

# Sphingolipid Signaling Pathways as Potential Therapeutic Targets in Gliomas

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**Abstract:** Glioblastoma multiforme (GBM) is a highly malignant brain tumor. The interconvertible bioactive sphingolipids sphingosine-1-phosphate (S1P) and ceramide have profound effects on GBM cells, with ceramide causing cell death and S1P leading to cell survival, proliferation and invasion. This review will examine the effects of ceramide and S1P on glioma cells and the therapeutic potential of these pathways.

**Key Words:** Glioma, glioblastoma multiforme, sphingolipids, sphingosine-1-phosphate, ceramide.

## INTRODUCTION

Gliomas are primary neoplasms of the central nervous system whose cells resemble glial cells. The most common glioma occurring in adult patients is the highly malignant, grade 4 astrocytoma, known as glioblastoma multiforme (GBM). GBM patients have a median survival of slightly less than one year, and current therapies only minimally improve the prognosis. The malignancy of these tumors is due to rapid cellular proliferation and diffuse invasion into surrounding brain, leading to inevitable recurrence of tumors even after radical resection. A greater understanding of the molecular mechanisms regulating growth and invasion of GBM will be necessary to develop more effective therapies.

In recent years a large amount of information has become available regarding the roles of sphingolipids as cell regulatory molecules. In particular, much work in this field has focused on the lipids ceramide and sphingosine-1-phosphate (S1P). The majority of these studies have shown that these two lipids have opposing effects on cells, with ceramide decreasing cell proliferation and leading to apoptosis, and S1P tending to stimulate cell proliferation and enhance survival. This led to the proposal by Spiegel's group of the existence of a sphingolipid rheostat, in which the balance between cellular levels of ceramide and S1P determines cell fate [1, 2].

These two lipids can be interconverted by a two step process in which ceramide is deacylated to sphingosine by ceramidase, and then phosphorylated to S1P by sphingosine kinase [3]. In the opposite direction, S1P can be dephosphorylated by specific phosphatases and then N-acylated by ceramide synthase (Fig. (1)) [4]. In addition, S1P can be broken down irreversibly by S1P lyase. Ceramide can be converted into a variety of more complex sphingolipids by further additions at the 1-OH position, including addition of phosphate to create ceramide-1-phosphate, phosphocholine to create sphingomyelin, and carbohydrates to create glycosphingolipids [3]. Thus, the activities of a variety of enzymes could be altered by cells to regulate the concentrations

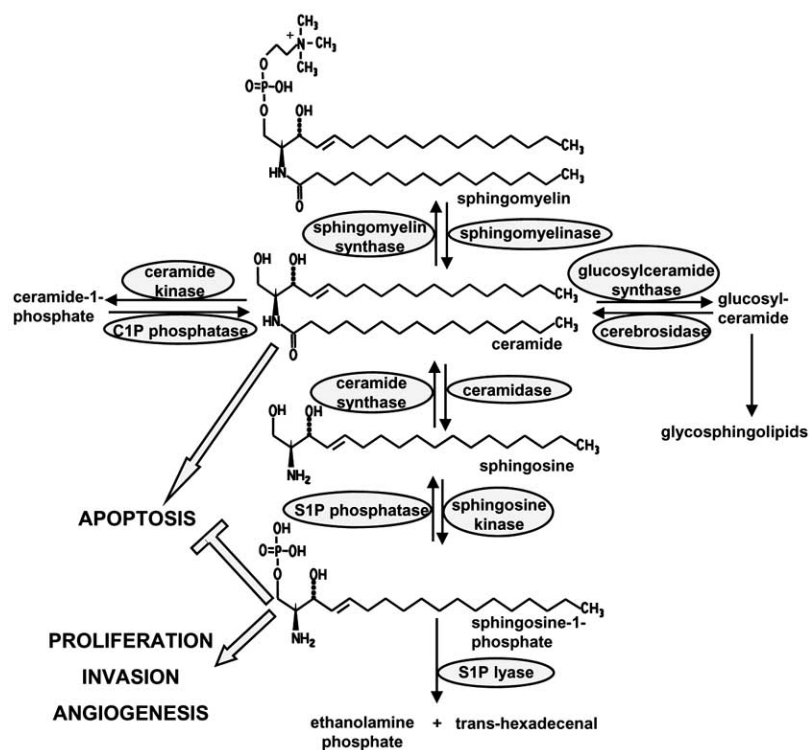
of ceramide and S1P. Interestingly, this also presents several potential points of intervention, in that drugs which alter the balance of ceramide and S1P levels, by modulating the activity of these enzymes, could be used to affect cell survival and proliferation. In this regard significant interest exists in drugs which could lead to increased levels of ceramide and/or decreased levels of S1P in cancer cells in order to induce apoptosis or sensitize the tumor cells to induction of apoptosis by standard chemotherapeutic or radiation therapies [5].

