REVIEW

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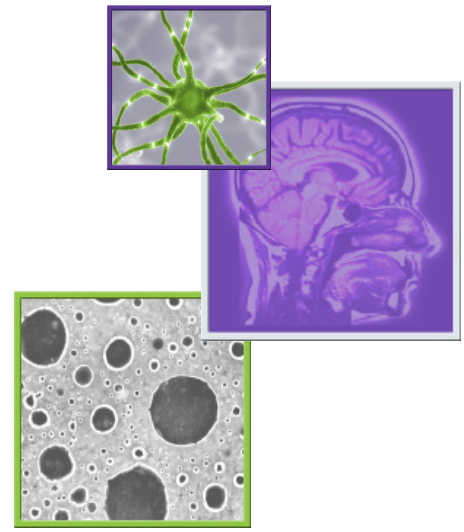
Targeting core (mutated) pathways of high-grade gliomas: challenges of intrinsic resistance and drug efflux



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Practice Points

- Glioblastoma multiforme (GBM) is characterized by a collection of mutated signaling pathways.
- Three core pathways are affected in a substantial fraction of patients.
- Therapies directly targeting just a single mutated pathway are unlikely to be successful.
- Instead, the combination of targeted therapeutics should be explored.
- Owing to its invasive character, GBM is a disease that affects the whole brain.
- Consequently, therapeutics against GBM must cross the blood–brain barrier (BBB) to also reach the more remote areas of the brain containing tumor cells.
- Most targeted agents have been designed for other cancers rather than for GBM, and many of them will not meet the requirement of sufficient BBB penetration.
- Candidate agents that are not or are very weak substrates of ABC transporters have an advantage with regard to BBB penetration.
- Alternatively, inhibitors of ABC transporters may be used to enhance the BBB penetration of substrate drugs.

SUMMARY High-grade gliomas are the most common type of primary brain tumor and are among the most lethal types of human cancer. Most patients with a high-grade glioma have glioblastoma multiforme (GBM), the most malignant glioma subtype that is associated with a very aggressive disease course and short overall survival. Standard treatment of newly diagnosed GBM involves surgery followed by chemoradiation with temozolomide. However, despite this extensive treatment the mean overall survival is still only 14.6 months

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and more effective treatments are urgently needed. Although different types of GBMs are indistinguishable by histopathology, novel molecular pathological techniques allow discrimination between the four main GBM subtypes. Targeting the aberrations in the molecular pathways underlying these subtypes is a promising strategy to improve therapy. In this article, we will discuss the potential avenues and pitfalls of molecularly targeted therapies for the treatment of GBM.

High-grade gliomas are the most common type of primary brain tumors and are among the most lethal types of human cancer [1]. High-grade gliomas are classified by the WHO in grade III as: anaplastic astrocytoma, anaplastic oligodendroglioma and anaplastic oligoastrocytoma; and grade IV: gliosarcoma and glioblastoma multiforme (GBM) [2]. Unfortunately, the majority of high-grade glioma patients are diagnosed with GBM, the most malignant subtype that is associated with a very aggressive course of disease and less than 3 months overall survival if left untreated [2]. Standard treatment of newly diagnosed GBM includes surgery followed by radiotherapy (30 × 2 Gy) plus temozolomide (75 mg/m^2; daily) for 6 weeks and maintenance temozolomide therapy (150–200 × 5 mg/m^2 per 28 days) for 6 months. However, despite this extensive treatment the mean overall survival is still only 14.6 months and more effective treatments are, thus, urgently needed [3].

In contrast to conventional chemotherapies that work by interfering with DNA synthesis or cell metabolism, targeted therapies work by inhibition of the deregulated cell signaling pathways in cancer cells by small molecules or antibodies. The underlying concept is that these signaling pathways are more critical for survival and growth of cancer cells than for normal cells. Consequently, targeted therapy holds the promise of being effective with less toxicity than conventional chemotherapies. Despite emerging success in some other tumor types, for example, imatinib for chronic myelogeneous leukemia [4] or vemurafenib in melanoma [5], the development of molecularly targeted therapy for gliomas appears to be much more challenging. Two small-molecule inhibitors of the EGF receptor (EGFR) tyrosine kinase that received regulatory approval for the treatment of lung cancer, erlotinib (Tarceva®, Genentech, Inc., CA, USA) and gefitinib (Iressa®, AstraZeneca, DE, USA), have been extensively evaluated for GBM treatment. The expectations were high since EGFR overexpression and mutations are common in GBMs. The results of the first Phase I studies with erlotinib were exciting [6,7] and a Phase II, single institution study demonstrated that treatment with erlotinib plus temozolomide before and after radiation significantly increased median survival of GBM patients to 19.3 months compared with 14.1 months in historical controls [8]. However, the results of subsequent clinical trials with EGFR inhibitors were all disappointing [9–14]. In particular, a randomized controlled Phase II study carried out by the European Organisation for Research and Treatment of Cancer (EORTC) demonstrated no clear benefit in progressive GBM patients treated with erlotinib compared with a control group receiving temozolomide or carmustine [10].

