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G protein-coupled receptors as oncogenic signals in glioma: emerging therapeutic avenues

Allison E Cherry^a and Nephi Stella^{a,b,c}

^aDepartment of Pharmacology, University of Washington 1959 NE Pacific St BB1538, Health Sciences Building Seattle, WA 98195 United States of America

^bPsychiatry & Behavioral Sciences, University of Washington 1959 NE Pacific St BB1538, Health Sciences Building Seattle, WA 98195 United States of America

Abstract

Gliomas are the most common malignant intracranial tumors. Newly developed targeted therapies for these cancers aim to inhibit oncogenic signals, many of which emanate from receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor (VEGFR). Unfortunately, the first generation treatments targeting these oncogenic signals provide little survival benefit in both mouse xenograft models and human patients. The search for new treatment options has uncovered several G protein-coupled receptor (GPCR) candidates and generated a growing interest in this class of proteins as alternative therapeutic targets for the treatment of various cancers, including GBM. GPCRs constitute a large family of membrane receptors that influence oncogenic pathways through canonical and non-canonical signaling. Accordingly, evidence indicates that GPCRs display a unique ability to crosstalk with receptor tyrosine kinases, making them important molecular components controlling tumorigenesis. This review summarizes the current research on GPCR functionality in gliomas and explores the potential of modulating these receptors to treat this devastating disease.

Keywords

Glioblastoma multiforme; Astrocytoma; G protein-coupled receptor; receptor tyrosine kinase

1. GBM: From histology to driver mutations

Gliomas are a brain neoplasm and account for 80% of primary central nervous system (CNS) tumors diagnosed between 2004 and 2008 in the United States (Central Brain Tumor Registry of the United States [CBTRUS], 2012). The largest subset, comprising well over

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^cCorresponding author nstella@uw.edu Phone: +1-206-221-5220 Address: 1959 NE Pacific St BB1538, Health Sciences Building Seattle, WA 98195 .
allison.cherry@gmail.com; nstella@uw.edu

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50% of gliomas, is the astrocytomas, which is so-named because these tumors share histological features with astrocytes. The World Health Organization (WHO) stratifies astrocytomas into four grades (I-IV) based on several histological criteria: cellularity, nuclear pleomorphism, mitotic activity of tumor and the presence of necrosis and neovasculture. Grade IV astrocytoma, or Glioblastoma Multiforme (GBM), is the most common primary glioma diagnosed in adults and occurs at an incidence of approximately 3 per 100,000 people per year in the United States alone (Central Brain Tumor Registry of the United States [CBTRUS], 2012). The median survival of patients diagnosed with GBM is only 14.2 months when treated with the standard of care, which consists of surgical resection followed by a combination of radiotherapy and the alkylating agent temozolomide (TMZ) (Stupp et al., 2005). With the exception of the addition of TMZ to the standard of care in 2005, which increased median survival of GBM patients by a mere 2 months, very little progress has been made to treat this cancer in decades (Stupp et al., 2005). The 5-year survival rate of GBM patients is only 4.7% owing to the inevitability of recurrence. The eventual resurfacing of this cancer is likely due in part to glioma stem cells (GSCs) within the tumor that resist radiation and chemotherapy (Bao et al., 2006; Liu et al., 2006) and are capable of propagating heterogeneous tumors (Lapidot et al., 1994; Bonnet and Dick, 1997; Reya et al., 2001; Johnson et al., 2014). Thus, the prognosis for patients diagnosed with GBM is dismal, and despite the clinical testing of several novel therapeutic approaches, little progress has been made to help these patients.

1.1. Classification of gliomas

The current classification system that divides gliomas by malignancy has not been effective in estimating prognosis, most notably between grades III and IV (Hartmann et al., 2010). Therefore, The Cancer Genome Atlas (TCGA) research network dedicated a significant amount of effort to stratify gliomas by driver mutations, first with GBM and more recently with lower grade gliomas (Cancer Genome Atlas Research Network, 2008; Guan et al., 2014). The initial genetic subclassification proposed by Verhaak et al. included four GBM subtypes: Classical, Mesenchymal, Proneural, and Neural (Verhaak et al., 2010).

The classical subtype is primarily defined by an amplification of the *epidermal growth factor receptor* (*EGFR*) and deletion of the tumor suppressor protein phosphatase and tensin homolog (*PTEN*) genes. The *EGFR* amplification in this subtype often co-occurs with a mutation that gives rise to a constitutively active receptor (*EGFRvIII*) (Sugawa et al., 1990; Ekstrand et al., 1991; Wong et al., 1992; Aldape et al., 2004). *EGFR* is an oncogenic receptor tyrosine kinase (RTK) that activates both pro-migratory and proliferative pathways, and when amplified or mutated, it plays a role in the development of various neoplasms (Zhang et al., 2007). The *EGFR* amplification, though not strictly defining the other genetic subtypes, is also commonly present in the proneural and neural subtypes, which makes it the most frequent genetic mutation in GBM. Thus, although 27% percent (Verhaak et al., 2010) of these cancers are characterized as the classical subtype, approximately 40% of all diagnosed GBMs contain an *EGFR* amplification, many of which also contain the *EGFRvIII* mutation (Libermann et al., 1985; Wong et al., 1987; Frederick et al., 2000; Aldape et al., 2004).

The mesenchymal and proneural subtypes comprise 29% and 28% of GBMs, respectively (Verhaak et al., 2010). The mesenchymal subtype is characterized by deletion or mutation of the tumor suppressors *neurofibromin 1 (NF1)* and *PTEN*. NF1 is a negative regulator of the oncogenic protein RAS, and PTEN antagonizes phosphoinositol-3-kinase (PI3K), which are two important signaling molecules downstream of RTKs (Figure 1). The proneural subtype typically have a *platelet derived growth factor receptor A (PDGFRA)* amplification and mutations in the tumor suppressor *TP53*. Like EGFR, PDGFRA is an RTK that activates mitogenic growth pathways.

Lastly, the neural subtype is the least common, and it comprises 16% of diagnosed GBMs. This subtype is not defined by a dysregulation of oncogenic or tumor suppressor pathways but instead defined by the presence of neuronal markers, such as neurofilament light polypeptide (NEFL), GABA receptor subunit alpha 1 (GABRA1), and synaptotagmin-1 (SYT-1).