Ceramide can be generated in two separate ways, by breakdown of sphingomyelin due to the action of sphingomyelinases or by *de novo* synthesis. Although these pathways can lead to ceramide formation in different cellular compartments, both mechanisms have been implicated in the apoptotic response to ceramide [6, 7]. Ceramide may cause its biological effects by either direct effects on signaling proteins such as ceramide-activated protein phosphatase, ceramide-activated protein kinases, and cathepsin D [3], or by alteration of membrane properties, particularly within rafts, leading to coalescence of rafts and clustering of apoptotic signaling molecules such as cd95/fas and TRAIL [8].

S1P functions to regulate signaling pathways in two separate ways, at the cell surface through a group of five G protein-coupled receptors, and intracellularly through a receptor-independent, incompletely understood mechanism [9]. S1P receptors are members of the endothelial differentiation gene (EDG) family, and are named S1P<sub>1</sub>/EDG-1, S1P<sub>2</sub>/EDG-5, S1P<sub>3</sub>/EDG-3, S1P<sub>4</sub>/EDG-6, and S1P<sub>5</sub>/EDG-8 [10]. Through these receptors, which couple with various preferences to G<sub>i</sub>, G<sub>q</sub> and G<sub>12/13</sub>, S1P links to a variety of signaling pathways including the MAP kinases ERK, p38 and JNK, PI3 kinase, increased [Ca<sup>2+</sup>]<sub>i</sub>, Rac, Rho, and decreased, and in some cases increased, cAMP [11]. As mentioned above, S1P is usually stimulatory toward cell proliferation and inhibitory of apoptosis. S1P can also regulate cell movement and invasiveness, either positively or negatively depending on the relative amounts of the various receptors present [12, 13]. Moreover, S1P is a potent enhancer of angiogenesis [14-16].

S1P is formed by the signal transduction enzyme sphingosine kinase (SphK), which is activated by a variety of

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**Fig. (1).** Diagram depicting interconversion of sphingolipids and the enzymes involved, as well as biological responses mediated by the two main bioactive sphingolipids, ceramide and sphingosine-1-phosphate, that may be important in glioma pathobiology.

stimuli including several growth factors [17, 18], immunoglobulin receptors [19, 20], and various G protein-coupled receptors [2]. Two SphK isotypes, SphK1 and SphK2, have been cloned [21-23]. While overexpression of SphK2 has recently been shown to induce apoptosis [24], SphK1 overexpression enhances cell survival and increases cell proliferation [25].

Several studies have linked S1P and enzymes involved in its metabolism to cancer. SphK1 overexpression transforms NIH 3T3 fibroblasts, suggesting a potential oncogenic role [26]. Overexpression of SphK1 was found in several human tumor types, including breast, lung and colon tumors, compared to matched normal tissue, suggesting that this enzyme may play a role in a wide variety of tumor types [27, 28]. SphK1 was also overexpressed in chemically-induced, rat colon adenocarcinomas [29] and a mouse leukemia model [30]. Overexpression of SphK1 in MCF-7 breast cancer cells caused enhanced proliferation, decreased apoptosis and led to formation of larger tumors in nude mice in an estrogen-dependent manner [31]. Conversely, a dominant-negative form of SphK1 inhibited estrogen-mediated mitogenic signaling in MCF-7 cells and decreased tumor formation in nude mice [32]. SphK1 has also been shown to mediate VEGF-induced Ras activation in bladder cancer cells by favoring inactivation of Ras-GAP [33]. Furthermore, SphK inhibitors decreased proliferation and increased apoptosis of a panel of cancer cell lines, and decreased growth of tumors derived from mammary adenocarcinoma cells in mice without significant toxicity [27]. SphK1 overexpression also prevents apoptosis induced by serum withdrawal or chemotherapeutic drugs in several cancer types [30, 34-36]. Fur-

