Glioblastoma

Swapan K. Ray
Editor

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Molecular Mechanisms of Pathogenesis and Current Therapeutic Strategies

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Preface

Glioblastoma is the most malignant brain tumor that even now remains incurable. It is such a deadly disease that patients in most cases do not survive more than a few months after the diagnosis. Our understanding of the cellular and molecular mechanisms of the formation of glioblastoma is rapidly advancing so as to provide us clues for devising rational therapeutic strategies for the treatment of this malignancy. It is important that we continue to increase our knowledge about the aberrant molecular markers for pathogenesis of this devastating disease and thereby explore new areas ultimately to find successful therapeutic strategies.

This book has a collection of chapters that include the latest understandings of histopathology, molecular genetics, aberrant signaling pathways, cytoskeleton dynamics, epigenetics, and role of stem cells in gliomagenesis, and also promising treatment strategies with the contributions from the prominent basic scientists and clinicians, who persistently work hard to make a difference and give fresh hope to the glioblastoma patients to survive longer. Various approaches such as sophisticated imaging techniques, improved surgical procedures, and new strategies for radiation therapy, combination chemotherapy, immunotherapy, chemoimmunotherapy, dietary therapy, and nanobiotherapy are being explored for the treatment of glioblastoma.

All the contributors to this book have explicated their earnest desire to make use of the latest knowledge about the pathogenesis of glioblastoma to indicate the novel targets and innovative therapeutic strategies. It is keenly contemplated that this book will be an important source of information on glioblastoma to the graduate students, postdoctoral fellows, principal investigators, and clinicians, who are interested and engaged in finding a cure for glioblastoma.

Columbia, SC — Swapan K. Ray

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Overview of the Molecular Genetics and Molecular Chemotherapy of GBM

Herbert B. Newton

Abstract Glioblastoma multiforme (GBM) remains refractory to conventional treatment approaches, including radiotherapy and cytotoxic chemotherapy. Molecular neurooncology has now begun to clarify the transformed phenotype of GBM and identify oncogenic pathways that might be amenable to small molecule and antibody "targeted" therapy. Growth factor signaling pathways are often upregulated in these tumors and contribute to oncogenesis through autocrine and paracrine mechanisms. Excessive growth factor receptor stimulation can also lead to overactivity of the downstream Ras signaling pathway. Other internal signal transduction pathways that may become dysregulated during transformation of GBM include Raf, MEK, PI3K, Akt, and mTOR. In addition, overactivity of vascular endothelial growth factor (VEGF) and other effectors leads to neoplastic angiogenesis. "Targeted" therapy against the growth factor signaling and Ras pathways include tyrosine kinase inhibitors (e.g., imatinib, erlotinib) and farnesyltransferase inhibitors (e.g., tipifarnib). Molecular therapeutic small molecules specific to Raf, PI3K, and mTOR include sorafenib, LY294002, and CCI-779, respectively. "Targeted" antiangiogenesis approaches include monoclonal antibodies to VEGF (e.g., bevacizumab) and vascular endothelial growth factor receptor tyrosine kinase inhibitors (e.g., vatalanib, sunitinib). Further development of "targeted" therapies designed to modulate the activity of these pathways, and evaluation of these new agents in clinical trials will be needed to improve survival and quality of life for patients with GBM.

Keywords Neurooncology • Molecular • Brain tumor • GBM • Signal transduction • Growth factors • Ras • Raf • PI3K • Akt • mTOR • Imatinib • Gefitinib • Erlotinib • Bevacizumab

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Introduction

Glioblastoma multiforme (GBM) and its variants are the most malignant forms of astrocytoma and are classified as WHO grade IV in the most recent neuropathological classification schemes (Newton 1994; Kleihues et al. 2002). It is the most common primary brain tumor (PBT) in adults, accounting for approximately 40% of all primary tumors and 80% of all high-grade primary central nervous system (CNS) neoplasms (Davis and McCarthy 2001, Wrensch et al. 2002). In North America, this corresponds to approximately 5 per 100,000 affected individuals per year. GBM can affect patients at any age, including young children, but has a peak incidence between ages 65 and 75. The tumor appears to have a gender predilection, with an incidence that is 40% higher in men than in women. In addition, GBM has a higher incidence in Caucasians than in other ethnic groups. Although GBM accounts for only about 1% of all adult cancers, it results in approximately 2% of all cancer-related deaths each year (American Cancer Society 2002).

The prognosis and survival of patients with GBM remains poor, with a median survival of approximately 12 to 14 months (Newton 1994; Davis and McCarthy 2001; Wrensch et al. 2002). It is obvious from these survival data that the current treatment of these patients (i.e., surgical resection, irradiation, conventional chemotherapy) is inadequate and unable to overcome the malignant biology of the tumor. Although cytotoxic chemotherapy has been demonstrated to provide a survival benefit to high-grade glioma patients in several recent meta-analyses, the results have been modest (Fine et al. 1993; Stewart et al. 2002). One prominent explanation for the poor results of conventional chemotherapy is the nonspecific, nontargeted nature of most of the currently used drugs (e.g., alkylating agents, DNA topoisomerase inhibitors, spindle poisons) (Newton et al. 1999; Newton 2006). Other potential causes for the poor treatment results in GBM patients include inadequate delivery of many drugs across the blood–brain and blood–tumor barriers (BBB, BTB), and the high degree of intrinsic chemotherapy resistance prevalent among high-grade brain tumors.

As mentioned above, conventional forms of treatment for GBM are not predicated on the biology of the malignant phenotype. It has become apparent that the transformed phenotype of brain tumor cells is highly complex and results from the dysfunction of a variety of interrelated regulatory pathways (Chung and Seizinger 1998; Maehama and Dixon 1998; von Deimling et al. 1995; Shapiro and Coons 1998). The transformation process involves amplification or overexpression of oncogenes in combination with loss or lack of expression of tumor suppressor genes. Oncogenes that have been demonstrated to be important for gliomagenesis include platelet-derived growth factor and its receptor (PDGF, PDGFR), epidermal growth factor and its receptor (EGF, EGFR), CDK4, mdm-2, Ras, Akt, and mTOR (mammalian target of rapamycin). Tumor suppressor genes of importance in glial transformation include p53, retinoblastoma (Rb), p16 and p15 (i.e., INK4a, INK4b), DMBT1, and PTEN. Most of these tumor suppressor genes function as negative regulators of the cell cycle, while others are inhibitors of important internal

signal transduction pathways. The net effect of these acquired abnormalities is a dysregulation of, and an imbalance between, the activity of the cell cycle and apoptotic pathways.

Due to the inadequacy of conventional therapeutic approaches, it is obvious that new treatment modalities must be developed that have a more molecular, "targeted" mechanism of action, with the ability to overcome the transformed phenotype of GBM (Mischel and Cloughesy 2004; Newton 2003; Newton 2004; Newton 2005; Rich and Bigner 2004; Tremont-Lukas and Gilbert 2003). Recent advances in growth factor and signal transduction biology are now providing the background for the development of small molecule and antibody-based "molecular therapeutics," a new class of drugs that manipulate and exploit these pathways. The following sections will delineate the molecular biology of the key transformation pathways in GBM and describe preclinical data and clinical trial results with "targeted," small molecule and monoclonal antibody-based molecular therapeutic agents.

Growth Factor Signaling – PDGF and PDGFR

PDGF is a 30-kDa protein that has four chains (A, B, C, D) (Shapiro and Coons 1998; Gilbertson et al. 2001; Guha et al. 1995; LaRochelle et al. 2001; Lokker et al. 2002; Maher et al. 2001; Westermark et al. 1995). Four separate genes control the expression of the different chains of PDGF. There is a similar organizational pattern within each gene, with the presence of seven exons. Each PDGF chain is synthesized as a higher molecular weight precursor protein that undergoes posttranslational modification. Active PDGF consists of disulfide-bonded homodimers or heterodimers of the various chains. There are also two forms of PDGFR, α and β, that are encoded by separate genes and also function as homodimers or heterodimers.

The PDGFR is a transmembrane glycoprotein that belongs to the protein-tyrosine kinase family of growth factor receptors (Shapiro and Coons 1998; Gilbertson et al. 2001; Guha et al. 1995; LaRochelle et al. 2001; Lokker et al. 2002; Maher et al. 2001; Westermark et al. 1995; Claesson-Welsh 1994; Hubbard and Till 2000). Binding of the divalent PDGF ligand to the receptor induces dimerization of two receptors and activation of the internal receptor complex. Receptor dimerization brings the catalytic domains of the internal tyrosine kinase regions into juxtaposition, allowing for autophosphorylation of the catalytic domain tyrosine residues and formation of attachment sites for internal signal transduction molecules. These signal transduction molecules include Src, Shc, Grb2, Nck, GTPase activating protein of Ras (Ras GAP), phosphatidylinositol-3′-kinase (PI3-kinase), phosphotyrosine phosphatase-1D, Janus kinases (JAK), STATs (signal transducers and activators of transcription), and phospholipase C-γ (PLC-γ) (Westermark et al. 1995; Claesson-Welsh 1994). Phosphorylation of these second messengers induces a complex cascade of activities within the cell, as downstream pathways become activated (see Fig. 1).