1.2. Current therapeutic strategies to treat GBM

During neoplastic transformation, cells typically acquire six attributes that allow for unchecked growth and spread: uncontrolled proliferative signaling, evasion of growth suppressors, activation of invasion and metastasis, replicative immortality, ability to induce angiogenesis, and resistance to cell death (Hanahan and Weinberg, 2011). The genetic characteristics that define the GBM subtypes feature common dysregulations in RTK signaling, which are major drivers behind several of the acquired hallmarks of malignancy. Accordingly, many of the newly developed GBM therapies are designed to target RTK signals. Three main classes of drugs have been developed to accomplish this task: vascular endothelial growth factor (VEGF) inhibitors, EGFR inhibitors, and non-specific tyrosine kinase inhibitors. Bevacizumab is an antibody designed to neutralize the signaling of the pro-angiogenic protein VEGF and was approved by the FDA for the treatment of GBM in 2009. Results were promising in early phase II clinical studies that involved small groups of patients; bevacizumab in combination with the topoisomerase inhibitor irinotecan or with TMZ and radiotherapy improved the 6-month progression free survival and overall survival (Friedman et al., 2009). However, in a recent large study involving 637 patients, bevacizumab had no significant effect on overall survival, and it prolonged progression-free survival by merely 3 months in patients with newly diagnosed GBM (Gilbert et al., 2014). Furthermore, evidence suggests that bevacizumab promotes tumor cell infiltration into the brain parenchyma (de Groot et al., 2010; Keunen et al., 2011). Efforts to treat GBM with small molecules targeting EGFR, such as erlotinib and gefitinib, have also fallen short of expectations in clinical trials. While gefitinib is used successfully to treat patients with non-small cell lung cancer, clinical trials evaluating this drug in GBM have failed to report any therapeutic benefit (Rich et al., 2004). Several clinical trials evaluating erlotinib in GBM patients have reported similar negative results (Rich et al., 2004; van den Bent et al., 2009; Reardon et al., 2010). Imatinib and sunitinib, both non-specific tyrosine kinase inhibitors that block PDGFR activity, have also have not produced therapeutic benefits in phase II trials (Wen et al., 2006; Razis et al., 2009; Kreisl et al., 2010).

These results suggest that exclusively targeting RTK signals is ineffective to treat GBM. Oncogenic signals that modulate glioma growth, migration, and angiogenesis may emanate from another class of receptors, the G protein-coupled receptors (GPCRs). Preclinical studies evaluating the efficacy of several GPCR-based therapies have produced encouraging results for glioma treatment. Therefore, new therapies aimed at targeting GPCRs independently or in conjunction with RTK-inhibiting drugs should be considered for GBM treatment.

2. GPCRs: a promising class of targets for treating GBM

Fifty to sixty percent of all marketed drugs target GPCRs (Pierce et al., 2002), but few such drugs have been tested for their therapeutic potential on GBMs. GPCRs control an array of signaling pathways that intimately interact with and modulate RTK signals that can control the migration and growth of tumors. One example of a GPCR-based therapy, the smoothened receptor antagonist vismodegib, has been clinically approved to treat basal cell carcinoma (Axelson et al., 2013). Examples of drugs targeting GPCRs that are undergoing clinical trials for the treatment of GBM include vismodegib and the CXCR4 antagonist plerixafor (Spectrum Health Hospitals and Phoenix Children's Hospital, ; Dana-Farber Cancer Institute et al., 2014).

In addition to affecting oncogenic signaling pathways, GPCRs are involved in regulating the differentiation state of cells, and can be overexpressed by GSCs (Aguado et al., 2007; Bar et al., 2007). A recent study aimed at profiling GPCR expression in GSCs, healthy astrocytes, neural stem cells, and GBM cell lines identified eight GPCRs that are exclusively expressed by GSCs (including LPHN2, GPR37, CALCRL, HRH2, GPR73, S1PR5, GPR128, and GPR103), revealing several new therapeutic targets to explore within the GPCR family (Feve et al., 2014). Accordingly, GPCRs represent valuable molecular modulators to control the stem cell phenotype. Below, we review some of the most promising GPCR subtypes that could be targeted for GBM therapies based on the current understanding of their roles in controlling tumor pathogenesis through molecular interactions with oncogenic signaling pathways.

2.1. Chemokine Receptors

CXCR4 and CXCR7 are related chemokine GPCRs that are best known for their roles in controlling the chemotactic response of T lymphocytes as well as the regulation of neurogenesis and neural progenitor cell proliferation and migration during development (Wu et al., 2009; Ma et al., 1998; Zou et al., 1998). These receptors are overexpressed by a number of cancer cells lines, including GBM and medulloblastoma cell lines, (Rubin et al., 2003) and by GBM patient tissues (Barbero et al., 2003; Calatuzzolo et al., 2011; Hattermann et al., 2010; Liu et al., 2013). CXCR4 expression correlates with tumor grade (Stevenson et al., 2008) and furthermore is overexpressed in GSCs compared with their more differentiated GBM counterparts, suggesting that this particular GPCR subtype may play a role in the maintenance of the GSC population (Ehtesham et al., 2009; Liu et al., 2013). In contrast, CXCR7 expression is induced locally in hypoxic areas of the tumor (Ehtesham et al., 2013). *In vitro* experiments have revealed that CXCR4 and CXCR7 mediate proliferative and migratory responses in GBM cell lines. For example, in an elegant

study by Ehtesham et al., subpopulations of cells from human GBM cell lines or primary tumor explants showed varying levels of invasiveness that correlated with CXCR4 expression levels (Ehtesham et al., 2006). Additionally, the ligand of these two chemokine receptors, CXCL12 (also known as stromal derived factor 1, SDF-1), activates mitogenic signaling in astrocytoma cell lines (Bajetto et al., 2006; Liu et al., 2013). Together, these studies suggest that CXCR4, CXCR7, and CXCL12 orchestrate a number of molecular pathways mediating the neoplastic development of GBM.