The failures of targeted therapy in the treatment of GBM are not limited to EGFR inhibitors. Inhibitors of mTOR have also been regarded as promising candidates for treating GBM, as the frequently deregulated PI3K–AKT–mTOR signaling pathway is considered to be a key mediator of GBM cell survival and growth. Rapamycin (sirolimus) and its analogs (rapalogs) temsirolimus (CCI-779) and everolimus (RAD001) are three mTOR inhibitors that have undergone extensive clinical evaluation for their therapeutic effect in GBMs [15–22]. Similar to the EGFR inhibitors, most trials with mTOR inhibitors as a single agent in GBM have failed to show any significant therapeutic benefit.

Despite these disappointing results, important lessons have been learned from translational studies with these agents. This article will focus on the recent development of targeted therapies on the core mutated pathways of GBM. Moreover, several major putative resistance mechanisms of GBM to the earlier studied targeted therapies will also be discussed.

Genetic alterations & classification of GBMs

The majority of patients with GBMs suffer from primary (or *de novo*) GBMs. In comparison with secondary GBMs, which evolve from low-graded gliomas, primary GBMs usually

Table 1. Classic classification of glioblastoma multiforme.

Subtype	Incidence (%)	Origin	Alterations (%)	Clinical history	Median overall survival (months)†
Primary or *de novo* GBM	95%	No recognizable precursor lesions	*LOH* 10q (70) *EGFR* amplification (36) *P16*^{INK4a} deletion (31) *TP53* mutation (28) *PTEN* mutation (25)	<3 months (68%) <6 months (84%)	4.7
Secondary GBM	5%	Developed from diffuse astrocytoma or anaplastic astrocytoma	*LOH* 10q (63) *EGFR* amplification (8) *P16INK4a* deletion (19) *TP53* mutation (65) *PTEN* mutation (4)	Low-grade astrocytoma origin: 5.1 years; anaplastic astrocytoma: 1.9 years	7.8

The classic classification includes primary and secondary GBM.
†Median overall survival without treatment.
GBM: Glioblastoma multiforme.

develop without pre-existing precursor lesions. Primary and secondary GBMs are histopathologically indistinguishable and are characterized by a high proliferative index, serpentine pseudopallisading necrosis and microvascular proliferation. However, primary and secondary GBMs are associated with differences in age of onset, clinical history, median survival and genetic changes (Tables 1 & 2).

Primary and secondary GBMs develop as a result of multiple genetic alterations that differ in the two types of GBM. Secondary GBM is more frequently a result of an early mutation in *P53*, whereas primary GBM more frequently harbors mutations in *EGFR* deletions within the *CDKN2* locus that codes for p14^{Arf}, p16^{Ink4a} and p15^{Ink4b}, and a homozygous loss of chromosome 10q23, which contains the *PTEN* gene. Overall, loss of chromosome 10q, *EGFR* amplification and deletion of p16^{Ink4a} have been demonstrated to be the most frequent genetic alterations in primary GBM [1,23–26].

The Cancer Genome Atlas project in GBMs

By implementation of large-scale multidimensional analytic platforms, a comprehensive characterization of the molecular basis of malignant gliomas recently became available. The Cancer Genome Atlas (TCGA) is a project that aims to catalog genetic mutations responsible for cancer. In 2008, TCGA published the results of their first cancer project on the analysis of genomic abnormalities in human GBM (mostly primary GBM) [27]. This work not only confirmed the common genetic aberrations reported previously, but also provided new insight into the roles of some known tumor-related genes, such as *ERBB2*/*HER2*, *NF1* and *P53*, and also uncovered new gene mutations. More importantly, it provided a network view of the pathways altered in the development of GBMs, which can be instructive for future therapeutic decisions and facilitate the search for more efficacious targeted therapies. As shown in Figure 1, frequent genetic alterations of GBM

Table 2. The Cancer Genome Atlas genomic classification of glioblastoma multiforme.

Subtype	Biomarker	Signature	Major alterations	Treatment response
Classical	Neuroembryonic stem cell	Astrocytic	*EGFR*, *CDKN2A/2B* and *PTEN*	Good
Mesenchymal	Mesenchymal markers	Astroglial	*NF1*, *PTEN*, *CHI3L1* and *MET*	Modest
Proneural	Oligodendrocytic development genes	Oligodendrocytic	*TP53*, *PDGFRA* or *PI3KCA/PIK3RI*, *IDH1* and *PTEN*	Poor or no response
Neural	Neural markers	Neuronal and astrocytic	*EGFR*	Marginal

The Cancer Genome Atlas classification reveals four clinically relevant subtypes based on the genomic profiles of glioblastoma multiforme and their correlations with biomarker expression, cellular lineages and response to standard aggressive chemoradiation therapy.
Data taken from [28].

occur in three core pathways; RTK/RAS/PI3K signaling, and P53 and Rb tumor suppressor pathways were mapped based on genetic analyses of 206 GBM samples.