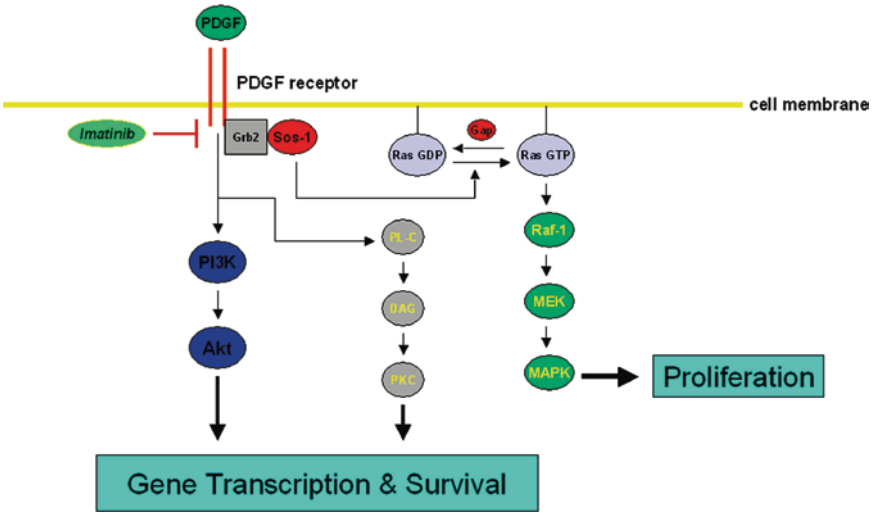

Fig. 1 PDGF binds to the extracellular portion of protein tyrosine kinase receptor PDGFR, inducing dimerization of two receptors and autophosphorylation of the catalytic domain tyrosine kinase residues. Receptor activity leads to attachment of molecules with SH2 domains, such as Grb2, PL-C, and PI3K, activating downstream signaling pathways that promote proliferation, gene transcription, and cellular survival. "Targeted" therapy of the pathway is depicted, using the receptor tyrosine kinase inhititor Imatinib mesylate (Gleevec). Adapted from reference (Newton 2003) and used with permission from Future Drugs, Ltd

PDGF and PDGFR are important oncogenic influences in the transformation process of glial tumors (Shapiro and Coons 1998; Gilbertson et al. 2001; Guha et al. 1995; LaRochelle et al. 2001; Lokker et al. 2002; Maher et al. 2001; Westermark et al. 1995). Studies in human glioma cell lines consistently demonstrate the expression of PDGF. The majority of glioma cell lines also concomitantly expressed variable amounts of PDGFR. Southern blot analyses have failed to demonstrate amplification or rearrangement of the PDGF or PDGFR genes in glioma cell lines. The enhanced expression of PDGF and PDGFR is most likely caused by aberrant regulation of structurally intact genes. Recent studies in cell lines have proved the presence of autocrine stimulation by PDGF and PDGFR (Guha et al. 1995; Lokker et al. 2002; Westermark et al. 1995). Analysis of human glioma tissue suggests that PDGF and PDGFR expression is present in tumors of all grades, and that the development of autocrine stimulatory loops is an early oncogenic transforming event. Extensive studies in resection specimens by Hermanson and colleagues demonstrated PDGF-A and PDGF-B expression in astrocytomas of all grades, with higher-level expression in more malignant tumors (Hermanson et al. 1992). Amplification of the PDGFR-α gene is very uncommon in resection specimens.

The demonstration of an important role for PDGF and PDGFR in the oncogenesis of gliomas has led to an aggressive search for strategies that can inhibit this pathway (Newton 2003; Adjei and Hidalgo 2005). The focus has been on the development of small molecule inhibitors of internal tyrosine kinase activity. The most

promising receptor tyrosine kinase inhibitor with activity against PDGFR is imatinib mesylate (i.e., Gleevec, STI571) (Newton 2003; Adjei and Hidalgo 2005). Imatinib blocks the ATP binding site of tyrosine kinase receptors, inhibiting this activity and interfering with the transduction of receptor-mediated signals to internal effectors (see Fig. 1). It has demonstrated significant in vitro activity against bcr-abl, c-kit, and PDGFR. In studies of GBM, imatinib was able to inhibit several different cell lines that had PDGF-mediated autocrine growth activity, as well as xenografted tumors (Kilic et al. 2000).

A phase I/II study of imatinib in patients with recurrent malignant gliomas is ongoing by the North American Brain Tumor Consortium (NABTC 99-08) (Wen et al. 2004). In the phase I portion of the study, 47 patients (GBM-32) have been accrued. Twenty-seven patients were receiving enzyme-inducing antiepileptic drugs (EIAED), while 20 patients were on nonenzyme-inducing antiepileptic drugs (NEIAED). The maximum tolerated doses (MTD) for the EIAED and NEIAED cohorts were 1,200 and 800 mg/day, respectively. For EIAED patients, the mean C_{max} and AUC of imatinib were decreased by more than 50%. There was only one patient with an objective response (complete responses, CR 0; partial responses, PR 1), along with six patients with stable disease (SD). The phase II portion of the study was restricted to NEIAEDs and enrolled a total of 48 patients. In the GBM cohort, there were 0 CR, 1 PR, and 6 SD, with a 6-month progression-free survival (PFS) of only 3%. For the AA/AO cohort, there were 0 CR, 0 PR, and 2 SD, with a 6-month PFS of 11%. A similar European phase II study also used single-agent imatinib (600 or 800 mg/day) for patients with recurrent GBM ($n=51$) and had more promising results (van den Bent et al. 2004). Responses included three patients with PR (10, 10, and 12+ months) and five patients with extended SD for more than 6 months. A phase II trial from a Brazilian group reports treating 20 patients with recurrent GBM that were selected on the basis of positive PDGFR expression via immunohistochemical analysis (Viola et al. 2007). Single-agent imatinib (800 mg/day) was administered until tumor progression; all patients were taking EIAED. The 6-month PFS rate was 52.2%, with a median PFS of 7.8 months. There were no CR or PR; 13 patients (65%) had stabilization of disease by MRI.

Several groups have attempted imatinib in combination with conventional and molecular forms of chemotherapy. In one report of an ongoing phase I trial, escalating doses of imatinib were used in combination with temozolomide (200 mg/m^2/day per cycle) (Sathornsumetee et al. 2006). A total of 56 patients had been accrued and stratified by usage of EIAED or NEIAED, including 46 with GBM and 9 with anaplastic gliomas. The MTD had not been reached for either stratum. There were four patients with PR, while another six patients had maintained SD for ten or more cycles. Thirty-two patients had progressive disease (57%) and discontinued treatment. The regimen was relatively well tolerated, with few cases of high-grade toxicity. In a phase II study of 30 patients with recurrent GBM after failure of temozolomide plus nitrosourea, imatinib (400 mg/day) was used in combination with hydroxyurea (500 mg twice daily), a ribonucleotide reductase inhibitor that disrupts DNA synthesis (Dresemann 2005). There was 1 CR, 5 PR, 11 SD, and 13 with progressive disease (PD). The 6-month and 2-year PFS rates were

32% and 16%, respectively. Median overall survival was 19 weeks, with a median TTP of 10 weeks. A follow-up study by the same group used imatinib (600 mg/day) and hydroxyurea (1000 mg/day) as maintenance treatment for a cohort of 30 GBM patients with SD (Dresemann et al. 2007). All of the patients had received prior irradiation and at least one form of chemotherapy (temozolomide – 21). The 6-, 12-, and 18-month PFS rates were 60%, 40%, and 30%, respectively. Overall survival at 12 and 18 months was 67% and 53%, respectively. The regimen was well tolerated, with minimal grade 3 and 4 toxicity. A similar study reported by Reardon and colleagues treated recurrent GBM patients with imatinib and hydroxyurea (Reardon et al. 2005). Thirty-three patients received imatinib at a dose of either 500 mg twice a day (on EIAED) or 400 mg/day (on NEIAED), in combination with hydroxyurea at 500 mg twice a day. The 6-month PFS rate was 27%, with a median PFS of 14.4 weeks. Three patients (9%; 1 CR, 2 PR) had objective responses by MRI, while 14 patients (42%) had stabilization of disease. The clearance of imatinib appeared to be accelerated by the concomitant use of hydroxyurea. Using the same dosing regimen, the Duke group has also applied imatinib and hydroxyurea to recurrent or progressive low-grade gliomas (Bota et al. 2007). Twenty-seven patients have been treated thus far (17 astrocytoma, 10 oligodendroglioma). There were no CR or PR; 17 patients (85%) have had stabilization of disease, while 3 (15%) had rapid progression. Further follow-up is needed before TTP and survival data can be determined. Although the majority of patients receiving imatinib and hydroxyurea appear to tolerate the regimen without significant complications, one report suggests that hematologic toxicity can be very severe and may correlate with disease control (Shah et al. 2006). In a series of 14 patients with recurrent high-grade glioma treated with imatinib (400 mg/day) and hydryoxyurea (500 mg bid), four responsive patients (2 PR, 2 SD) were noted to have significant hematological toxicity. However, none of the patients with PD had therapy-limiting toxicity. The authors concluded that responsive patients with significant myelosuppression might be candidates for peripheral stem cell harvesting and autologous stem cell rescue.