Adding to the evidence that CXCR4 functionality may promote GBM tumorigenesis, the treatment of intracranial xenograft tumors with the CXCR4 antagonist AMD3100 markedly slowed the tumor growth (Rubin et al., 2003). Interestingly, AMD3100 significantly decreased tumor volume, tumor cell invasion, and neovascularization while the non-specific RTK inhibitors vatalanib and sunitinib increased tumor volume and cell invasion, respectively (Ali et al., 2013). Moreover, concurrent treatment of intracranial xenograft tumors with radiation and AMD3100 prevented tumor recurrence and neovascularization more effectively than the VEGF neutralizing antibody, DC101 (Kioi et al., 2010). These results are bolstered by the favorable safety profile of AMD3100 in humans (Hendrix et al., 2000), and there is an early stage clinical trial examining AMD3100 (also known as plerixafor) either alone or in combination with bevacizumab to treat high-grade gliomas (Dana-Farber Cancer Institute et al., 2014). Together, this evidence provides an initial indication that targeting the GPCR CXCR4 may be effective for treating GBM.

2.2. Lysophospholipid Receptors

Lysophospholipids are important signaling mediators that exert a wide-range of biological effects. Two well-studied examples are lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), both of which exist at nanomolar to micromolar concentrations in the blood (Eichholtz et al., 1993; Okajima, 2002) and signal via two related subtypes of GPCRs, LPA and S1P receptors.

2.2.1. LPA Receptors—Evidence for LPA acting as a mediator of tumorigenesis includes studies showing that LPA stimulates mitogenesis, protects from apoptosis, activates cytoskeletal rearrangements, and stimulates tumor cell invasion (Imamura et al., 1993; Manning et al., 2000; Mills and Moolenaar, 2003). LPA is produced in the extracellular milieu by the secreted phospholipase D enzyme autaxin (ATX), which was originally identified as an autocrine factor that stimulated melanoma cell migration and was associated with tumor progression (Stracke et al., 1992; Tokumura et al., 2002). Importantly, ATX upregulation has been observed in many cancers, including GBM, and it leads to an increase in autocrine LPA signaling and increased invasion of these tumors (Hoelzinger et al., 2005; Hoelzinger et al., 2008). There are six molecularly identified LPA receptors (LPAR₁-LPAR₆) that are differentially expressed and exhibit overlapping coupling to similar signaling pathways (Choi et al., 2010). Among these receptors, LPAR₁, LPAR₂ and LPAR₃ are expressed by GBM cell lines, and LPAR₁ and LPAR₃ are overexpressed in GSCs (Annabi et al., 2009). By activating its cognate GPCRs, LPA increases tumor growth by potently activating DNA synthesis and stimulates robust chemotactic migration and invasion of GBM cells. Accordingly, LPA has been referred to as a *bona fide* growth factor (van

Corven et al., 1989) and thus, together with its GPCRs, is an important regulator of tumorigenesis.

2.2.2. S1P Receptors—S1P shares many of the same biological activities as LPA, but its effects on tumor cells appear more complex, as it can act as either a pro- or anti-proliferative signal depending on the type of cancer (Goetzl et al., 1999; Balthasar et al., 2006). Blood-borne S1P is generated by sphingosine kinases either in the cytosol of platelets, which release S1P upon activation, or in the extracellular space by sphingosine kinase-1 excreted from endothelial cells (Yatomi et al., 2000; Ancellin et al., 2002). High levels of sphingosine kinase in GBM tumors correlate with a greater than 3-fold shorter survival time (Van Brocklyn et al., 2005). There are five S1P receptors (S1PR₁-S1PR₅), and among these, S1PR₁, S1PR₂ and S1PR₃ are expressed in GBM cell lines and tissues (Van Brocklyn et al., 2003; Van Brocklyn et al., 2005). S1PR₁ expression has been found to inversely correlate with tumor grade and to be predictive of patient survival (Yoshida et al., 2010a; Yoshida et al., 2010b). In line with this evidence, exogenous overexpression of S1PR₁ decreased the growth of U87MG intracranial xenograft tumors (Yoshida et al., 2010b). Despite these encouraging results, the precise role of S1PR₁ in GBM pathogenesis is the subject of some debate, as one study showed that exogenous S1PR₁ overexpression in GBM cell lines *increased* cell proliferation (Young and Van Brocklyn, 2007). The use of different cell lines expressing distinct driver mutations or components of the intracellular signaling network downstream of S1PR₁ could account for this discrepancy. Additionally, the finding that S1PR₁ overexpression decreases tumor size *in vivo* could indicate that this receptor mediates anti-survival cues rather than antiproliferative cues. Therefore, S1PR₁ could increase cell-cycle progression while decreasing cell survival.

The role of S1P receptors in the control of cell migration also appears to be complex. S1PR₁ and S1PR₂ activation have differential effects on *in vitro* astrocytoma cell migration, where activation of S1PR₁ increased cell motility and invasion while activation of S1PR₂ decreased cell motility (Malchinkhuu et al., 2005; Young and Van Brocklyn, 2007). Despite the reduction in cell migration, S1PR₂ activation increases cellular invasion (Young and Van Brocklyn, 2007). Here, it is important to make the distinction between *in vitro* cell migration assays that test the overall mobility of a cell and cell invasion assays that test the ability of a cell to penetrate an extracellular matrix. Thus, the balance between S1PR₁ and S1PR₂ expression can dictate the migratory capacity of a cell (Young and Van Brocklyn, 2007) however, high expression of either receptor is sufficient to promote tumor cell invasion.

The differing biochemical consequences of activating LPA and S1P receptors provide further evidence that lysophospholipid signaling is quite intricate. To our knowledge, there are currently no studies that have examined the effects of LPA or S1P receptor inhibition in intracranial GBM xenograft models, making the therapeutic importance of targeting this particular group of GPCRs difficult to predict. Given the complexity resulting from eleven combined receptors with different pharmacological profiles interacting with two signaling lipids, it is unlikely that targeting just one GPCR subtype will significantly affect clinical outcomes. Using selective drugs for each subtype might represent one route by which the complexity of this system can be leveraged for therapeutic benefits.