Another important finding based on the TCGA is the molecular classification of GBM [28]. Four GBM subtypes: proneural; neural; classical; and mesenchymal subtypes, described in this study showed strong correlations with GBM cells of origin, clinical characteristics and response to standard chemoradiation therapy. For example, the proneural subtype was

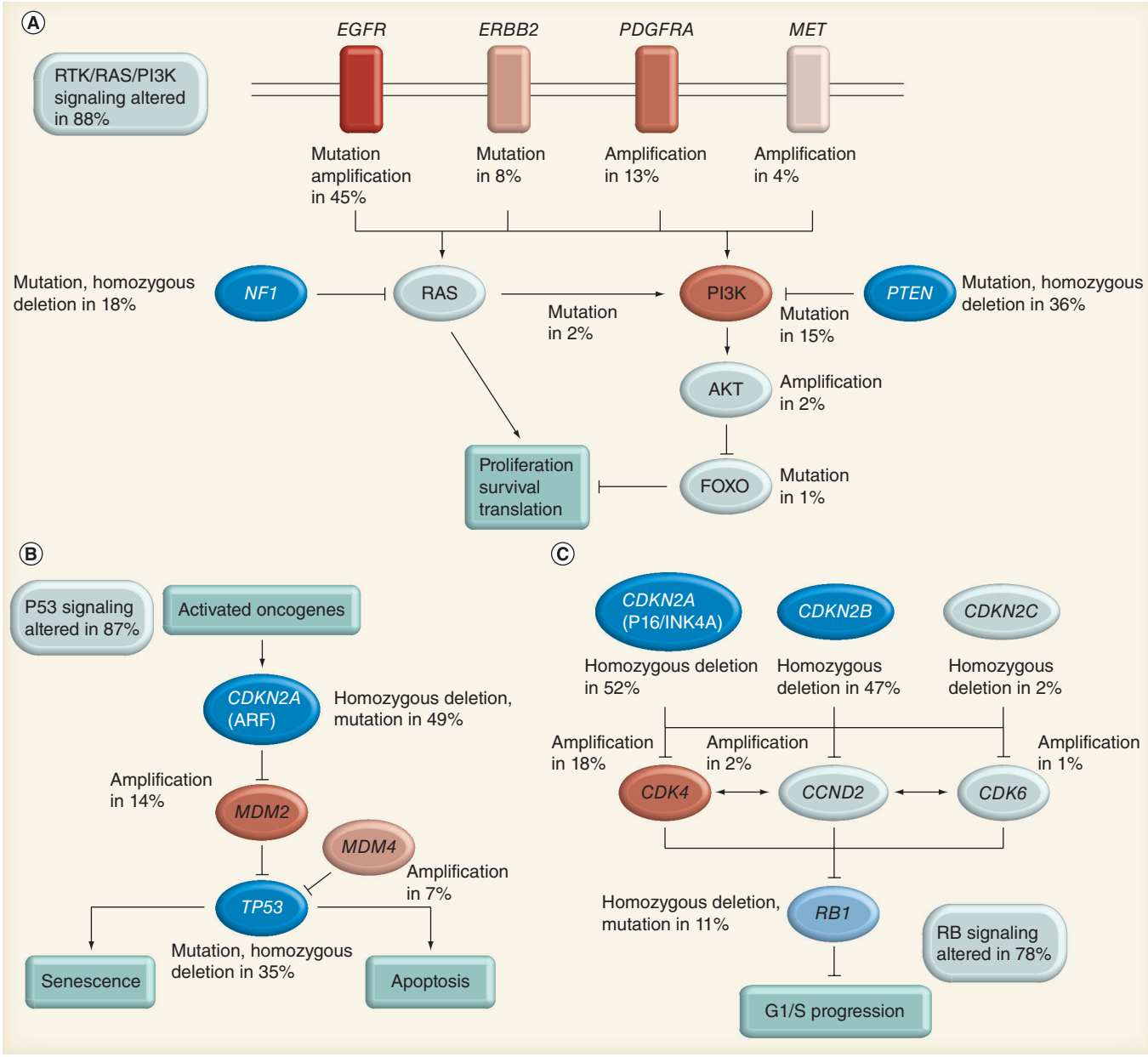

Figure 1. The Cancer Genome Atlas of glioblastoma multiforme. Primary sequence alterations and significant copy number changes for components of the **(A)** RTK/RAS/PI3K, **(B)** P53 and **(C)** Rb signaling pathways are shown. Red indicates activating genetic alterations, with frequently altered genes showing deeper shades of red. Conversely, blue indicates inactivating alterations, with darker shades of blue corresponding to a higher percentage of alteration. For each altered component of a particular pathway, the nature of the alteration and the percentage of tumors affected are indicated. Boxes contain the final percentages of glioblastoma multiformes with alterations in at least one known component gene of the designated pathway.
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associated with younger age and *IDH1* and *P53* mutations with a trend toward longer survival for these patients. Intriguingly, however, patients with proneural subtype GBM did not have an improved survival when receiving aggressive treatment. On the contrary, patient's with the classical subtype GBM, usually harboring *EGFR* amplification and homozygous deletion of *CDKN2* and *PTEN*, demonstrated the greatest benefit from standard treatment among all subtypes (Tables 1 & 2). Given the fact that each subtype harbors specific aberrations in molecular pathways, one may expect that targeting these pathways by specific inhibitors may provide new avenues for developing improved therapies.