Based on the tolerability and efficacy of the imatinib/hydroxyurea combination, several groups have added a third, "targeted" drug to the regimen, in an attempt to further boost efficacy. In one phase I trial, vatalanib (PTK787/ZK22584; 250–500 mg twice daily), a vascular endothelial growth factor receptor (VEGFR) inhibitor, was added to imatinib and hydroxyurea, with dosing stratified for EIAED versus NEIAED (Sathornsumetee et al. 2007). Thirty-five patients with recurrent GBM have been treated thus far. MTDs have not been reached for either stratum. Neuroimaging evaluation has demonstrated 10 PR (29%), with 9 patients continuing to remain on study. Toxicity has been mild in the majority of patients. In another phase I study with a similar dose escalation and stratification scheme, RAD001 (2.5 mg/day), an oral inhibitor of mTOR (see below), was added to imatinib and hydroxyurea (Desjardins et al. 2006). Twenty-eight patients with recurrent GBM have been enrolled. There has been 1 PR noted by MRI; 15 patients remain on study. The regimen has been well tolerated by the majority of patients.

Growth Factor Signaling – EGF and EGFR

EGF is a 6.5-kDa polypeptide that is composed of 53 amino acids and functions as a monomeric ligand (Shapiro and Coons 1998; Maher et al. 2001; Carpenter and Cohen 1979). The mature form of EGF is liberated from a 130-kDa precursor molecule following posttranslational modification. EGF binds to the EGFR, which has only one active form. The EGFR (ErbB1) is a 170-kDa transmembrane glycoprotein of the ErbB family of receptor tyrosine kinases, which also includes ErbB2 (HER2 or Neu), implicated in the pathogenesis of breast cancer (Maher et al. 2001; Hubbard and Till 2000; Arteaga 2001; Hackel et al. 1999; Normanno et al. 2005; Woodburn 1999; Yarden 2001). In addition to EGF, the receptor can also bind and become activated by other monomeric ligands (e.g., TGF-α). Binding of the ligand to the extracellular region of the receptor (domains I–IV) induces receptor dimerization. Crystal structure analyses at 2.8 and 3.3 A resolution have demonstrated that initial ligand binding promotes a large rearrangement of the dimerization interface, thus exposing a protruding β-hairpin arm from each domain II which can interact and mediate dimerization (Ferguson et al. 2003; Ogiso et al. 2002). After receptor dimerization is complete, activation of the cytoplasmic regions occurs, with autophosphorylation of catalytic-domain tyrosine residues. The phosphorylated tyrosine residues serve as docking sites for signal transducers and adaptor molecules. Activated cytoplasmic signal transducers and pathways are similar to PDGFR (see Fig. 2) and include PLC-γ, Ras GAP, PI3-K and Akt, the JAK-STAT system, Src, Shc, Grb2, and stress-activated protein kinases.

The EGF-EGFR signaling pathway has a critical role in the oncogenesis and transformation process of many solid tumors, including GBM (von Deimling et al. 1995; Normanno et al. 2005; Arita et al. 1989; Frederick et al. 2000; Hurtt et al. 1992). This was suspected following the discovery that several viral products were able to mimic or modify EGFR function, including the avian erythroblastosis virus v-erbB oncogene and E5, a product of the human papillomavirus.

In resting, nontransformed cells, EGFR concentration and signaling activity are tightly controlled and regulated. In gliomas and other solid tumors, the membrane density of EGFR is often excessive, and the signaling pathway is hyperactive (Maher et al. 2001; Arteaga 2001; Frederick et al. 2000). High-grade gliomas, especially GBM, are known to overexpress EGF, TGF-α, and EGFR, consistent with autocrine and paracrine stimulatory loops. Amplification of wild-type EGFR is the most common oncogenic alteration in GBM, occurring in 40–50% of all primary tumors (Maher et al. 2001; Frederick et al. 2000). EGFR amplification is less common and has a smaller gene dosage in lower-grade gliomas. In GBM, amplification of the wild-type gene appears to be a precursor to subsequent mutations of EGFR, which usually involve intragene deletions (Frederick et al. 2000). These mutations further augment receptor signaling activity, promoting tumor growth. The most common mutation of EGFR is the vIII mutant (also known as ΔEGFR), which occurs in 67% of EGFR-positive tumors, and involves a 5′ deletion of codons 6-273 (Frederick et al. 2000; Ekstrand et al. 1994; Wong et al. 1992).

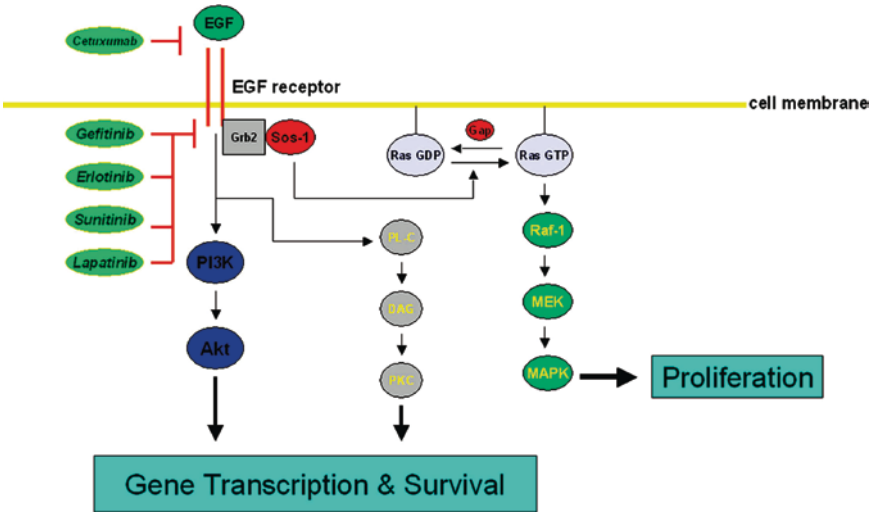

Fig. 2 EGF binds to the extracellular portion of protein tyrosine kinase receptor EGFR, inducing dimerization of two receptors and autophosphorylation of the catalytic domain tyrosine kinase residues. Receptor activity leads to attachment of molecules with SH2 domains, such as Grb2, PL-C, and PI3K, activating downstream signaling pathways that promote proliferation, gene transcription, and cellular survival. "Targeted" therapy of the pathway is depicted, using the receptor tyrosine kinase inhititors Gefitinib (Iressa), Erlotinib (Tarceva), Sunitinib (Sutent) and Lapatinib, as well as the anti-EGFR monoclonal antibody Cetuximab (IMC-225). Adapted and modified from reference (Newton 2003) and used with permission from Future Drugs, Ltd

This mutation eliminates exons 2–7 of the EGFR mRNA, thereby removing the ligand-binding domain of the receptor protein and allowing for ligand-independent, constitutive activity. Unlike wild-type EGFR, these constitutively active mutants are not downregulated and processed for lysosomal degradation.

Amplification and mutation of EGFR, with subsequent alteration of EGFR expression, has multiple effects on GBM cells that promote growth and survival (Shapiro and Coons 1998; Maher et al. 2001; Hackel et al. 1999; Barker et al. 2001; Biernat et al. 1997; Chakravarti et al. 2002; Lal et al. 2002; Nagane et al. 1998; Nishikawa et al. 1994; Penar et al. 1997). The presence of EGFR mutations in glioma cells enhances tumorigenicity by increasing proliferative capacity and reducing apoptosis. Activation of Bcl-X_L, an antiapoptotic prosurvival factor, is the presumed mechanism by which mutants reduce apoptotic activity (Nagane et al. 1998). Tumor cells containing mutations also become more invasive. Effectors of tumor invasion, such as matrix metalloproteases and serine proteases, are upregulated in cells with mutant EGFR (Lal et al. 2002). Methods to reduce EGFR activity (neutralizing antibodies, antisense molecules, EGFR inhibitors) can reduce cellular motility and invasiveness (Maher et al. 2001; Penar et al. 1997). The presence of mutant EGFR confers resistance to radiation and chemotherapy (e.g., cisplatin). In general, the presence of EGFRvIII is associated with a poor prognosis. Patients with GBM overexpressing EGFRvIII have shorter overall survival times and a more rapid time to tumor progression (Feldkamp et al. 1999a).