2.3. Cannabinoid Receptors

Activation of the cannabinoid GPCRs also has the remarkable ability to regulate several fundamental components of tumorigenesis, namely arresting the cell cycle, reducing cell viability by controlling autophagy and apoptosis, preventing tumor cell invasion, and affecting GSC differentiation (Bifulco and Di Marzo, 2002; Aguado et al., 2007; Pisanti et al., 2013) without toxically affecting healthy cells (Galve-Roperh et al., 2000). There are two molecularly identified cannabinoid GPCRs, CB₁ and CB₂, that are activated by the endogenous cannabinoids (eCB) arachidonylethanolamine (AEA, also known as anandamide) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). In addition to the two eCB, CB₁ and CB₂ are activated by naturally occurring compounds, such as ⁹-tetrahydrocannabinol (THC) that are derived from the plants in the *Cannabis* genus (Matsuda et al., 1990; Munro et al., 1993), and by synthetic compounds, such as CP-55940 and WIN55,212-2 (CP and WIN, respectively). CB₁ and CB₂ activation reduces the ability of GSCs to form tumors *in vivo* (Aguado et al., 2007), and CB₂ expression correlates with glioma malignancy (Sanchez et al., 2001). This evidence suggests that cannabinoid receptor signaling may play a role in tumorigenesis.

Perhaps more striking is the ability of cannabinoid compounds to induce tumor cell death. The tumor-killing properties of cannabinoid compounds were identified in 1975 when THC treatment of lung adenocarcinoma tumors implanted in mice inhibited tumor growth by 75% and increased lifespan by 36% compared with controls (Munson et al., 1975). Since then, cannabinoid treatment has proven effective in a number of xenograft tumor models (summarized in (Velasco et al., 2012)). In a landmark study published by Galve-Roperh et al., rats implanted intracranially with C6 rat glioma cells were treated with THC or WIN. The results were striking; 1/5 (THC) and 1/3 (WIN) of the treated animals had complete tumor resolution, while the remaining animals had significantly smaller and less vascularized tumors. Remarkably, there was no detectable cytotoxicity in the healthy brain tissue surrounding the site of original tumor growth, suggesting that these drugs selectively kill malignant cells (Galve-Roperh et al., 2000). GBM regression was also observed with a selective CB₂ agonist *in vivo* (Sanchez et al., 2001). In addition, a small clinical trial was performed to address the safety and possible therapeutic efficacy of intratumoral THC injection in nine patients with recurring GBM (Guzman et al., 2006). These results revealed that THC was well tolerated and that overt psychoactive effects were minimal. The median survival of these nine patients was 5.7 months after treatment initiation. In contrast, the median survival of patients with recurrent GBM is 7.4 months after re-operation of the recurrent tumor (Park et al., 2010); however, two patients who received THC lived for approximately 12 months. These studies laid the foundation for examining the potential of drugs targeting cannabinoid receptors and this signaling network mediating the selective killing of tumor cells.

2.4. Other notable GPCRs: formyl peptide, neurokinin-1, and smoothened receptors

2.4.1. Formyl peptide receptors—The formyl peptide receptor (FPR) is a GPCR that was discovered in phagocytic leukocytes induced by bacterial N-formyl-methionyl-leucyl-phenylalanine (fMLF). FPR is overexpressed in primary human gliomas and glioma cell

lines, and an increase in its expression level correlates with tumor grade (Le et al., 2000; Zhou et al., 2005; Chen et al., 2006). There are three known endogenous ligands for this GPCR: Annexin 1 (Anx A1) (Babbin et al., 2006), Cathepsin G (Sun et al., 2004), and mitochondrial formyl peptides (Rabiet et al., 2005). Necrotic GBM cells release Anx A1, which stimulates tumor growth, invasion, and the release of angiogenic factors through its action on FPR (Zhou et al., 2005; Yao et al., 2008; Yang et al., 2011). Accordingly, Anx A1 knockdown significantly decreased the growth of xenograft GBMs in mice and increased their survival. Double knockdown of Anx A1 and FPR enhanced this effect, and some mice survived more than 100 days longer than those injected with wild-type tumor cells (Yang et al., 2011). The small-molecule FPR inhibitor Nordy effectively inhibits chemotaxis, proliferation, and VEGF production *in vitro*. Intracranial xenograft tumors treated with Nordy were half the size and significantly less vascularized than vehicle-treated tumors (Chen et al., 2007). This evidence suggests that the Anx A1/FPR signaling axis appears to play a role in tumorigenesis, especially in necrotic areas. Thus, blocking this signaling pathway may inhibit tumor growth stimulated by Anx A1 emanating from the necrotic tumor core.

2.4.2 Neurokinin-1 receptors—The Neurokinin-1 receptor (NK1R), also known as the Substance P receptor, is expressed in the central and peripheral nervous systems as well as in vascular endothelial and immune cells, where it promotes cell motility and angiogenesis in healthy individuals (Satake and Kawada, 2006). In addition to substance P, NK1R is activated by the tachykinin hemokinin-1 (HK-1) (Kurtz et al., 2002). Evidence from the last three decades indicates that activation of this GPCR subtype in cancer cells might control tumorigenesis by mediating mitogenic and pro-survival signaling that lead to activation of transcription factors and DNA synthesis (Eistetter et al., 1992; Christian et al., 1994; Luo et al., 1996; Lieb et al., 1997; Akazawa et al., 2009). Accordingly, both substance P and NK1R levels are increased in a number of cancers, including breast carcinomas, metastatic melanomas, thyroid carcinoma, and GBM (Hennig et al., 1995; Khare et al., 1998; Harford-Wright et al., 2014; Munoz et al., 2005; Satake and Kawada, 2006). In gliomas, NK1R expression correlates with tumor grade; in fact, it may possess some oncogenic activity, as a truncated form of this receptor that is thought to be constitutively active causes malignant transformation when expressed in non-malignant model systems (Fong et al., 1992; Hennig et al., 1995; Patel et al., 2005). In addition to activating pro-growth and survival pathways, NK1R stimulation increases GBM cell migration and invasion (Mou et al., 2013). Accordingly, *in vitro* blockade of this receptor in GBM cells results in a reduction in the migration in the presence of HK-1 (Mou et al., 2013) as well as an increase in apoptosis in the absence of any additional chemotherapeutic agents (Akazawa et al., 2009). Therefore, the NK1R antagonist aprepitant, which is FDA approved for treatment of chemotherapy-induced nausea and vomiting, could be considered for cancer indications. Aprepitant is highly effective in reducing tumor size in xenograft models of hepatoblastoma, osteosarcoma, and brain metastases of melanoma (Berger et al., 2014; Harford-Wright et al., 2014; Munoz et al., 2014). Demonstrating the efficacy of aprepitant in xenograft mouse models of GBM could provide a foundation to examine its potential as a new cancer treatment.

