compared to estrogen-receptor positive cells. In addition, the triple-negative subtype was associated with increased GA activity and was also most sensitive to CB-839 treatment. In two xenograft models, CB-839 mediated significant anti-tumour activity. CB-839 may therefore be a promising novel therapeutic molecule for targeting glutamine-dependent tumours in patients, as well for treating cancer-induced pain or inflammatory pain linked to increased glutamate levels in the CNS, meriting further investigation and clinical testing.

### Inhibition of TRPV1

TRPV1 has emerged as an attractive target for pharmacological intervention in pathological conditions associated with pain, including cancer-induced bone pain [185, 205]. Desensitization of TRPV1 on peripheral afferent terminals renders these termini insensitive to a wide range of agonists that induce nociception through channel activation, including glutamate. TRPV1 antagonism has been an active area of medicinal chemistry, resulting in the synthesis of novel antagonists (reviewed in [206]). Some of these compounds display only modest efficacy in reducing nociceptive behaviours associated with chronic pain, potentially due to the multi-modal nature of TRPV1 sensitization [207]. However, A-425619, AMG 9810, AMG 517, and AMG 8163 display antagonism against heat-, proton- and capsaicin-induced TRPV1 activation, demonstrating enhanced abilities to reduce pain [206]. JNJ-17203212 has been shown to relieve pain symptoms in an osteolytic sarcoma model, specifically implicating TRPV1 antagonism with reduced cancer-induced bone pain [185].

The effectiveness of a potential TRPV1-targeted therapeutic agent for treating pain may vary given the array of stimuli that modulate TRPV1 activity. Targeting TRPV1 also poses the risk of impairing the perception of noxious stimuli to such an extent as to evoke pathological changes in core body temperature and increasing the risk of burn-related injuries [208, 209]. Recently, a study aimed at elucidating the mechanism controlling the physical opening of the TRPV1 channel in response to extracellular stimuli has implicated its hydrophobic interaction with lipid rafts [210]. Novel pharmacological developments could potentially aim to target this particular interaction in an effort to better regulate TRPV1 activity.

### SUMMARY

The uncontrolled proliferation of cancer cells is promoted by significant metabolic adaptations that accommodate an increased demand for energy and metabolic intermediates. This is reflected by GA up-regulation in cancer cells, promoting the production of glutamate, an essential metabolic substrate. With the energetic requirements in place to support rapid growth, cancer cells must be able to clear increased levels of ROS that accompany elevated metabolic rates, which otherwise would impair their survival due to oxidative stress. The need to maintain redox balance is met by up-regulating the system x<sub>c</sub><sup>-</sup> cystine/glutamate antiporter,

which provides cancer cells with a source of cysteine, the rate-limiting substrate of GSH synthesis. In order to produce adequate levels of GSH, high levels of glutamate are exported by system  $x_c^-$  in exchange for cystine across the plasma membrane. The increasing extracellular levels of glutamate may be what ultimately drive the propagation of noxious stimuli through the activity of receptors located on the terminal ends of primary afferent sensory neurons. Metabotropic and ionotropic glutamate receptors relay sensory information through proximal TRPV1 receptors, thereby linking peripheral sensory information to the CNS and initiating a pain response. This hypothesis is depicted in Fig. (3). Together, GA, system  $x_c^-$ , and TRPV1 underlie the pathological role of dysregulated glutamatergic signalling associated with cancer-induced pain. Each acts as a potential therapeutic target to diminish the production of and response to glutamate in the periphery. This offers the potential for developing novel interventions that limit dysregulated glutamatergic signalling and the associated debilitating side-effects related to cancer-induced pain that compromise patient quality of life.

### CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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