There has been an intense effort to develop "targeted" therapies to the EGF/EGFR signaling pathway (Adjei and Hidalgo 2005). The two most promising approaches are monoclonal antibodies (mAb) to the EGFR and small molecule inhibitors of EGFR tyrosine kinase activity (see Fig. 2). The rationale for mAb therapy was to develop highly specific, high-affinity agents that can interfere with normal ligand binding and inhibit internal signal transduction (Dicks and Rutka 1997). Several mAbs to EGFR have been developed and are undergoing clinical trials for solid tumors (Arteaga 2001; Woodburn 1999; Yarden 2001; Baselga and Arteaga 2005; Dillman 2001; Grünwald and Hidalgo 2003; Herbst and Shin 2002; Mendelsohn 2002; Mendelsohn and Baselga 2003; Nathoo et al. 2004). One of the most active and promising antibodies is IMC-C225 (Cetuximab), a chimeric human/mouse version of mAb 225 that uses the mouse variable region heavy and light chains in combination with human IgG_1 in the constant region (Sathornsumetee et al. 2006; Baselga and Arteaga 2005). IMC-C225 binds to EGFR with higher affinity than EGF or TGF-α, prevents ligand binding, blocks ligand-induced tyrosine kinase activation, and stimulates receptor internalization (see Fig. 2). In addition, IMC-C225 can induce apoptosis and inhibit angiogenesis in cultured tumor cells and tumor xenografts, and is able to augment the effects of irradiation in EGFR-amplified GBM (Nathoo et al. 2004; Baselga 2001; Eller et al. 2002; Eller et al. 2005). Another well-studied monoclonal antibody is mAb 806, which was raised against the EGFRvIII form of the receptor (Dillman 2001). It is now known that mAb 806 recognizes both the EGFRvIII variant and a subset of wild-type EGFR when overexpressed in tumor cells, but does not bind to wild-type EGFR expressed in normal tissues (Luwor et al. 2001). The antibody appears to bind to an epitope of the receptor (a disulfide-bonded cysteine loop) that is only present in a "transitional" configuration, which occurs as the receptor changes from its inactive tethered conformation to a dimeric untethered form (Johns et al. 2004). When glioma cell lines and mouse xenografts that overexpress EGFRvIII are treated with mAb 806, a significant, dose-dependent inhibition of growth is noted (Luwor et al. 2001). Furthermore, treatment of human tumor xenografts with mAb 806 in combination with more traditional anti-EGFR monoclonal antibodies (e.g., mAb 528) results in additive and, in some cases, synergistic antitumor activity (Perera et al. 2005). No clinical trial data are available yet for the use of IMC-C225 or mAb 806 in the treatment of malignant gliomas.

The most thoroughly studied new form of "targeted" therapy against EGFR is small molecule inhibitors of EGFR-mediated tyrosine kinase activation (Arteaga 2001; Baselga and Arteaga 2005; Grünwald and Hidalgo 2003; Mendelsohn 2002; Mendelsohn and Baselga 2003; Nathoo et al. 2004; Fry and Bridges 1995; Levitzki 2002; Levitzki and Gazit 1995; Renhowe 2002; Nicholas et al. 2006). Initial screening for drugs with EGFR inhibitory activity resulted in the discovery of several classes of compounds that varied in their potency and specificity against EGFR, and physicochemical properties (e.g., indolinethiones, quinazolines) (Bridges 1999). Newer generation inhibitors have since been developed that are competitive inhibitors of the Mg-ATP binding site of the catalytic domain of the EGFR tyrosine kinase (see Fig. 2). Drugs vary in their affinity for EGFR and in their extent of kinase inhibition. Specific and reversible EGFR inhibitors include gefitinib (ZD1839, Iressa), erlotinib

(OSI-774, Tarceva), and PKI-166 (Levitzki and Gazit 1995; Renhowe 2002; Nicholas et al. 2006). These drugs all have a low molecular weight, allowing better tumor site penetration, and can be administered orally. Numerous phase I and II trials of these drugs have been completed in systemic solid tumors (Arteaga 2001; Baselga and Arteaga 2005; Fry and Bridges 1995). They are generally well tolerated; the most common toxicities have been skin rash, diarrhea, and nausea.

Gefitinib has been tested against high-grade gliomas in animal models and early phase I and II trials. When used for the treatment of intracerebral brain tumors in athymic mice that overexpressed EGFR, gefitinib was able to ablate EGFR phosphorylation and increase median survival by 88–105% (Heimberger et al. 2002). However, in tumors expressing the EGFRvIII mutant, gefitinib was not able to significantly inhibit EGFR phosphorylation or tumor growth. A combined phase I/II study by the Radiation Therapy Oncology Group (RTOG 0211) has used gefitinib in combination with external beam irradiation for newly diagnosed GBM patients, stratified for anticonvulsant therapy (Chakravarti et al. 2006). A total of 178 patients were enrolled: 31 in the phase I portion and 147 in the phase II segment. The MTD from the phase I cohort was 500 mg/day in patients on NEIAED. The median overall survival was 11 months, with an overall PFS of 5.1 months. In comparison to historical RTOG control patients, the median overall survival was not statistically significantly different (11.5 vs. 11 months; $p=0.14$). In a phase I/II study of 40 patients with recurrent malignant gliomas, the NABTC stratified dosing of gefitinib based on anticonvulsant usage (Lieberman et al. 2003). Partial responses were noted in four GBM and one anaplastic oligodendroglioma. However, responses were brief, with median time to progression (TTP) of 8–15 weeks. In a phase II study of GBM patients after the first relapse, the Duke group used gefitinib dosing of 500–1,000 mg/day (Peery et al. 2003). Thus far, there has been 1 PR of 36+ weeks' duration and 22 patients with SD. A phase II study from an Italian group applied gefitinib (250 mg/day) to 28 patients with progressive malignant glioma (GBM 16, AA 9, AO 3) (Franceschi et al. 2007). There were no CR or PR; five patients had stabilization of disease (GBM 3, AA 1, AO 1). The 6- and 12-month PFS were 14.3% and 7.1%, respectively, with a median TTP of 8.4 weeks. The median overall survival was 24.6 weeks, with overall survival rates at 6 and 12 months of 50% and 14.2%, respectively. Clinical responses to gefitinib could not be predicted on the basis of amplification or expression of EGFR, or the expression of phospho-Akt. Gefitinib has also recently been applied to pediatric patients with refractory solid tumors, including gliomas (Freeman et al. 2006). In a phase I trial of refractory solid tumors, gefitinib (150–500 mg/m^2) was administered on a daily basis. The MTD was 400 mg/m^2/day, with rash as the DLT, similar to adults. Of interest, two patients with brainstem gliomas had stabilization of disease for 40 and 60+ weeks.

Several authors have begun clinical trials of gefitinib in combination with rapamycin (sirolimus; Rapamune), an inhibitor of mTOR signaling (see below). Rich and associates have begun a phase I trial of gefitinib plus rapamycin, and have enrolled 23 patients with recurrent malignant gliomas (GBM 20) (Rich et al. 2005). The regimen has been well tolerated, with a single DLT event consisting of grade 3 mucositis. The MTD has not yet been reached for patients on EIAED or NEIAED. Two objective

responses have been noted thus far. A similar study by Badruddoja and coworkers has enrolled 21 GBM patients to date, 18 of which were assessable for response (Badruddoja et al. 2006). The combination of gefitinib (500 mg/day) and rapamycin (2 mg/day, titrated to plasma level of 4–12 μg/mL) has been generally well tolerated, with a few incidents of grade III/IV toxicity including diarrhea, rash, and wound infection. Treatment responses thus far include 1 MR and several patients with SD. The mean TTP was 3 months, with a 6-month PFS rate of 16.6%. A pilot study by Doherty and colleagues used a regimen of gefitinib (500 mg/day) and rapamycin (4 mg/day) for the treatment of nine patients with recurrent malignant gliomas (Doherty et al. 2006). There was one patient with a PR and five with SD. The median TTP was 12 weeks, with a 6-month PFS rate in the GBM cohort of 25%. A recent report from the Memorial Sloan-Kettering group described a pilot study using gefitinib in combination with RAD-001 (Everolimus), a soluble analog of rapamycin that also inhibits mTOR (see below) (Nguyen et al. 2006). Nineteen patients with recurrent GBM on NEIAED were enrolled, and received gefitinib (250 mg/day) and rapamycin (30–70 mg weekly). There were six patients with objective responses on MRI (32%; PR 2, MR 4), while another three patients (16%) had stabilization of disease. The median overall survival was 6.5 months, with a median TTP of 2.6 months. The regimen was relatively well tolerated; the most frequent grade III and IV toxicities were elevated liver transaminases and lymphopenia.

Preclinical and phase I data for use of erlotinib are more limited, although there is extensive experience in systemic solid tumors (Grünwald and Hidalgo 2003; Nathoo et al. 2004; Nguyen et al. 2006; Hidalgo and Bloedow 2003). In a phase I study of patients with malignant glioma, the UCSF group used erlotinib alone or in combination with temozolomide (150–200 mg/m^2/day × 5 days, q28d) (Prados et al. 2003). Patients were stratified by use of EIAED. Erlotinib was started at 100 mg/day and titrated by 50 mg increments. Thus far, the MTD has not been reached. Exposure to erlotinib and its active metabolite were markedly reduced (50–75%) when used concomitantly with EIAED or EIAED plus temozolomide. This cohort of patients has since been further studied, in an attempt to correlate response to erlotinib with downstream molecular markers (Haas-Kogan et al. 2005). Brain tumor specimens from each patient were analyzed for the expression of EGFR, the presence of the EGFRvIII mutant, EGFR gene amplification, PTEN mutations, and the phosphorylation status of Akt. Response to erlotinib was associated with EGFR expression and amplification, especially in the GBM cohort. An even stronger association was noted for lack of response to erlotinib and significant amounts of phosphorylated Akt. None of the 22 tumors with high levels of phospho-Akt responded to treatment, while 8 among 18 tumors with low levels of phospho-Akt responded to erlotinib ($p < 0.001$). In addition, the levels of phosphorylated Akt were inversely associated with TTP ($p < 0.001$).