2.4.3. Smoothed receptors and hedgehog signaling—Sonic hedgehog (SHH) signaling, normally vital to tissue patterning during development and to neural stem cell maintenance, promotes the formation of GSC populations, which are responsible for resistance to therapy and tumor recurrence in GBM (Yan et al., 2014). The SHH ligand functions through two transmembrane proteins known as patched (PTCH) and smoothed (SMO), the latter being a GPCR. Binding of SHH to PTCH activates SMO, which signals through the transcription factor glioma-associated oncogene-1 (Gli-1) and regulates gene expression. The hyperactivity of this pathway is well described in a subset of medulloblastomas; however, only in the last decade have we increased our understanding of SHH signaling in the context of glioma biology (Goodrich et al., 1997; Kool et al., 2012; Pugh et al., 2012). While multiple studies have shown that SHH signaling is activated in glioma grades II and III, whether it is activated in GBM is still debatable (Bar et al., 2007; Ehteshami et al., 2007). The discrepancy is likely due to the heterogeneous nature of GBM combined with the possibility that SHH signaling is confined to small populations of GSCs that may not be enriched in the tumor tissues sampled (Bar et al., 2007). Nonetheless, inhibition of SMO by the antagonist cyclopamine reduces GSC sphere formation, growth, and tumor engraftment into nude mice (Bar et al., 2007). Furthermore, cyclopamine treatment conferred a survival benefit to mice implanted with primary high-grade gliomas (Sarangi et al., 2009). The concept of targeting SMO for treatment of glioma is relatively new. However, because this signal transduction pathway is hyperactive in populations of GSCs that are resistant to conventional therapies, its promise as a therapeutic target has continued to increase with our understanding of SHH signaling in GBM.

3. GPCR signaling to oncogenic pathways

3.1. Canonical and non-canonical signaling through G protein and RTK activation

The GPCR family is composed of over 900 members divided into six classes. All GPCRs share a common heptahelical structure, and different GPCR classes are defined by sequence homology (Pierce et al., 2002). In the canonical GPCR signaling pathway, the activation of the receptor by its ligand leads to binding and activation of a heterotrimeric G protein ($G\alpha\beta\gamma$). The $G\alpha$ subunit is activated by GDP/GTP exchange that results in the dissociation of the $G\beta\gamma$ subunit, and both subunits activate downstream second messengers to elicit a cellular response. The $G\alpha$ subunit is divided into four main subtypes: $G\alpha_s$, which stimulates cAMP production; $G\alpha_{i/o}$, which inhibits cAMP production; $G\alpha_{q/11}$, which stimulates phospholipase C (PLC) and calcium mobilization; and $G\alpha_{12/13}$, which regulates actin cytoskeleton remodeling through the activation of small GTPases. The $G\beta\gamma$ subunit can also directly affect cAMP production, tyrosine kinase and RTK activation, and ion channel activation, which add an additional level of complexity to GPCR signaling (Pierce et al., 2002).

The G protein signal is terminated when $G\alpha$ hydrolyzes GTP to GDP and re-associates with $G\beta\gamma$. In addition, GPCRs can be deactivated or internalized through the actions of kinases and β -arrestins. Phosphorylation of serine and threonine residues on the receptor by protein kinase A (PKA), protein kinase C (PKC), or GPCR regulating kinases (GRKs) provides a docking site for β -arrestin. This protein sterically hinders further G protein coupling and

mediates the internalization of the GPCR. Therefore, loss of activity or expression of either GRKs or β -arrestins could lead to an increase in GPCR signaling. GRK3, one of the kinases that phosphorylate CXCR4, is downregulated specifically in the classical subtype of GBMs, and this loss of GRK expression is thought to be associated with sustained oncogenic signaling of CXCR4 (Woerner et al., 2012). Further supporting this hypothesis, the loss of β -arrestin 1 activity is positively correlated with glioma malignancy (Mandell et al., 2009). Alternatively, β -arrestins can initiate mitogenic signaling by serving as an adapter protein for mitogen activated protein kinase (MAPK) signaling components, such as ERK, JNK, and p38 (DeWire et al., 2007). GRK5 is upregulated in GBM and GSCs, and knockdown of this protein reduces the proliferation of these cells, a phenomenon that could result from increased MAPK signaling through recruited β -arrestins (Kaur et al., 2013). Because of the complexity of GPCR signaling, these receptors are often referred to as “molecular hubs” that control a wide array of downstream effectors.

Recent advances in our understanding of GPCR signaling mechanisms have led to the realization that these receptors are involved in multiple non-canonical signaling pathways. For example, the transactivation of RTKs, such as EGFR, by GPCRs is instrumental in controlling cellular processes, such as growth, migration, and angiogenesis, and this crosstalk has been shown to contribute to the progression of many cancers (Filardo et al., 2000; Hart et al., 2005; Thomas et al., 2006). RTKs are single pass membrane proteins that dimerize upon ligand binding, allowing each monomer to transphosphorylate key tyrosine residues on the receptor. These phosphorylated residues serve as docking sites for adaptor proteins containing a Src Homology 2 (SH2) or phosphotyrosine binding domain (PTB) that anchor downstream signaling components of mitogenic pathways, such as MAPK, RAS, and PI3K (Figure 1). GPCRs activate growth factor receptors via two mechanisms: 1) extracellularly, by promoting the release of growth factors that provide autocrine or paracrine stimulation or 2) intracellularly, through phosphorylation cascades (Figure 1) (Daub et al., 1997; Pierce et al., 2001; Bhola and Grandis, 2008). In the first scenario, GPCR signaling leads to the activation of the matrix metalloproteinase (MMP) tumor necrosis factor α -converting enzyme (TACE), which then cleaves growth factors from the membrane and results in the autocrine activation of growth factor receptors that can mediate a variety of tumorigenic responses (Prenzel et al., 1999; Gschwind et al., 2003; Schafer et al., 2004; Hart et al., 2005). Alternatively, in the second scenario, GPCRs activate non-receptor tyrosine kinases, such as SRC, by direct interaction with by $G\alpha$ or $G\beta\gamma$ (Ma et al., 2000; Piiper and Zeuzem, 2004), which can phosphorylate tyrosine residues on RTKs (Huang et al., 2007). The activation-state of the RTK that results from intracellular phosphorylation by downstream GPCR signaling does not necessarily involve all the phosphotyrosine sites that extracellular activation requires. For example, in the human GBM cell line U87MG, EGF treatment promotes the phosphorylation of EGFR at residues Y845, Y1173, Y1068, and Y992, whereas activation of the GPCR FPR results in the phosphorylation of EGFR only at residue Y992. Nonetheless, this single phosphorylation event enhances both chemotaxis and proliferation induced by fMLF (Huang et al., 2007). This evidence indicates that through a combination of classical G protein signaling and RTK transactivation, GPCRs can regulate several crucial oncogenic pathways.