thermore, SphK1-overexpressing prostate cancer cells formed larger tumors in nude mice that were more resistant to treatment with the chemotherapeutic agent docetaxel, thus demonstrating the protective effect of SphK1 *in vivo* [35]. Conversely S1P lyase, which irreversibly degrades S1P, is down regulated in human colon cancer, and its overexpression enhances cancer cell apoptosis [37].

## CERAMIDE IN GLIOMAS

As discussed above, most studies on ceramide have shown that this lipid has growth inhibitory and apoptosis-inducing properties. Significant evidence exists that ceramide composition is altered in gliomas and that ceramide may play a role in regulating cell growth, apoptosis of tumor cells, or resistance to apoptosis induced by chemotherapeutic drugs in a variety of tumor cell types [2, 38], including glioma cells.

Ceramide levels are lower in human glioma tissue compared to normal surrounding brain tissue [39]. Moreover, the decrease in ceramide in gliomas is proportional to histological grade and patient survival [39], suggesting that gliomas may down regulate ceramide as a means of avoiding apoptotic cell death. In addition, the fatty acid composition of ceramides within gliomas may be important, as different ceramide species are commonly present in gliomas compared to normal brain tissue. Thus, ceramides in GBMs contain an overabundance of mono-unsaturated fatty acids (C18:1) in comparison to normal brain and low grade astrocytomas [40]. Differences were also detected between several glioma cell lines with regard to the fatty acids present in ceramides using liquid chromatography tandem mass spectrometry

[41]. Unfortunately, little is known regarding the differences in biological effects of various ceramide species, and thus, the biological or pathological significance of these observations is not currently clear.

Ceramide appears to be a common mediator of glioma cell death in response to a variety of stimuli. For example, ceramide liberated from sphingomyelin by the action of sphingomyelinases mediates apoptotic death of glioma cells induced by cytokines such as TNF- $\alpha$  [42, 43] and Fas [44]. In addition, exogenously added ceramide and Fas ligand have a synergistic effect on glioma cell apoptosis [45]. Ceramide also mediates glioma cell apoptosis in response to treatments such as gamma irradiation [46], the topoisomerase inhibitor etoposide [47] and chemotherapeutic drugs such as cisplatin [48]. In addition to mediating apoptotic signals, ceramide may also enhance apoptosis of glioma cells indirectly by inhibiting pro-survival signals. Thus, ceramide caused dephosphorylation and inactivation of the pro-survival kinase Akt through a ceramide-activated protein phosphatase in U-87 MG glioma cells [49].

A major goal of therapy for glioma patients is the induction of tumor cell apoptosis without affecting normal glia and neurons. Interestingly, ceramide mediates glioma cell apoptosis in response to a group of bioactive lipids known as cannabinoids, a promising group of compounds for glioma therapy, that signal through G protein-coupled cell surface receptors [50]. While cannabinoids are anti-proliferative and apoptotic for glioma cells, they spare or even protect non-tumor brain cells. Cannabinoids have been shown to induce apoptosis of glioma cells but not primary astrocytes or neurons [51, 52]. Several cannabinoids including the endocannabinoids anandamide, and 1-arachidonoyl glycerol, non-psychoactive synthetic cannabinoids, stearoylethanolamide and cannabidiol also kill glioma cells and decrease growth of glioma xenografts in nude mice with minimal toxicity [53-58].