Several groups are now investigating the use of erlotinib in combination with irradiation for newly diagnosed tumors. Krishnan and colleagues are conducting a phase I trial of single-agent erlotinib for patients with GBM (Krishnan et al. 2005). The MTD has not yet been reached for patients on EIAED (up to 200 mg/day) or NEIAED (up to 150 mg/day). Thus far, the median TTP and survival were 23 and

55 weeks, respectively. No objective responses have been noted, although nine patients have had SD. A similar phase II study of erlotinib in combination with temozolomide and irradiation has recently been finalized at the Cleveland Clinic (Peereboom et al. 2005). Twenty-eight patients with newly diagnosed GBM have received erlotinib (50–150 mg/day), temozolomide (75 mg/m^2/day), and irradiation. After RT, patients continued treatment with adjuvant erlotinib and standard temozolomide. The median PFS was 3.6 months; six patients remained alive and stable at 4.4+, 8.3+, 11.5+, 14.7+, 15.8+, and 21.8+ months. No objective responses were noted on MRI follow-up. The regimen was associated with considerable grade III and IV toxicity, including *Pneumocystis carinii pneumonia*, nonneutropenic sepsis, and refractory bone marrow aplasia.

In the setting of recurrent disease, several investigators have begun to evaluate the activity of single-agent erlotinib. Raizer and associates have reported the results of a phase I trial in a cohort of 32 patients with recurrent malignant glioma (GBM 21) who were all on EIAED (Raizer et al. 2005). The MTD was determined to be 650 mg/day for patients on EIAED; no treatment response data were available. In a phase II study, Vogelbaum and colleagues used erlotinib (150 mg/day) for 24 patients with recurrent GBM who were all on NEIAED (Vogelbaum et al. 2004). Responses thus far include five patients with PR and five with SD. However, the responses have not been durable, with a median TTP of only 22 weeks. A similar phase II study evaluated the activity of erlotinib in 48 patients with recurrent GBM at first relapse (Cloughesy et al. 2005). Dosing was individualized until DLT; patients were grouped according to EIAED or NEIAED usage. Objective responses included 1 patient with a CR and 3 patients with PR, as well as another 18 patients with SD. The 6-month PFS was 17%, with a median survival of 10 months. Molecular correlational studies suggested a slight trend toward an improved outcome for patients with tumors expressing EGFR. A recent European report describes a randomized phase II trial of erlotinib versus control chemotherapy (temozolomide or BCNU) for patients with recurrent GBM (van den Bent et al. 2007). A total of 110 patients were randomized to receive either erlotinib (150–200 mg/day on EIAED; 300–500 mg/day on NEIAED), temozolomide (150–200 mg/m^2/day × 5 days every 28 days), or BCNU (60–80 mg/m^2 IV, days 1–3 every 8 weeks). Six patients had SD while receiving erlotinib; there were no objective responses. Two patients on the control arm had tumor shrinkage. The 6-month PFS rates for erlotinib and the control arm were 12% and 24%, respectively. Overall survival rates at 6 and 12 months for the erlotinib cohort were 61% and 24%, while for the control cohort they were 63% and 26%. Responses to erlotinib did not correlate with EGFR expression, EGFR amplification, or the presence of the EGFRvIII mutation.

Studies have also begun to evaluate the use of erlotinib in combination with cytotoxic chemotherapy and other molecular agents for patients with recurrent high-grade gliomas. The group at MD Anderson have used the combination of erlotinib (150–200 mg/day) and intravenous carboplatin (target AUC of 6 mg × mL/min; day 1 of monthly cycle) in a phase II trial of recurrent GBM that had failed first-line temozolomide therapy (DeGroot et al. 2007). Thus far, 17 evaluable patients have been enrolled. The median TTP is 15.2 weeks, with a range of 8.0–28.4 weeks.

Neuroimaging responses and molecular pathological correlates (e.g., PI3K, Akt, PTEN) have not been reported thus far. In the pilot study by Doherty and colleagues, erlotinib (150 mg/day) and rapamycin (4 mg/day) were used for the treatment of 17 patients with recurrent malignant gliomas (Doherty et al. 2006). There was 1 patient with a PR and 5 with SD. The median TTP was 12 weeks, with a 6-month PFS rate in the GBM cohort of 25%. The NABTC has begun a phase I/II trial of erlotinib (150 mg/day) in combination with weekly intravenous CCI-779 (Temsirolimus; 15–50 mg), another mTOR inhibitor (see below) (Robins et al. 2007). Twenty-two patients with recurrent gliomas (GBM 15, AA/AO 7) have been enrolled thus far. There was an unexpectedly high incidence of grade III/IV rash and mucositis. The MTD is estimated to be 150 mg/day for erlotinib and 15 mg/day for CCI-779. Neuroimaging responses and survival data have not been published as yet.

Ras Signaling

Ras is a key intermediate in the signal transduction pathways linking membrane-bound receptor tyrosine kinases to downstream cascades of protein kinase effector molecules (Boguski and McCormick 1993; Lowy and Willumsen 1993). Ras signaling is involved in a wide variety of cellular processes including growth, differentiation, cytoskeletal organization, membrane trafficking, and apoptosis. Ras has several different isoforms; the H-Ras, K-Ras, and N-Ras oncogenes are located on chromosomes 11p, 12p, and 1p, respectively, and encode 21-kDa proteins. The Ras proteins contain 188 or 189 amino acids, exhibit high sequence homology, and are localized on the inner surface of the plasma membrane. Ras functions as a molecular switch and cycles between an inactive GDP-bound form and an active GTP-bound form. However, before Ras can attach to the inner cell membrane and become active, it must undergo several post-translational modification steps at the C-terminus to increase its hydrophobicity (see Fig. 3). The first and most critical step is the farnesylation step, catalyzed by farnesyltransferase (FTase). FTase catalyzes the transfer of a 15-carbon farnesyl isoprenoid group to the cysteine residue of the CAAX sequence.

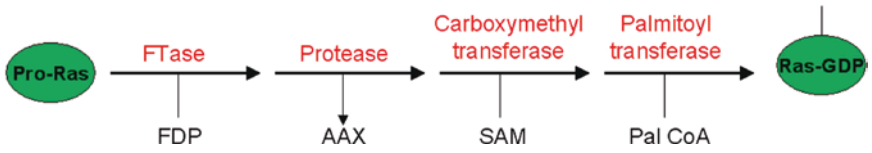

Fig. 3 Before Ras can attach to the inner cell membrane and become activated, it must undergo extensive posttranslational modification. The first and most critical step for Pro-Ras is farnesylation of cysteine residues by FTase. Subsequent modifications include proteolytic cleavage of the AAX peptide by protease, carboxymethylation of the new C-terminal by carboxymethyl transferase, and the addition of a palmitoyl group to a cysteine residue upstream of the CAAX motif by palmitoyl transferase. Adapted from reference (Newton 2003) and used with permission from Future Drugs, Ltd

Ras-GDP is rapidly and transiently converted to Ras-GTP in response to the binding of numerous extracellular ligands to cell surface receptors. Important ligands include EGF, PDGF, FGF, IGF-I, TGF-α, interleukin-2, and interleukin-3 (Boguski and McCormick 1993; Lowy and Willumsen 1993). The cell-surface receptors are usually receptor tyrosine kinases. After autophosphorylation and activation of the receptor active site, "adapter proteins," such as Grb2, bind to the receptor and then recruit Ras activator proteins such as Sos-1, which function as Ras guanine-nucleotide-exchange factors (GEF). The GEF binds to Grb2 and mediates the exchange of GDP for GTP, thereby activating Ras. GTPase activator protein (GAP; NF-1, neurofibromin) promotes the hydrolysis of Ras-bound GTP to GDP, converting Ras to its inactive form and inhibiting further signal transduction. Activated Ras-GTP stimulates several downstream effectors, including Raf, Rac and Rho, MEKK, PI3K, and phospholipase C (see Fig. 4), to induce gene expression that promotes cellular proliferation and survival.