3.2. GPCR-mediated control of tumor cell growth

Tumor growth results from a dysregulation of the balance between cell proliferation and cell death. With the exception of cannabinoid receptors and possibly S1PR₁, the GPCRs discussed above enhance glioma cell proliferation. Proteins known as cyclin-dependent kinases (CDKs) are the principal controllers of cell proliferation. Each CDK subtype is activated by a different cyclin, and individual cyclin-CDK pairs coordinate the progression through different stages of the cell cycle. Cyclin-CDK pairs are inhibited by CDK inhibitors (CDKIs), and cell cycle progression is the result of a balance between “on” signals (cyclins) and “off” signals (CDKIs). The transcription of both of these mediators is tightly regulated by upstream signals, including GPCRs; thus, the consequence of GPCR stimulation on cell cycle progression depends on various competing mechanisms. Briefly, GPCRs can activate five main signaling pathways leading to the transcription of cyclins and CDKIs (Figure 1). Gα_i and Gα_s modulate cyclic-AMP (cAMP) levels, which stimulate PKA to phosphorylate the transcription factor CREB. Gα_{q/11} acts through PKC to activate the transcription factor NFκB. Alternatively, GPCRs activate PI3K/AKT and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways through SRC stimulation or RTK transactivation. Lastly, GPCRs can activate ERK signaling through RTKs. A detailed overview of how GPCRs regulate cell cycle progression has been reviewed elsewhere (New and Wong, 2007).

The modulation of cell survival by GPCRs is also complex. The primary mechanism by which these receptors elicit a pro-survival response is through AKT and NFκB activation (Figure 2). AKT prevents apoptosis by phosphorylating and inactivating the pro-apoptotic Bcl-2 family protein BAD. NFκB promotes cell survival by altering the transcription of gene products involved in apoptosis and by blocking the transcription of pro-apoptotic genes; however, this response may depend on upstream signals involved in its activation (Liu et al., 2012). For example, LPAR stimulation, which activates NFκB, protects ovarian cancer cells from programmed cell death by reducing cell surface expression of the Fas receptor, thus reducing Fas ligand-activated apoptosis (Meng et al., 2005). LPA and S1P are also known to protect against apoptosis through Gα_i-mediated AKT activation in various cancers (Radeff-Huang et al., 2004), although the molecular details of this mechanism are still being elucidated.

One of the most promising biological responses linked to the activation of some GPCRs, such as the cannabinoid receptors, is the ability to promote apoptosis in cancer cells (Galve-Roperh et al., 2000). Our current understanding of the molecular components mediating selective CB₁/CB₂-induced toxicity in tumor cells includes the involvement of ceramide lipid accumulation, which triggers an endoplasmic reticulum stress response. This response drives the transcription of the stress protein p8, which results in the inactivation of AKT and subsequently leads to the activation of caspase 3 (Figure 2). The detailed molecular mechanism allowing CB₁ and CB₂ receptors to increase ceramide production in tumor cells remains to be elucidated, but initial evidence points to the involvement of serine palmitoyltransferase, the rate-limiting step in *de novo* ceramide synthesis (Sanchez et al., 1998a; Sanchez et al., 1998b; Blazquez et al., 2000; Cudaback et al., 2010). The ability of these receptors to couple to apoptosis is inversely correlated with their expression levels,

which contributes further complexity to cannabinoid-induced cytotoxicity. Presumably, this phenomenon is due to an increase in pro-survival AKT signaling in gliomas expressing high levels of CB₁ or CB₂, which is important to consider when developing cannabinoid-based therapies for GBM (Cudaback et al., 2010).

3.3. GPCR-mediated control of cancer cell invasion

Tumor cell invasion is a multistep process involving changes in cell adhesion, degradation of the surrounding extracellular matrix (ECM) by MMPs, and increased motility or chemotaxis (Figure 3). The first step of cell migration is the alteration of cell adhesion. Dynamic cell-ECM attachment sites known as focal adhesions (FA) facilitate movement by integrating mechanical and chemical signals that control the adhesive state of the cell during migration. These sites can contain over 100 different proteins that are centered on clusters of activated integrins (Zamir et al., 1999). Extracellular adhesive cues are also sensed by members of the adhesion class of GPCRs. This class is so-named because of the common presence of adhesion domains on the N-terminus, and it is emerging as an important group of regulators in tumor biology. Similar to integrins, adhesion GPCRs have been described as “context recognizers”, sensing and integrating chemical, cellular, and ECM cues that help determine whether a cell will undergo a particular action, such as migrate or divide (Langenhan et al., 2013). Adhesion GPCRs are unique because although they consist of a classical heptahelical structure, their long N-terminus contains functional domains usually found in adhesive proteins or RTKs. Thus, they can be considered as bi-functional proteins because they signal intracellularly like a GPCR, but they are capable of transmitting stimuli through contact with other cells or ECM. Several adhesion GPCRs have been implicated in cancer cell adhesion and motility (reviewed in (Lin, 2012). For example, GPR56 is upregulated in GBM tissues, and the N-terminal domain was found to mediate glioma cell adhesion (Shashidhar et al., 2005).