Several studies have shown that the mechanism of cannabinoid induced apoptosis in glioma cells involves generation of ceramide [59]. Cannabinoids activate sphingomyelin hydrolysis in C6 rat glioma cells [51], resulting in sustained ceramide accumulation [60]. In addition, cannabinoid-induced apoptosis of glioma cells is blocked by an inhibitor of ceramide synthesis fumonisins-B1 [61]. Thus, cannabinoids appear to increase ceramide levels through both degradative and synthetic pathways in glioma cells. Interestingly, cannabinoids also block proliferative and survival signals by the extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase and Akt and activate the pro-apoptotic function Bad by decreasing its phosphorylation in C6 glioma cells [62], although it is unclear whether these effects were mediated by ceramide. It should be noted that ceramide itself, as opposed to cannabinoids, is toxic for astrocytes. In fact, cannabinoids protect primary astrocytes from apoptosis induced by exogenously added ceramide [63]. This suggests that treatment with ceramide itself would not be a viable therapeutic strategy, but rather treatment with agents that increase ceramide in tumor cells, such as cannabinoids, would be more effective and specific. Moreover, it may be possible to enhance the effectiveness of such agents, or even of conventional chemotherapeutic or radiation treatments, with

agents that block conversion of ceramide to less toxic molecules. For example, siRNA targeting acid ceramidase, was recently shown to enhance the apoptotic effect of daunorubicin on hepatoma cells, and *in vivo* use of this siRNA decreased tumor growth in a mouse hepatoma model [64].

Nevertheless, some glioma cells are resistant to the cytotoxic actions of ceramide. It may be possible to enhance ceramide sensitivity however, as siRNA blocking expression of the x-linked inhibitor of apoptosis in ceramide-resistant glioma cell lines sensitized these cells to ceramide-induced cell death [65]. Thus, while drugs that enhance ceramide levels may be useful against gliomas, in some cases it may be necessary to combine such drugs with other therapies that enhance tumor cell sensitivity.

Although most studies of the toxic effects of ceramide have focused on apoptosis induction a few have shown that ceramide can cause non-apoptotic death of glioma cells.

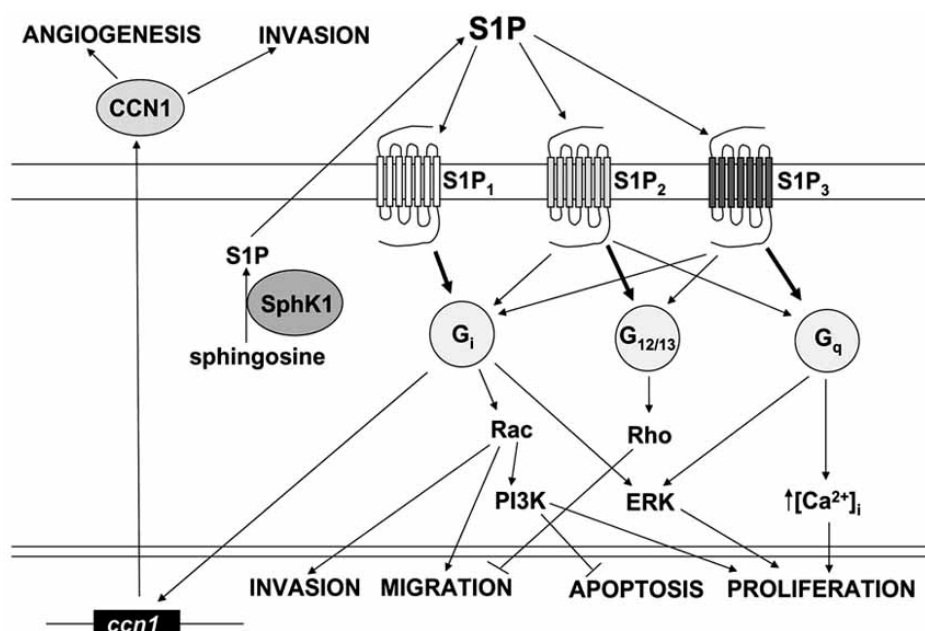
Ceramide causes death of several human glioma cell lines in the absence of caspase activation or loss of mitochondrial membrane potential [66, 67]. Moreover, ceramide-induced death of U-373 MG and T98G human glioma cells was due to induction of autophagy [68].