Blood–brain barrier & drug efflux transporters

The brain is often referred to as a pharmacological sanctuary site since most drugs are unable to cross the blood–brain barrier (BBB) [29–31]. The BBB represents one of the major challenges to the efficacy of chemotherapy against GBMs. The BBB is formed by endothelial cells that are closely linked by tight junctions, disabling the paracellular movement of substances. Moreover, in contrast to most endothelial cells in the rest of the body, endothelial cells in the BBB lack fenestra and have low endocytic activity. Consequently, entry of substances into the brain can only occur by transcellular passage through the endothelium. Moreover, the pericytes and astrocytes intimately surrounding the endothelial cells form a secondary lipid layer, which further enforces the impermeability of the BBB [32]. Entry of essential nutrients (e.g., glucose) is strictly regulated by a range of uptake transporters. Other substances can only enter the brain by passive diffusion across the BBB, and the ability to do so is determined by a series of molecular parameters such as sufficient lipid solubility (octanol:water partition coefficient), molecular weight, degree of ionization, plasma protein binding and tissue binding. Nonetheless, even compounds that have molecular characteristics in favor of passive diffusion demonstrate much lower brain penetration than expected due to the activity of drug efflux transporters [31].

ABC drug transporters expressed at the BBB have well-known roles in the restriction of therapeutic agents into the brain [33]. Of all the efflux transporters present in the BBB, two transporters are mainly responsible for the efflux of anticancer agents back into the blood capillaries. These proteins are ABCB1 and ABCG2 (Figure 2B).

ABCB1

ABCB1 (also called P-gp or MDR1) is a 170-kDa membrane-associated protein expressed at high levels in normal human tissues, including the brain capillaries (Figure 2C). It was first discovered by its ability to confer multidrug resistance in cultured tumor cells [34]. ABCB1 is a highly promiscuous transporter, which recognizes an amazing range of drugs. Like all members of the ABC transporter superfamily, energy for the active transport of compounds is provided by hydrolysis of ATP at the nucleotide binding domains [31,35].

In addition to affecting cellular drug accumulation in tumor cells, ABC drug efflux transporters also actively affect the drug disposition by its expression at various barrier sites (BBB, intestinal epithelium and blood–testis barrier) [36–40]. ABCB1 was the first drug efflux transporter showing a remarkable impact on the brain delivery of substrate agents. Mice have two genes that are equivalent to *ABCB1*, namely *Abcb1a* and *Abcb1b* of which *Abcb1a* is the subtype that is expressed in the BBB. *Abcb1a*-deficient mice demonstrate a dramatic sensitivity to the neurotoxic pesticide ivermectin and to the cytotoxic drug vinblastine [41]. The role of ABCB1/Abcb1a in limiting drug brain penetration has been extended to a plethora of agents, including many novel targeted agents.

ABCG2

ABCG2 (murine subtype Abcg2), also known as BCRP, is a 72-kDa ABC transporter. Similar to ABCB1, it plays an important role in drug disposition and distribution in the body (Figure 2D). ABCG2 is expressed in many tissues of the body, including the apical side of the intestinal lumen, the bile canaliculus in liver hepatocytes and the capillaries of the BBB. In addition, ABCG2 transports a broad range of endogenous and exogenous compounds [31,35]. However, pharmacokinetic studies using *Abcg2*-knockout mice showed little effect on the brain penetration of drugs, with a few exceptions. This is most likely due to most drugs being substrates of both ABC transporters and the fact that the accumulation of these substances by the brain is limited by *Abcb1*, which is still present in *Abcg2*-knockout mice. The absence of both Abcb1 and Abcg2, however, results in a profound increase in brain uptake compared with the absence of each transporter alone [42]. Due