Once a cell has sensed its environment and detached from the ECM and surrounding cells, it must break down ECM proteins to move through this dense environment. This process is essential to tumor cell invasion and is mediated by MMPs, which degrade components of the extracellular milieu. MMPs are tightly regulated at both the transcriptional and post-translational levels. GPCR signaling can upregulate MMP transcription by activating transcription factors that bind to the MMP promoters. For example, stimulation or overexpression of CXCR4 increases MT2-MMP and MMP-9 expression (Zhang et al., 2005; Ehtesham et al., 2006). This effect of CXCR4 may be dependent on activation of the transcription factor FOXM1, which has been shown to increase transcription of MMPs and invasion of glioma cells (Figure 3) (Dai et al., 2007; Wu et al., 2008; Wang et al., 2014). Once MMP expression is increased, these proteins must be activated. In general, GPCRs can activate MMPs via SRC-family kinases, albeit through a poorly understood mechanism (Luttrell and Luttrell, 2004). However, CXCL12-mediated activation of MMP-2 in GBM cells is dependent on PI3K/AKT and ERK signaling, suggesting that the SRC-dependent activation of MMPs may occur through these pathways (Wu et al., 2008).

The last step in tumor cell invasion is the activation of cell motility processes that facilitate cell movement. The contribution of GPCR signaling to chemotaxis and cell migration has

been studied for several decades. Briefly, cell migration results from orchestrated signaling events that are activated spatiotemporally to polarize the cell, drive the leading edge forward, and retract the trailing edge. The signaling events that control cell polarization and movement are coordinated primarily by members of the Rho family of small GTPases, including RHO, RAC, and CDC42, which can be activated by either GPCRs or RTKs (Figure 3) (Raftopoulou and Hall, 2004). Each small GTPase controls its own “microdomain” of cytoskeletal machinery. RAC regulates the formation of a lamellum, which ruffles at the leading edge of a cell forming a lamellipodium (Ridley et al., 1992). In contrast, RHO is associated with the formation of actin stress fibers, which not only aid in cellular retraction of the trailing edge but also help anchor the leading edge to the ECM by putting mechanical stress on the leading edge FAs (Ridley and Hall, 1992; Kuo, 2013). Lastly, CDC42 activation leads to filopodia formation (Kozma et al., 1995). Importantly, RAC and RHO activity exist in a balance, where the activity of each GTPase inhibits the activity of the other (Raftopoulou and Hall, 2004). Therefore, GPCR signaling to specific GTPases can act as a “tipping point” to initiate different stages of cell migration.

Ligands acting through GPCRs modulate the activity of critical effector proteins controlling each modality of cell migration. GBM cell migration can be regulated either through direct GPCR stimulation of GTPases or through RTK transactivation. In the case of LPA-induced migration, RAC and CDC42 are activated independently of EGFR or PDGFR stimulation (Malchinkhuu et al., 2005; Malchinkhuu et al., 2009). Alternatively, many GPCRs require RTK activity to elicit a migratory response. For example, CXCR4-induced chemotaxis depends on PDGFR activation in GBM cells, as blocking the activity of either receptor reduces the chemotactic response to both ligands (Sicacaluga et al., 2013). A similar relationship exists between FPR and EGFR (Chen et al., 2007; Huang et al., 2007). However, the molecular crosstalk between GPCRs and RTKs occurs bi-directionally, as many RTKs require GPCR activity to induce chemotaxis. For example, CXCR4 is essential for EGF-induced chemotaxis of GBM cells, but EGFR activation is not necessary for CXCL12-induced chemotaxis, highlighting the intimate and often complex association between these two signaling systems (Sicacaluga et al., 2013).

3.4. GPCR-mediated communication with the tumor microenvironment

Tumors are not autonomous entities. Bi-directional communication between the tumor and the surrounding tissues, known as the tumor microenvironment, is essential to tumor growth processes, such as immune system evasion, cancer stem cell niche maintenance, and new blood vessel growth. For example, Yan et al. discovered that the SHH ligand released by endothelial cells promoted the de-differentiation of glioma cells to GSCs in perivascular niches through the action of SMO (Yan et al., 2014). This response is of particular relevance to the development of new GBM treatments because of the difficulty in eradicating these chemo- and radiotherapy-resistant cells.

Another pertinent illustration of GPCR-mediated tumor-stromal communication occurs during angiogenesis. The molecular mechanisms involved in angiogenesis are similar to the mechanisms required for cell proliferation and invasion; thus, it is not surprising that GPCRs play a role in this important process. The initiation of angiogenesis occurs in multiple steps:

1) ECM degradation, 2) disruption of cell-cell and cell-matrix adhesions, 3) endothelial cell proliferation, and 4) endothelial cell migration to newly forming blood vessels (Richard et al., 2001). These steps, originally believed to be mediated primarily by VEGF and fibroblast growth factor (FGF) receptor signaling, are partially controlled by GPCR signaling (Ferrara and Alitalo, 1999). An excellent example is again found in the signaling between CXCR4 and CXCL12. Tumor cells release CXCL12 in response to hypoxia, which stimulates the chemotactic migration of bone marrow-derived cells and endothelial cells toward hypoxic areas where they build new vasculature (Ceradini et al., 2004; Aghi et al., 2006; Grunewald et al., 2006; Jin et al., 2006). This concept is particularly relevant in the context of GBM tumors treated with radiation because most GBM recurrences initiate within the radiation-treated area, which induces transient hypoxia and subsequent re-vascularization of the recurrent tumor (Kioi et al., 2010). Conversely, GBM angiogenesis is enhanced by GSCs recruited to the sites of newly forming vessels by CXCR4/CXCL12 signaling. These GSCs are then stimulated to transdifferentiate into vasculature-supporting cells known as pericytes (Cheng et al., 2013). Therefore, in addition to increasing the release of pro-angiogenic factors and activating MMPs, GPCRs localize angiogenesis to hypoxic areas and increase the structural integrity of newly forming blood vessels.

4. Concluding remarks

Significant advances in the standard of care for cancer treatment have occurred through the addition of therapies targeting driver mutations. Indeed, this effort fueled the development of breakthrough cancer treatments, including imatinib for chronic myelogenous leukemia and tamoxifen for breast cancer. Because RTKs function as prominent oncogenes in GBM, significant efforts have been dedicated to developing therapies that target these overactive signaling pathways to treat this cancer. Unfortunately, these efforts have yet to produce remarkable increases in survival. Owing to the ability of GPCRs to feed into RTK signaling and other parallel signaling pathways, the knowledge accumulated by the studies reviewed here indicate that GPCRs represent a promising new class of therapeutic targets for the treatment of GBM. Similarly, the signaling redundancies between GPCRs and RTKs present a strong case for considering combined treatments that target both protein classes.