### SPHINGOSINE-1-PHOSPHATE IN GLIOMAS

Several studies have examined signal transduction and biological effects of S1P on glioma cells. A simplified diagram depicting likely signaling pathways utilized and biological effects of S1P on glioma cells is shown in Fig. (2). Several studies have used the rat glioma model C6. S1P activates a wide variety of signaling pathways in these cells including phospholipase C /  $Ca^{2+}$  signaling, ERK MAP kinase, protein kinase C (PKC) and phospholipase D [69]. C6 cells express high levels of the S1P receptor  $S1P_2$  [69], which couples to  $G_i$ ,  $G_q$ , and  $G_{12/13}$  [13], a small amount of the receptor  $S1P_1$ , which couples exclusively to  $G_i$  [70], and  $S1P_5$  [71], which couples to  $G_i$  and  $G_{12}$  [72]. Through these receptors and signaling pathways, S1P stimulates expression of several immediate early genes. S1P-stimulates  $G_i$  and PKC signaling leading to ERK activation, EGR-1 expression and FGF-2 expression in C6 cells [73]. S1P also enhances c-fos expression [74]. The effect of S1P on ERK / EGR-1 / FGF-2 is mediated by  $S1P_1$ , while  $S1P_2$  mediates activation of phospholipase C, increased  $[Ca^{2+}]_i$  and phospholipase D [75].

S1P has important biological effects on C6 glioma cells as well. Consistent with activation of the above pathways, overexpression of SphK1, the enzyme which forms S1P, in C6 cells drives cell proliferation in the absence of serum [76]. Moreover, S1P is released extracellularly by C6 glioma cells [77] as well as astrocytes [78, 79]. Thus, the potential for an autocrine loop of S1P signaling that enhances growth and/or survival of glioma cells exists.

Our lab has examined the role of S1P and SphK in human glioma cells. Human glioma cell lines and glioma tissue commonly express  $S1P_1$ ,  $S1P_2$  and  $S1P_3$  receptors, with no  $S1P_4$  and only very low levels of  $S1P_5$  detectable [80, 81]. We have found that exogenously added S1P is mitogenic for 50% of human glioma cell lines examined, by signaling through ERK MAP kinase and PI 3-kinase  $\beta$  [82]. The mitogenic response to S1P is strong in some glioma cell lines and



**Fig. (2).** Diagram depicting S1P signaling through its receptors in glioma cells, and potential links to cell proliferation, migration, invasion, survival and angiogenesis. Bold arrows indicate preferential G protein coupling of S1P receptor subtypes. Further details of the linkage of individual receptors to various pathways and biological responses are discussed in the text.

weak in others [82, 83]. The potency of S1P on these cells may be determined by the relative S1P receptor expression in these cells, as cell lines which express low levels of the S1P<sub>1</sub> receptor did not respond mitogenically to S1P, suggesting that this receptor is crucial for proliferative responses [81]. Interestingly, overexpression of any of the three widely expressed S1P receptors, S1P<sub>1</sub>, S1P<sub>2</sub>, or S1P<sub>3</sub>, in a human glioma cell line that normally expresses low S1P receptor levels enhances S1P-stimulation of proliferation, however, in agreement with results discussed above, S1P<sub>1</sub> is the most potent [84]. Similarly, knockdown of any of the three S1P receptors in a human glioma cell line that normally expresses high S1P receptor levels enhances S1P-induced proliferation, again with S1P<sub>1</sub> being the most effective [84].