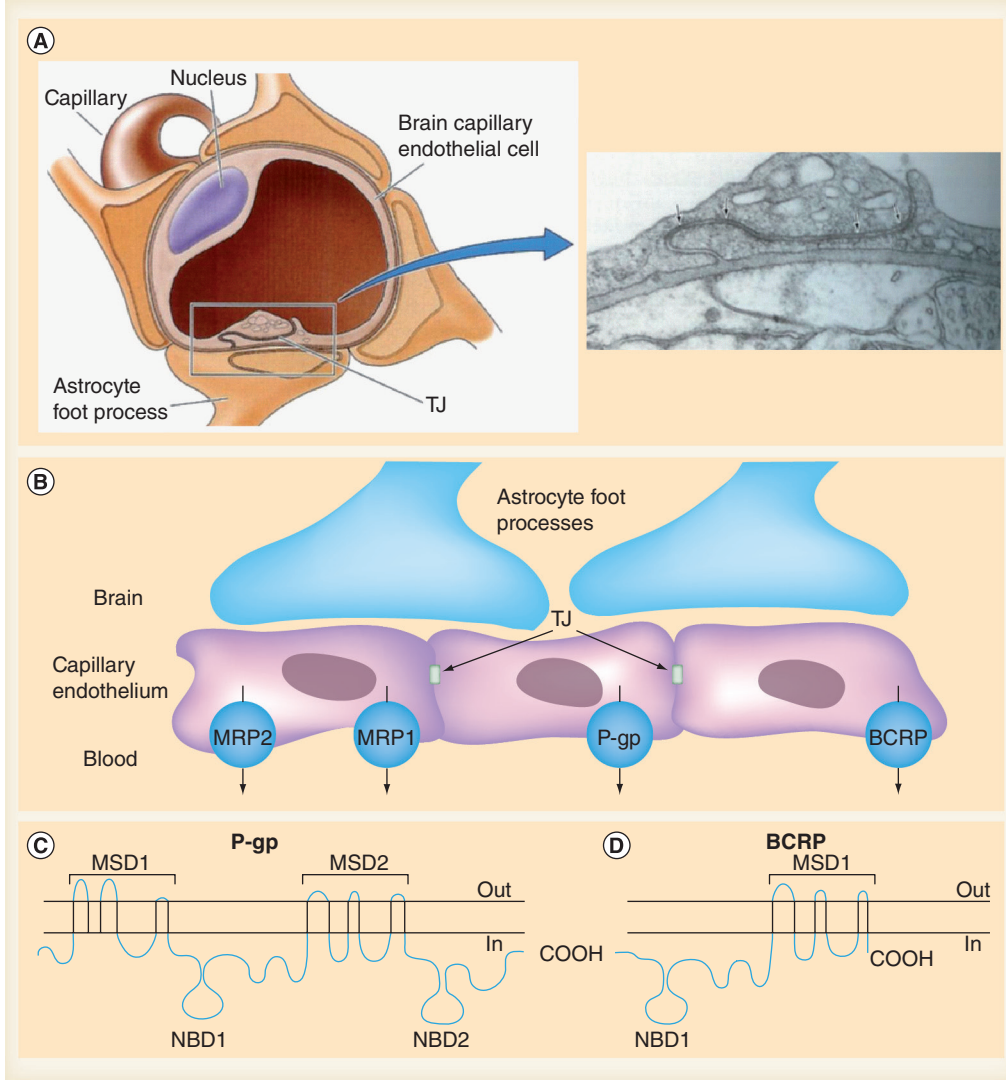

Figure 2. The blood–brain barrier. (A & B) Blood–brain barrier and ABC drug efflux transporters at the blood–brain barrier. **(C & D)** Secondary structures of P-gp and BCRP, respectively.
MSD: Membrane-spanning domain; NBD: Nucleotide-binding domain; TJ: Tight junction.
Right-hand panel in **(A)** reproduced with permission from [110] © Oxford University Press (1991).
Left-hand panel in **(A)** and **(B)** adapted with permission from [111].
(C & D) Adapted with permission from [35].

to the extremely broad substrate specificities of these two transporters, the concerted action of ABCB1 and ABCG2 is not restricted to only a few drugs, but represents a common mechanism to limit the brain entry of many drugs and, thus, potentially confers resistance to brain tumor chemotherapies (Table 3).

Targeting EGFR & lessons learned from erlotinib trials in GBM

The EGFR (ERBB1) is a member of the ERBB family of transmembrane RTKs and binds to at least six different ligands, including EGF and TGF-α. After binding a ligand, dimerization of EGFR takes place and the complex is activated and recruits PI3K. This activates the PI3K–AKT–mTOR pathway, transducing a proliferation signal to the cell. In tumor cells, *EGFR* amplification is often present as small fragments of extrachromosomal DNA (double minutes) and is often associated with structural mutations in the *EGFR* gene, of which several variants have been identified. *EGFRvIII* (i.e., *ΔEGFR*) is the most commonly occurring mutation in

GBMs derived by a nonrandom 801 bp in-frame deletion of exons 2–7, and codes for a truncated and constitutively activated protein [43–45]. Overall, *EGFRvIII* expression in the presence of *EGFR* amplification plays an important role in enhanced tumorigenicity and indicates a poor survival prognosis in GBM patients [46].

Although *EGFR* amplification and mutation is considered to be an important factor, none of the currently tested EGFR inhibitors have shown any clinical efficacy against GBM. The contrast between the more successful application of EGFR inhibitors in other types of cancer such as lung cancer and failure of EGFR inhibitors in GBM have been extensively studied. These studies suggest that the lack of response to EGFR inhibitors in GBM is multifactorial. A first issue is that it is not clear whether glioma cells will be exposed to therapeutic levels of erlotinib (i.e., can a therapeutic level of erlotinib be reached in the glioma tissue?). Erlotinib is a substrate of both ABCB1 and ABCG2, and the two drug efflux transporters together resulted in a sevenfold reduction of brain:plasma ratio in wild-type compared with *Abcb1*- and *Abcg2*-knockout mice [47,48]. Thus, the limited BBB penetration of erlotinib caused by ABCB1 and ABCG2 may be at least partly responsible for the resistance of GBM to erlotinib treatment. Unfortunately, the fact is that ABCB1 and ABCG2 have a long list of overlapping substrates, including most RTK inhibitors, such as gefitinib [49], sunitinib [50], dasatinib [51,52], imatinib [53] and lapatinib [54], and the brain penetration of these compounds is also markedly restricted by these two transporters (Table 3).

Table 3. Impact of Abcb1 and Abcg2 on the brain penetration of targeted agents as demonstrated in Abcb1- and/or Abcg2-deficient mice.