Approximately 30% of all human cancers harbor at least one mutation of a Ras gene (Boguski and McCormick 1993; Lowy and Willumsen 1993; Adjei 2001; Bos 1989; Rowinsky et al. 1999). These mutations are single-point mutations that affect

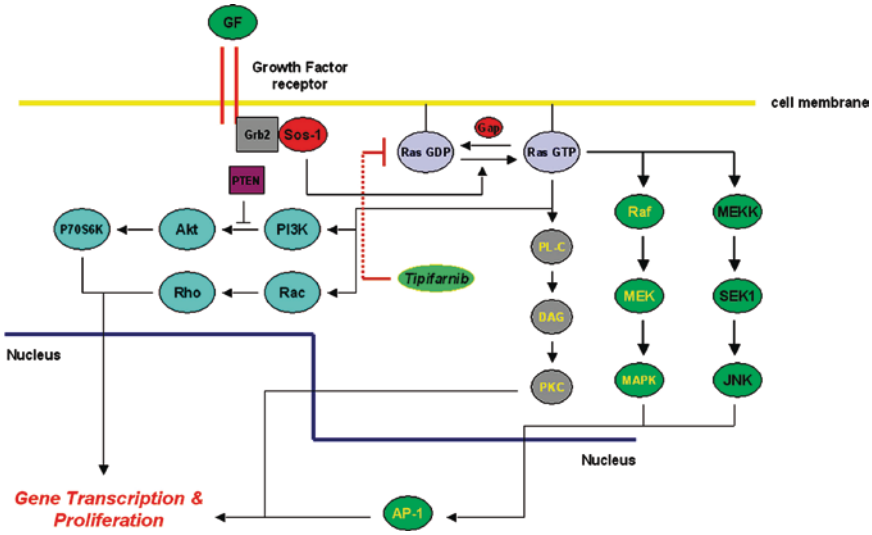

Fig. 4 After attachment of mature Ras-GDP to the inner cell membrane, binding of ligand (i.e., PDGF, EGF, TGF-α, FGF, IGF-I) to growth factor receptors results in receptor kinase activity, and binding of adapter proteins (e.g., Grb2) and Ras activator proteins (e.g., Sos-1). Sos-1 and other guanine-nucleotide-exchange factors bind to the SH2 domain of Grb2 and mediate the exchange of GDP for GTP, thereby activating Ras. Activated Ras is converted to its inactive form by GTPase activator protein (GAP), which mediates the hydrolysis of Ras-bound GTP to GDP. Ras-GTP stimulates downstream effector molecules, including Raf, Rac, MEKK, PI3K, and phospholipase C, which induce gene expression and promote cellular proliferation and survival. Molecular "targeted" therapy of the Ras signaling pathway is also demonstrated, using the farnesyltransferase inhibitor Tipifarnib (R115777). Adapted from reference (Newton 2003) and used with permission from Future Drugs, Ltd

only a few critical sites in the gene. These alterations of protein structure render mutant Ras insensitive to GAP-induced GTP hydrolysis, with subsequent constitutive activation of downstream signaling effectors. Although activating Ras mutations are frequent in other solid tumors, they appear to be quite uncommon in gliomas (Bos 1989; Guha 1998; Guha et al. 1997; Woods et al. 2002). However, despite the lack of mutations, increased expression and high levels of Ras-GTP have been demonstrated in GBM cell lines and tumor resection specimens in comparison to normal brain and lower-grade astrocytomas (Guha et al. 1997; Feldkamp et al. 1999b). The importance of increased Ras activity in GBM is supported by studies demonstrating reduced growth of preclinical GBM if treated with dominant-negative Ras mutants or Ras pathway inhibitor drugs. Overall, current data suggest that activation of the Ras signaling pathway in malignant gliomas is due to the aberrant expression and overactivity of membrane tyrosine kinase receptors, including EGFR, PDGFR, FGFR, and IGF-IR.

The importance of Ras signaling in oncogenesis and transformation has led to the development of "targeted" therapeutic strategies, such as antisense oligonucleotides, FTase inhibitors (FTI), Raf kinase inhibitors, MEK inhibitors, and geranylgeranyl transferase type 1 inhibitors (Adjei 2001; Rowinsky et al. 1999; Pincus et al. 2000). Of these various approaches, FTI have had the most rapid and extensive clinical development (Adjei 2001; Rowinsky et al. 1999; Sebti and Hamilton 2000; Singh and Lingham 2002). FTI block the farnesylation of Ras in a dose-dependent manner. FTase inhibition downregulates signaling pathways activated by Ras, such as Raf, MEK, MAPK, PI3K, and VEGF (see Fig. 4). Several FTI (e.g., R115777, SCH66336) have demonstrated the ability to inhibit tumor growth in culture and xenograft models of solid tumors, including gliomas (Rowinsky et al. 1999; Sebti and Hamilton 2000; Singh and Lingham 2002; Feldkamp et al. 1999b; Feldkamp et al. 2001; Fels et al. 2000). It appears that FTI can be effective against glioma cells regardless of the Ras mutational status of the tumor (Fels et al. 2000). In cells without Ras mutations, but with high levels of Ras-GTP secondary to excessive growth factor receptor stimulation, FTI are still able to inhibit activation of Ras and downstream effectors.

Several FTI have been aggressively advanced into clinical trials against solid tumors (Adjei 2001; Rowinsky et al. 1999; Sebti and Hamilton 2000; Singh and Lingham 2002). The most extensively studied FTI is R115777 (Tipifarnib), which has been well tolerated in phase I trials (see Fig. 4). In an NABTC phase I trial of R115777 in patients with recurrent malignant gliomas who are on EIAED, doses started at 300 mg bid, 21 days on and 7 days off (Cloughesy et al. 2005). The MTD was 600 mg bid, with DLT of grade III/IV skin rash at 700 mg bid. There were no objective responses, but three patients had disease stabilization for greater than 6 months (GBM 2, AO 1). The median PFS of the evaluable cohort was only 7 weeks. The concomitant use of an EIAED significantly affected the AUC of R115777, reducing it by 5.3-fold in comparison to historical control patients. In a phase II study of R115777 by the NABTC, 42 patients with recurrent malignant gliomas received 300 or 600 mg bid, 21 days on and 7 days off, depending on their anticonvulsant status (i.e., NEIAED vs. EIAED) (Cloughesy et al. 2006). A total of 89 patients were enrolled, including 67 with GBM and 22 with anaplastic glioma. On neuroimaging analyses, there were five patients with PR, all with GBM (four on

NEIAED, one on EIAED); no CR was noted. The median PFS for the GBM cohorts on NEIAED and EIAED was 9 and 6 weeks, respectively. If the PFS data of the NEIAED and EIAED cohorts are compared and analyzed, there is a statistically significant difference (NEIAED vs. EIAED; $p = 0.01$). For the total cohort of 89 patients, the 6-month PFS rate was 11.2%. In an attempt at neoadjuvant chemotherapy, the New Approaches to Brain Tumor Therapy (NABTT) consortium used preirradiation R115777 in a cohort of newly diagnosed GBM patients with residual enhancing tumor (Lustig et al. 2005). The majority of patients progressed rapidly through R115777 and required the initiation of radiotherapy. There were no objective responses and the median overall survival was only 7.5 months.

Raf/MEK Signaling

As outlined above, the Ras signaling pathway has been strongly implicated in the transformation process and malignant phenotype of many solid tumors, including high-grade gliomas. Because Ras is the focal point of multiple, redundant signaling complexes and has proved difficult to inhibit in numerous clinical studies of FTI drugs, some investigators are evaluating downstream effectors of the Ras-MAPK (mitogen-activated protein kinase) pathway as possible therapeutic targets, such as Raf and MEK (Sebolt-Leopold and Herrera 2004; Beeram et al. 2005; Roberts and Der 2007). Raf is a 68- to 74-kDa cytosolic protein, which has three major isoforms (A-Raf, B-Raf, C-Raf) and functions as the first downstream effector in the Ras signaling pathway (see Fig. 4). In the cytosol, C-Raf is part of a 300- to 500-kDa protein complex, which consists of C-Raf, heat shock protein 90, and the dimeric protein cofactor 14-3-3. The 14-3-3 protein binds to two specific phosphoserine residues of C-Raf (S259 and S621), thereby masking the kinase domain and inactivating the protein. The serine/threonine kinase function of Raf is activated by GTP-bound Ras, through a series of phosphorylation steps and conformational changes. Raf can also be activated via Ras-independent pathways, including Src, Janus kinase 1, interferon-β, protein kinase C-α, Bcl-2, and various scaffolding proteins (Beeram et al. 2005). Once GTP-bound Ras binds to the Ras-binding domain (RBD) and cysteine-rich domain (CRD) of Raf, on the amino terminal end of the protein, Raf is recruited to the inner cell membrane. The binding of Ras displaces the 14-3-3 protein, allowing access to critical residues involved in Raf activation, such as serine residue S259. After displacement of protein 14-3-3, S259 is dephosphorylated by protein phosphatase 2A and other phosphatases. In additon, phosphorylation of other residues (e.g., S338, Y341) by Ras, growth factors, and integrins is also necessary for the complete activation of Raf, by removing the inhibitory control of the regulatory domain on the kinase domain. Once Raf is fully activated, it propogates the signal within the pathway by phosphorylating the two dual-specificity MAPK kinases, MEK1 and MEK2 (also referred to as MKK1 and MKK2; see Fig. 5) (Sebolt-Leopold and Herrera 2004; Beeram et al. 2005; Roberts and Der 2007). MEK1 and MEK2 are 56- to 57-kDa proteins with dual kinase activity – they can phosphorylate both serine/threonine and tyrosine residues. The two forms of MEK have 80%