The studies reviewed herein represent examples of the most thoroughly studied therapeutic GPCR candidates for GBM treatments. Because the search for new targets to treat these malignant tumors is ongoing, an exhaustive study published by Feve, Saliou, and Zeniou et al. examined the GPCR expression profile between primary GSCs, U87MG cells, neural precursor cells, and healthy astrocytes to identify new GPCR candidates that are differentially expressed and could be used for targeted therapies (Feve et al., 2014)

Moreover, many fundamental questions remain unanswered. One intriguing phenomenon that requires a more detailed examination is the selective toxicity of cannabinoid-based drugs on tumor cells. There are multiple signaling pathways downstream of GPCRs, and receptor bias towards a particular pathway may depend on the presence or absence of downstream components within the receptor complex. Therefore, one testable hypothesis for the selective toxicity induced by cannabinoid receptor activation is that the acquisition of a malignant phenotype alters the molecular coupling within the cannabinoid receptor complex,

resulting in changes in downstream signaling. Understanding the details of this signaling bias could provide clues into how malignancy reshapes the molecular landscape of tumor cells and how this selective process of cell death can be hijacked to create better-targeted therapies.

New avenues to treat GBM are desperately needed. GPCRs hold the remarkable quality of a multifaceted signaling capacity that can control several modalities of the malignant state and the resulting growth and spread of cancer. GPCRs hold a promising and exciting therapeutic potential that should be considered for developing novel cancer therapies.

Abbreviations

GPCR	G protein-coupled receptor
GBM	Glioblastoma Multiforme
GSC	Glioma stem cell
TMZ	Temozolomide
EGFR	Epidermal growth factor receptor
VEGFR	Vascular endothelial growth factor receptor
RTK	Receptor tyrosine kinase
TCGA	The Cancer Genome Atlas
PTEN	phosphatase and tensin homolog
NF1	Neurofibromin 1
PI3K	Phosphoinositol-3-kinase
PDGFRA	Platelet derived growth factor receptor 1A
S1P	Sphingosine-1-phosphate
LPA	Lysophosphatidic acid
ATX	Autaxin
THC	⁹ -tetrahydrocannabinol
WIN	WIN55,212-2
FPR	Formyl peptide receptor
fMLF	N-formyl-methionyl-leucyl-phenylalanine
NK1R	Neurokinin-1 receptor
Anx A1	Annexin 1
HK-1	Hemokinin-1
SHH	Sonic hedgehog
PTCH	Patched receptor

SMO	Smoothened receptor
Gli-1	glioma-associated oncogene-1
MMP	Matrix metalloproteinase
ECM	Extracellular matrix
FA	Focal adhesion
CDK	Cyclin-dependent kinase
CDKI	CDK inhibitor
JAK	Janus kinase
STAT	Signal transducer and activator of transcription

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Highlights

- Exciting GPCR therapeutic targets have been identified for GBM.
- GPCR signaling affects tumor growth, metastasis and angiogenesis.
- GPCRs modulate oncogenic tyrosine kinase pathways.

Figure 1. GPCR-mediated control of the cell cycle

GPCRs regulate five main pathways leading to the transcription of cyclins and CDKIs that regulate cell cycle progression. (1) G_{α_s} and $G_{\alpha_{i/o}}$ proteins activate and inhibit cAMP production, respectively. cAMP subsequently activates Protein kinase A (PKA), which stimulates the transcription factor CREB. Therefore, G_{α_s} proteins increase CREB stimulation while $G_{\alpha_{i/o}}$ proteins decrease CREB stimulation. (2) $G_{\alpha_{q/11}}$ activates protein kinase C (PKC) through the activity of phospholipase C β (PLC β). PKC can indirectly lead to the activation of NF κ B. (3-5) G_{α} proteins activate three additional pathways through the direct action of SRC tyrosine kinase or RTK transactivation. RTKs are transactivated by the intracellular phosphorylation by SRC-family kinases or by autocrine signaling activated by MMP-dependent cleavage of growth factors from the plasma membrane. (3) PI3K/AKT signaling activated by SRC leads to the inhibition of the transcriptional inhibitor GSK3. Additionally, PI3K signaling activates NF κ B. The commonly mutated protein PTEN normally antagonizes PI3K signaling. (4) STAT transcription factors are activated and mobilized by JAK proteins that are phosphorylated through SRC-dependent or RTK-dependent mechanisms. (5) RTK-dependent activation of RAS leads to the stimulation of

PLC β (**2**) and PI3K (**3**) and can additionally stimulate ERK. The commonly mutated protein NF1 normally antagonizes RAS signaling.

Figure 2. GPCR control of cell-survival

(1) Anti-survival signaling mediated by GPCRs, such as the cannabinoid receptors, is dependent upon an increase in ceramide following the induction of serine palmitoyltransferase, which is the rate-limiting step in *de novo* ceramide production. Ceramide activates an ER-stress response, causing the upregulation of the stress protein p8, which inhibits the pro-survival factor AKT. **(2) Pro-survival** signaling elicited by GPCRs is generally dependent on AKT-mediated inhibition of the pro-apoptotic Bcl-2 family protein BAD. Thus, activation of AKT leads to the inhibition of cytochrome c release from the mitochondria and subsequent activation of caspase 3. In an alternate pathway, GPCRs activate NF κ B, which can reduce pro-apoptotic gene transcription, such as the Fas receptor, leading to a reduction in apoptotic potential.

Figure 3. GPCR control of migration and invasion

GPCRs control glioma cell infiltration by activating MMPs to degrade the ECM and by stimulating mobility factors within the cell, such as RHO, RAC, and CDC42. MMP transcription is activated downstream of GPCRs, potentially by the FOXM1 transcription factor in gliomas. MMP activation is mediated by PLCβ or the SRC family kinases. Rho GTPases are stimulated by Gα proteins or by RTK transactivation. These proteins act in concert to spatiotemporally regulate the formation of migratory features, such as stress fibers, lamellipodia, and filopodia.