Agent	Target protein(s)	Brain penetration limited by Abcb1?	Brain penetration limited by Abcg2?	Ref.
Sirolimus	mTOR	Yes	No	[Lin *et al.*, Unpublished data]
Palomid 529	mTOR	No	No	[112]
Erlotinib	EGFR	Yes	Yes	[47,113]
Gefitinib	EGFR	Yes	Yes	[49]
Sunitinib	VEGFR-2 and -3, c-Kit, FLT3 and PDGFR	Yes	Yes	[50]
Cediranib	VEGFR	Yes	Yes	[114]
Axitinib	VEGFR	Yes	Yes	[115]
Sorafenib	c-Kit, PDGFR and Raf	Yes	Yes	[102]
Dasatinib	BCR–ABL, c-Kit, PDGFR, SRC	Yes	Yes	[52]
Vemurafenib	B-Raf^{V600E}	Yes	Yes	[116,117]
Dabrafenib	B-Raf^{V600E}	Yes	Yes	[118]
Imatinib	BCR–ABL, c-Kit, PDGFR	Yes	Yes	[53,105]
Lapatinib	HER2 (ERBB2), EGFR	Yes	Yes	[54]
GDC-0941	PI3K	Yes	Yes	[69]
Tandutinib	c-Kit, FLT3 and PDGFRβ	Yes	Yes	[119]

EGFR: EGF receptor; PDGFR: PDGF receptor; VEGFR: VEGF receptor.

A second issue is that deregulated components downstream of EGFR could abolish the effects of EGFR inhibition. For example, Mellinghoff *et al.* reported that PTEN loss in GBM cells would cause resistance to erlotinib [55]. However, this is not the only reason, since the randomized EORTC study also found tumors with expression of PTEN, and EGFR and/or EGFRvIII that responded poorly [10]. There was only a weak relationship between the levels of phosphorylated AKT and the response to erlotinib. As we know, PTEN is not the only key factor controlling the signaling downstream of EGFR. In addition, PI3K mutation and AKT amplification can lead to activation of the PI3K pathway. Furthermore, there is active crosstalk between the PI3K and RAS pathways [56,57], and activation of the RAS–RAF–MEK–ERK pathway is common in GBM [58]. This pathway activation can be caused by a mutation or deletion of *NF1* or (more rarely) by mutation of *RAS*. In addition, mutation and amplification of other parallel RTKs, such as *ERBB2*, *PDGFR* and *c-MET*, could also activate signaling via the PI3K–mTOR and RAS pathway, thereby conferring resistance to EGFR inhibition [59]. Last but not least, another explanation for the disappointing clinical activity of erlotinib in GBM versus lung cancer was delivered by a recent study by Vivanco *et al.* [60]. Vivanco *et al.* demonstrated that distinct types of *EGFR* mutations in lung cancer and GBM responded differently to EGFR inhibitors. Importantly, they also found that in

lung cancer, the first-generation EGFR inhibitor erlotinib effectively inhibits EGFRs carrying mutations in the kinase domain, whereas it performs very poorly against EGFRs with mutations or deletions in the extracellular domain as in GBM [60]. The putative resistance of GBM to erlotinib caused by drug efflux transporters and/or intrinsic molecular mechanisms are demonstrated in Figure 3.

Targeting the PI3K–AKT–mTOR pathway

The PI3K–AKT–mTOR pathway, activated by extracellular survival signaling factors via RTKs, is a major cell signaling pathway involved in regulating a variety of cellular processes, including cell proliferation, survival, growth, glucose metabolism and protein synthesis [61]. The most frequent alteration responsible for the deregulation of this pathway in GBM is the loss of *PTEN* (36%). In addition, mutation of the *PI3KCA* (15%) gene, and occasionally *AKT* amplification (2%) or *FOXO* mutation (1%), also contribute to the activation of downstream signaling (Figures 1 & 4) [27,62,63]. Constitutive PI3K–AKT–mTOR pathway activation is a hallmark of GBM.