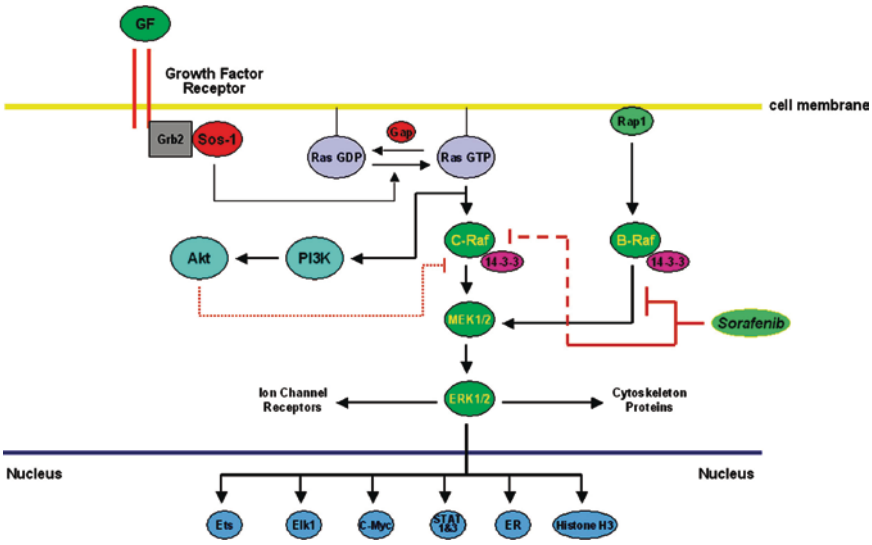

Fig. 5 C-Raf is stimulated by Ras-GTP, after activation of the Ras pathway by various growth factor receptors. Ras-GTP also activates the PI3K/Akt pathway, which is inhibitory to C-Raf activity. B-Raf is activated by Rap1. The 14-3-3 proteins are constitutively inhibitory to both C-Raf and B-Raf. Once activated, C-Raf and B-Raf phosphorylate MEK1 and MEK2, respectively, which then activate ERK1 and ERK2. After activation, ERK1/2 interact with ion channel receptors and cytoskeletal proteins while in the cytoplasm, and then translocate into the nucleus, where they activate several effectors, including Ets, Elk1, C-Myc, STAT1 and STAT3, ER, and Histone H3. Molecular "targeted" therapy of the Raf/MEK/ERK signaling pathway is also demonstrated, using the Raf inhibitor, Sorafenib (BAY 43-9006), which inhibits C-Raf and B-Raf. Adapted from references (Roberts and Der 2007; LaRocca et al. 1989; Mawrin et al. 2003)

amino-acid homology and almost identical three-dimensional structures. MEK1 and MEK2 are highly specific, and have no known substrates except for MAPK1 and MAPK2, respectively (also referred to as ERK1 and ERK2 – extracellular signal-regulated kinases 1 and 2) (Beeram et al. 2005). Both MEK proteins sequentially phosphorylate ERK1 and ERK2 at two sites – tyr185, followed by thr183. After phosphorylation and activation by MEK, ERK1 ($p44^{MAPK}$) and ERK2 ($p42^{MAPK}$) are translocated to the nucleus where they induce the transcription of numerous cytoplasmic and nuclear regulatory proteins, including Elk-1, c-Fos, Jun, AP-1, c-Myc, STAT 1/STAT3, and Ets (see Fig. 5). The net result of this transcriptional activity is to upregulate cyclin D1, cyclin E, and cdc-25, and downregulate $p27^{kip-1}$ and other cyclin-dependent kinase inhibitors, promoting progression through the cell cycle, aberrant growth, dedifferentiation, and cell survival.

Raf mutations and/or overexpression have been demonstrated in numerous solid tumors, including melanoma, lymphoma, and carcinomas of the breast, thyroid, kidney, liver, biliary tract, head and neck, and lung, as well as in GBM (Sebolt-Leopold and Herrera 2004; Beeram et al. 2005; Roberts and Der 2007; LaRocca et al. 1989). Similarly, activation and overexpression of MEK and ERK have been demonstrated in gliomas and other solid tumors (Sebolt-Leopold and Herrera 2004; Roberts and Der 2007; Mawrin et al. 2003). Several small molecule inhibitors

of Raf have been identified, including sorafenib (BAY 43-9006), L-779450, SB203580, RAF265, and PLX4032 (Sebolt-Leopold and Herrera 2004; Beeram et al. 2005; Roberts and Der 2007). Sorafenib has been studied most extensively and has undergone testing in phase I, II, and III trials as a single agent and in combination with other drugs (Beeram et al. 2005). It is an orally available compound that was approved by the FDA in 2005 for the treatment of advanced renal cell carcinoma. Sorafenib is a potent inhibitor of ATP binding in the catalytic domain of C-Raf, wild-type B-Raf, and the ^{V599E}B-Raf mutant. It does not inhibit MEK1/2, ERK1/2, erbB1, or erbB2. However, further studies of sorafenib have determined that it is a potent inhibitor of other important receptor tyrosine kinases involved in angiogenesis, such as VEGFR-2, VEGFR-3, PDGFR-β, Flt-3, c-Kit, and FGFR-1 (Beeram et al. 2005; Roberts and Der 2007). Thus far, the results of clinical trial analyses of sorafenib have not yet clarified whether or not inhibition of Raf has been of clinical value. It is possible that the antitumor activity may be more related to the ability of sorafenib to inhibit angiogenesis. Further analysis of clinical trial data will be needed to clarify this important issue. Sorafenib has now been applied to malignant gliomas in early clinical trials; these results will be presented below, in the section on Multitargeted Agents. Preclinical studies and preliminary clinical trials are ongoing for L-779450, SB203580, RAF265, and PLX4032. However, none of these drugs have been applied to malignant gliomas as yet.

Several small molecule MEK inhibitors have now been identified, including CI-1040 (PD184352), PD0325901, and ARRY-142886 (AZD6244) (Sebolt-Leopold and Herrera 2004; Roberts and Der 2007; Messersmith et al. 2006). As a class, MEK inhibitors usually have a noncompetitive mechanism, and do not bind to the ATP-binding site of the kinase. Instead, structural analyses of MEK have revealed a unique inhibitor binding site adjacent to the ATP site (Roberts and Der 2007; Messersmith et al. 2006). Binding of an inhibitor to this site results in stabilization of the inactive conformation of the kinase, with reduction of kinase activity. CI-1040, PD0325901, and ARRY-142886 have all demonstrated activity against systemic solid tumors in preclinical testing and are now in early phase I and II trials (Sebolt-Leopold and Herrera 2004; Beeram et al. 2005; Messersmith et al. 2006). None of these drugs have published results against malignant gliomas in the preclinical or clinical trial setting. However, CI-1040 has been tested against three malignant schwannoma cell lines derived from neurofibromatosis patients (Mattingly et al. 2006). CI-1040 was able to inhibit the proliferation of all three cell lines; the inhibition was closely correlated with a reduction in the activity of ERK1 and ERK2. In addition, CI-1040 was able to induce apoptosis in cells from all three cell lines.

PI3K/Akt Signaling

Stimulation of growth factor receptors and the Ras pathway leads to the activation of numerous signal transduction molecules, instigating a cascade of downstream effectors that mediate growth and survival of tumor cells (Shapiro and Coons 1998;

Newton 2003; Maher et al. 2001). One of the most important signaling pathways for tumor transformation involves PI3K, Akt, and mTOR (Downward 1998; Martin and Blenis 2002; Schmelzle and Hall 2000; Vivanco and Sawyers 2002; Wymann and Pirola 1998). The primary event in this pathway is activation of PI3K, which can occur from numerous agonists and receptors, including PDGFR, EGFR, VEGFR, interleukin receptors, interferon receptors, integrin receptors, and the Ras pathway (Adjei 2001; Downward 1998; Schmelzle and Hall 2000; Vivanco and Sawyers 2002; Wymann and Pirola 1998). The PI3K family of kinases catalyzes the transfer of the γ–phosphate of ATP to the D3 position of the head group of phosphatidylinositols (PtdIns), a form of membrane lipid. The PI3Ks can be grouped into three classes, based on substrate preference and sequence homology. The substrate preference of the Class I PI3K are PtdIns, PtdIns(4)P, and PtdIns(4,5)P. Class I PI3K are heterodimers of approximately 200 kDa that are composed of a regulatory subunit (p55 or p85) and a catalytic subunit (p110).

The favored substrate for Class IA PI3K is PtdIns(4,5)P, with the production of PtdIns(3,4,5)P after phosphorylation of the 3′-hydroxyl group (Martin and Blenis 2002; Vivanco and Sawyers 2002; Wymann and Pirola 1998). After its production, PtdIns(3,4,5)P recruits the serine/threonine kinase Akt to the juxta-membrane region (see Fig. 6). Simultaneously, PtdIns(3,4,5)P activates other membrane-associated kinases, PDK1 (PtdIns(3,4,5)P-dependent kinase-1) and PDK2 (PtdIns(3,4,5)P-dependent kinase-2). Once Akt is associated with the membrane and is bound to PtdIns(3,4,5)P, a conformational change occurs that allows phosphorylation at the threonine 308 and serine 473 positions by the catalytic domains of PDK1 and PDK2, respectively. Phosphorylation of both sites is required for full activation of Akt and must occur before Akt can detach from the membrane and interact with downstream effectors in the cytosol.