While S1P receptors likely mediate at least some of the downstream effects of S1P in glioma cells, expression levels of S1P receptors did not correlate with tumor malignancy or patient survival. However, measurement of SphK transcript levels in GBM tissue by real time PCR analysis revealed that high levels of SphK1 expression correlate with significantly shorter patient survival [80]. Patients whose tumors were among the highest one third with regard to SphK1 expression survived a median 102 days, while those within the lower two thirds survived a median 357 days. Thus, high SphK1 expression could be useful as an indicator of poor prognosis for GBM patients. Furthermore, we showed that SphK1 is important for proliferation of glioma cell lines, as a SphK inhibitor or knock down of SphK1 expression with siRNA decreased proliferation by preventing entry into the cell cycle [80]. SphK2 knock down has a similar effect, however expression of this isoform did not correlate with patient survival [80], suggesting other functions for SphK1 besides driving cell proliferation, that may contribute to GBM malignancy. Thus, inhibition of SphK1 or knock down of SphK1 expression by siRNA could be useful to decrease

malignant behavior of GBMs. In addition, agents that block signaling through S1P receptors might be useful as most of the cellular responses to S1P are thought to be receptor mediated.

Enhanced GBM malignancy in relation to SphK1 expression may be related to tumor cell invasion. S1P stimulates approximately 50% of human glioma cells to migrate and invade through Matrigel [81]. The motility/invasion response to S1P was less clearly correlated with receptor subtype expression than the mitogenic response, however, glioma cells in which S1P does not stimulate enhanced motility tended to express higher proportions of S1P<sub>2</sub> [81]. This result is expected, as S1P<sub>2</sub> has previously been shown to block cell motility induced by S1P<sub>1</sub> and S1P<sub>3</sub> [85]. In agreement, migration of some glioma cell lines is inhibited by S1P through S1P<sub>2</sub> signaling [83, 86]. Thus, cell lines expressing high levels of S1P<sub>2</sub>, are inhibited from migration by S1P, while those expressing low levels are stimulated, and the contrasting effects of these receptors on cell migration were confirmed by overexpression of S1P<sub>1</sub> or S1P<sub>2</sub> in glioma cells, thus favoring stimulation or inhibition of migration respectively [83]. Glioma cell lines [81] and glioma tissue [80] commonly express all three of the S1P receptors discussed above. Utilizing tissue from two GBM cases, Malchinkhuu *et al.* suggested that S1P<sub>2</sub> receptor expression is upregulated in astrocytoma cells in comparison to normal astrocytes [86]. However, we recently examined expression levels of S1P<sub>1</sub>, S1P<sub>2</sub>, and S1P<sub>3</sub> by real time PCR analysis in 48 cases of GBM in comparison to 20 cases of the relatively benign pilocytic astrocytoma. We found that receptor expression was highly variable among different tumors, however, no significant difference in expression of S1P<sub>1</sub>, S1P<sub>2</sub>, or S1P<sub>3</sub> between these two groups was detected [80]. Moreover, in most tumors examined S1P<sub>2</sub> expression was lower than that of S1P<sub>1</sub>, or S1P<sub>3</sub>, when data were expressed as overall expression

level or as a percentage of the three S1P receptors. Thus, while S1P<sub>2</sub> may lead to inhibition of glioma cell motility in a few cases, this can not be generalized to GBMs as there is no consistent up regulation of S1P<sub>2</sub> expression in these tumors.

In a variety of different cell lines the S1P<sub>2</sub>-mediated decrease in cell migration is due to inhibition of the small GTPase Rac [85, 87, 88]. However, in glioma cell lines, although S1P<sub>2</sub> decreased cell migration as expected, it did not decrease Rac activity [83]. We have recently confirmed this finding using U-118 MG glioma cells overexpressing S1P<sub>2</sub> [84]. Thus S1P<sub>2</sub> inhibition of glioma cell migration appears to be mediated by a different mechanism. This may involve excessive activation of Rho leading to stress fiber formation [84] and, in some cells, cell rounding mediated through Rho kinase [83].