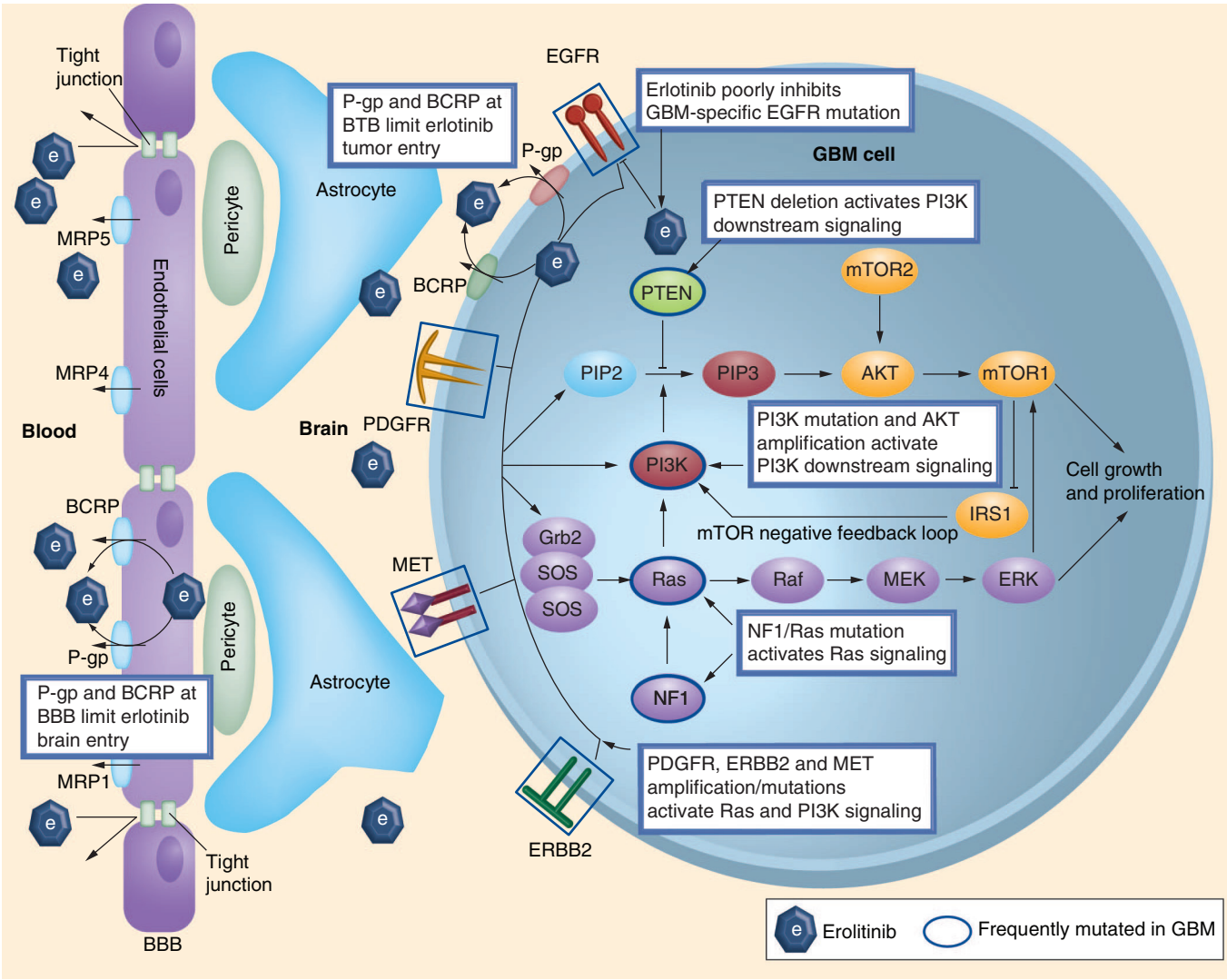

Figure 3. Putative mechanisms of glioblastoma multiforme resistance to erlotinib treatment. Erlotinib acts on the EGFR causing downstream signaling of the PI3K–mTOR and Ras–MEK–ERK pathways. However, the glioblastoma multiforme-specific mutation *EGFRvIII* is less susceptible to erlotinib, and redundant receptor tyrosine kinases (PDGFR, ERBB2 and MET) may also cause downstream signaling. Moreover, the activity of the drug transporters (ABCB1 and ABCG2) located at the BBB and in the tumor cell may cause insufficient entry of erlotinib to elicit target inhibition.
BBB: Blood–brain barrier; BTB: Blood–tumor barrier; EGFR: EGF receptor; GBM: Glioblastoma multiforme; PDGFR: PDGF receptor.