Akt is a 56 kDa serine/threonine protein kinase with three human isoforms (Akt1, Akt2, Akt3) that have significant sequence homology to protein kinase C and protein kinase A (Vivanco and Sawyers 2002; Datta et al. 1999; Sekulic et al. 2000). Following its activation by PDK 1 and PDK2, Akt has several downstream targets that mediate its ability to promote cell survival and growth (see Fig. 6). Activated Akt phosphorylates numerous downstream effectors, including glycogen synthase kinase 3 α and β (GSK3α, GSK3β), BAD, 6-phosphofructo-2-kinase (PFK-2), GLUT-4, $p70^{S6K}$, E2F, mTOR, and others. The most important signal responsible for cell survival is mediated through BAD, a proapoptotic member of the Bcl-2 family (Vivanco and Sawyers 2002; Datta et al. 1999; Datta et al. 1997; Kajiwara et al. 2003). Akt phosphorylates BAD, impairing its ability to heterodimerize with, and inhibit, the prosurvival proteins Bcl-2 or Bcl-xL. The net effect of this interaction is to inhibit apoptosis and promote cell survival. Other Akt-mediated prosurvival mechanisms involve modifying cell cycle function and enhanced cell proliferation. For example, Akt is known to phosphorylate and inhibit the cyclin-dependent kinase inhibitors, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Gesbert et al. 2000; Zhou et al. 2001). Phosphorylation of MDM2 by Akt promotes the degradation of p53, leading to enhanced cell cycle activity at the G_1/S interface (Mayo and Donner 2001). Akt also plays an important role in the activation of mTOR, which is reviewed in detail in the next section.

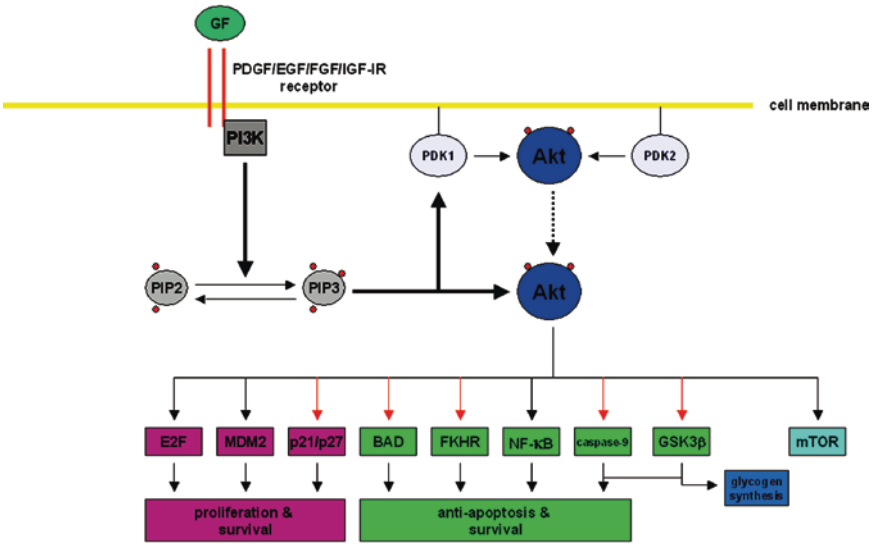

Fig. 6 PI3K is activated by growth factor receptors after stimulation by the various ligands (e.g., PDGF, EGF, IGF, FGF), as well as by the Ras pathway. Activity of PI3K leads to the production of PtdIns(3,4,5)P (PIP3), after phosphorylation of PtdIns(4,5)P (PIP2). PIP3 binds Akt and translocates it near the cell membrane, where it can be phosphorylated and activated by PDK1 and PDK2. After the activation of Akt, it interacts with, and phosphorylates, several downstream effectors that promote proliferation and survival, including MDM2, p21/p27, BAD, FKHR, NF-κB, caspase-9, GSK3β, and mTOR. Adapted from reference (Newton 2004) and used with permission from Future Drugs, Ltd

The PI3K/Akt signaling pathway is overactive in GBM, which can be partially explained by upstream activation from growth factor receptors (e.g., EGFR, PDGFR) and Ras (Choe et al. 2003; Sakata et al. 2002). When cultured astrocytoma cells are implanted into mice, the derived tumors resemble GBM if the cells constitutively express Akt (Stein 2001). Implanted cells without activated Akt produce tumors that are not as aggressive. Increased activity of PI3K and Akt has also been shown to correlate with increased invasiveness and gelatinase activity in malignant gliomas (Kubiatowski et al. 2001). Although the above data suggest a direct role for PI3K and Akt in the transformation of some tumors, it is more common for the PI3K/Akt signaling pathway to become overactive through abnormalities of regulation.

The PI3k/Akt pathway has several forms of regulation, but the most important is the activity of the tumor suppressor gene PTEN (Lustig et al. 2005). The PTEN gene is located on chromosome 10q23.3 and encodes a 403 amino acid cytoplasmic protein (Li et al. 1997; Cantley and Neel 1999; Steck et al. 1997; Vazquez and Sellers 2000). PTEN has lipid phosphatase activity and is able to dephosphorylate PtdIns(3,4,5)P with specificity for the phosphate group at the D3 position of the inositol ring. This activity forms the basis for its designation as a tumor suppressor gene. PTEN directly antagonizes PI3K activity and reduces the concentration of

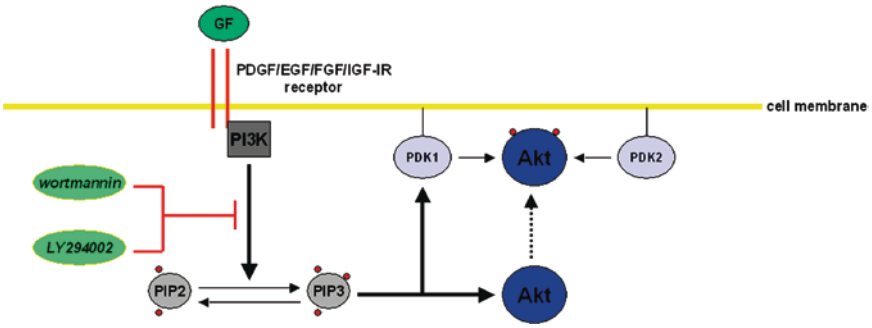

Fig. 7 "Targeted" therapy of the PI3K signaling pathway includes the drugs wortmannin and LY294002, which inhibit the ATP binding site of PI3K. Adapted from reference (Newton 2004) and used with permission from Future Drugs, Ltd

PI3K-dependent downstream effectors. In tumors and PTEN-deficient tumor cell lines high basal levels of PtdIns(3,4)P, PtdIns(3,4,5)P, and phospho-Akt are noted. PTEN mutations and loss of function are frequent in high-grade astrocytomas, and are responsible for the abnormally high levels of activity in the PI3K/Akt signaling pathway (Vazquez and Sellers 2000; Fults and Pedone 2000; Knobbe et al. 2002; Li et al. 1999; Rasheed et al. 1997; Zhou et al. 1999). In gliomas, the spectrum of PTEN mutations affects the entire span of the gene. More than 50% of the mutations in gliomas result in premature stop codons and the production of a truncated protein. There is a strong predilection for PTEN mutations to occur in high-grade tumors, with a reported frequency of 15–40% in primary or de novo GBM. Secondary GBM are less likely to harbor PTEN mutations.

An intense effort is now under way to develop "targeted," molecular-based chemotherapy approaches to inhibit the PI3K/Akt/PTEN signaling pathway. Several compounds have been identified that are inhibitory to PI3-kinases, including wortmannin, LY294002, and staurosporine (see Fig. 7) (Stein 2001; Powis et al. 1994; Vlahos et al. 1994; Walker et al. 2000; Wymann et al. 1996). Wortmannin is an irreversible inhibitor of all PI3-kinases and other members of the PI3K superfamily. Although wortmannin is a very potent inhibitor of PI3-kinases, it is quite nonselective. LY294002 also inhibits PI3-kinases via interaction at the ATP binding site, as a pure competitive inhibitor (Wymann et al. 1996). Similar to wortmannin, LY294002 is nonselective and can inhibit the entire class of PI3-kinases. Wortmannin and LY294002 have been applied to various cultured cells and are able to inhibit growth in concentrations expected to inhibit class Ia PI3-kinases (Vignot et al. 2005).

Wortmannin and LY294002 have been applied to brain tumor cultures and animal models (Newton 2004; Knobbe et al. 2002; Chernikova et al. 1999; Klingler-Hoffman et al. 2003; Kubota et al. 2000; Su et al. 2003). Both drugs are able to induce growth inhibition as single agents and appear to function as radiation sensitizers when used in combination with irradiation. The mechanism of growth inhibition remains unclear, but may be partially due to increased expression of the cell