Interestingly, we recently found that, although S1P<sub>2</sub> overexpression decreases glioma cell motility, it actually enhances invasion of glioma cells through Matrigel by a mechanism involving stimulation of expression of the secreted matricellular protein CCN1/Cyr61 and cell adhesion [84]. Induction of CCN1 expression was mediated by S1P<sub>1</sub> through Gi signaling and S1P<sub>2</sub> through unknown pathways [84]. Interestingly, CCN1 is known to be a pro-angiogenic protein [89]. Furthermore, overexpression of CCN1 in GBM correlates with short survival time of patients [90]. In addition, microarray analysis of S1P-stimulated glioma cells revealed that S1P up regulates several genes that may enhance glioma cell invasiveness [91]. Current work is focusing on examination of the roles played by several of these genes in S1P-stimulated glioma invasion. Clearly, regulation of glioma cell migration and invasion by S1P is a complex phenomenon involving several S1P receptors and multiple signaling pathways. It is possible that additional complexities such as heterodimerization of S1P receptors with each other [92] or with other related receptors [93] could modulate the responsiveness of glioma cells to S1P.

S1P has also been shown by several groups to stimulate angiogenesis in a variety of *in vitro* and *in vivo* assay systems [14, 15, 94]. In addition, an antibody specific for S1P effectively blocks angiogenesis in several mouse tumor models, although brain tumor models were not investigated [95]. GBMs are highly angiogenic tumors, and angiogenesis is thought to be an important aspect of the highly malignant behavior of these tumors. Thus, stimulation of angiogenesis could contribute to the enhancement of GBM malignancy by S1P produced by SphK. However, contrary to its stimulatory effects on most endothelial cells, S1P inhibits migration and tube formation by several different brain-derived endothelial cells [96]. Nevertheless, S1P could enhance angiogenesis by stimulation of expression of other pro-angiogenic factors, for example CCN1/Cyr61 as discussed above. In agreement, microarray analysis of S1P-stimulated glioma cell lines revealed that S1P up regulates expression of several genes that may be involved in induction of glioma angiogenesis [91].

## CONCLUSIONS

Sphingolipids regulate a wide variety of cellular responses including cell proliferation, survival and migration as well as complex biological processes such as angiogene-

sis, all of which are important aspects of tumor cell behavior. The ability for these lipids to be interconverted along with the contrasting effects of ceramide and S1P, provides potential targets for therapeutically manipulating these processes. Drugs that stimulate sphingomyelinase or *de novo* sphingolipid synthesis in order to increase ceramide concentrations could induce tumor cell apoptosis. Combining such treatments with other drugs to inhibit SphK and/or enzymes that convert ceramide into more complex sphingolipids could be useful to enhance the effectiveness of such treatments. The studies summarized in this review emphasize the possibility that such therapies may be particularly useful for the highly malignant GBM.

Strategies for blocking conversion of ceramide into less toxic sphingolipids have been recently reviewed, including pharmacologic inhibition of ceramidase, and antisense-mediated knockdown of other enzymes such as glucosylceramide synthase [5]. SphK inhibitors currently available include DL-threodihydrosphingosine, dimethylsphingosine [97] and 2-(p-Hydroxyanilino)-4-(p-chlorophenyl) thiazole [27]. The last of these has been shown to decrease growth of a mammary adenocarcinoma model in mice [98]. Additional strategies may involve blocking the function of S1P receptors. Although several research groups and pharmaceutical companies are currently working on developing S1P receptor antagonists, such compounds, particularly ones that show specificity for individual S1P receptors, are not currently available. Finally, it will be necessary to better understand the mechanisms by which S1P enhances GBM malignancy. For example, what are the relative contributions of S1P-driven tumor cell proliferation, survival and invasion as well as tumor angiogenesis, and which S1P receptors are involved in which of these processes. Knock out mice or mice deficient in many of the enzymes discussed above [104-107], as well as the S1P<sub>2</sub> and S1P<sub>3</sub> receptors [108] exist. Thus, it is possible that future work with such mice could help to elucidate the roles of various members of these pathways in gliomagenesis and glioma malignancy. In addition, GBM tumor stem cells, which appear to be the cells that drive tumor formation, have recently been discovered [99, 100]. These cells form tumors in mice that closely resemble actual GBMs with regard to histology, invasiveness and angiogenesis, while traditional glioma cell lines do not [101-103]. It will be interesting to determine whether sphingolipids play similar roles in these cells, and whether targeting sphingolipid pathways is effective therapeutically against these tumors.

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