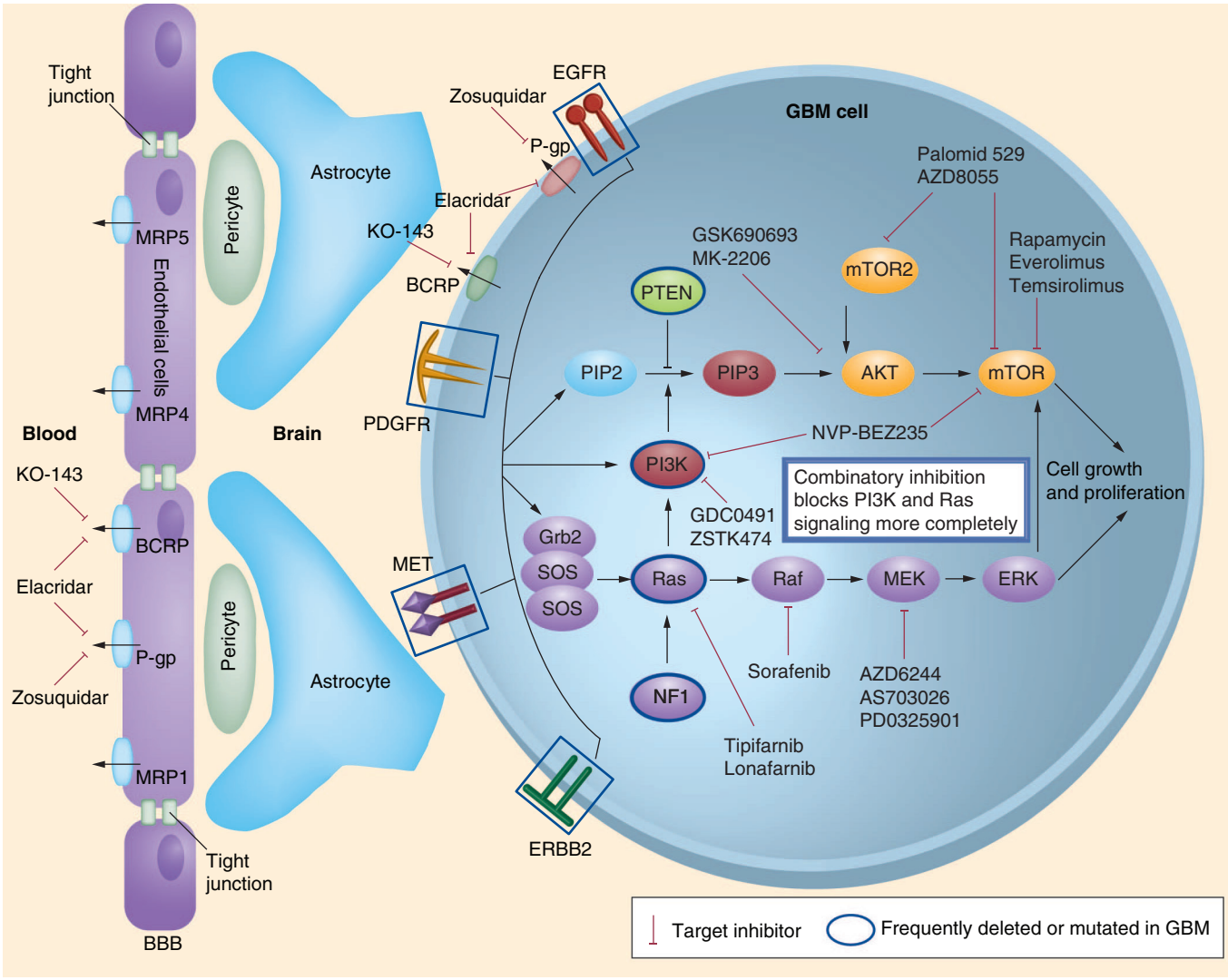

Figure 4. Core pathways involved in glioblastoma multiforme. RTK–PI3K–AKT–mTOR and RTK–RAS–RAF–MEK–ERK signaling pathways and putative inhibitors.
BBB: Blood–brain barrier; EGFR: EGF receptor; GBM: Glioblastoma multiforme; PDGFR: PDGF receptor.

The class IA PI3K is a heterodimer composed of an 85-kDa regulatory subunit (P85α) and a 110-kDa catalytic subunit (P110α). Once RTK recruits PI3K to the cellular membrane, the PI3K subunit converts inactive PIP_2 into active PIP_3, which then recruits AKT to the membrane together with PDK1. Furthermore, PTEN counteracts PI3K by converting PIP_3 back into PIP_2, functioning as a tumor suppressor. Unlike other components of cellular pathways with multiple protein family members, there is no PTEN-related protein present in the cells that can compensate for its loss. Therefore, it is not surprising that the loss of PTEN function plays a pivotal role in tumorigenesis [64].

PI3K inhibitors

Due to the high mutation rates of *PTEN* and *PI3KCA* (the gene that encodes the catalytic subunit P110α of PI3K), and the importance of this pathway in GBM, PI3K, and especially its subunit P110α, provides an attractive drug target. The first generation of PI3K inhibitors (LY294002 and wortmannin) showed *in vivo* antitumor efficacy, but were associated with poor stability or solubility, undesirable toxicities and crossover inhibition of other lipid and protein kinases [65,66]. Therefore, clinical trials with these compounds have not been initiated. Since the crystal structure of PI3K was elucidated, the development of new PI3K inhibitors has been accelerated. More selective PI3K inhibitors have been developed, with

promising antitumor efficacy and low toxicity in preclinical research. For example, GDC-0941 is a potent and selective ATP-competitive PI3K inhibitor. It inhibits the PI3K P110α subunit with an inhibitory concentration at 50% inhibition (<10 nM) and inhibits the phosphorylation of AKT with an inhibitory concentration at 50% inhibition (28 nM) [67]. GDC-0941 treatment has led to an increase in apoptosis and inhibition of growth in a subset of xenograft tumor cell lines [68]. *In vivo* antitumor activity with daily oral dosing at 150 mg/kg of GDC-0941 achieved 98% growth inhibition in subcutaneous U87MG xenografts [65,68]. Unfortunately, GDC-0941 is also a substrate of both ABCB1 and ABCG2. After intravenous or oral administration, the GDC-0941 brain-to-plasma ratio in *Abcb1*- and *Abcg2*-knockout mice was approximately 30-fold higher than in wild-type mice. The PI3K pathway was markedly inhibited as evidenced by 60% suppression of the phosphorylated AKT in the brains of Abcb1- and Abcg2- knockout mice, whereas no inhibition was detected in the brains of wild-type mice [69]. Therefore, the potential efficacy of GDC-0941 as a targeted agent for treatment of GBM is limited due to ABCB1 and ABCG2.

AKT is a serine/threonine protein kinase that can be activated by phosphorylation at the threonine-308 by PDK1, or serine-473 by mTORC2. The mechanism by which the latter phosphorylation occurs is not fully understood; however, recent work suggests that activation of mTORC2 kinase activity is induced by EGFR-vIII in GBM cells, and that abnormal mTOR2 signaling can promote GBM growth and survival [70]. When phosphorylated, AKT in turn phosphorylates a variety of downstream effector proteins, of which mTORC1 is one of the most important ones. There are very few trials with AKT inhibitors in GBM. The planned clinical trial with MK-2206 has been canceled by Merck (NJ, USA) due to a reprioritization within their oncology program [201].

■ mTORC1 & mTORC2 inhibitors

mTORC1 is regarded as a central regulator of cell growth and has a critical role in tumor development. Via two major downstream targets, S6K (p70 S6 kinases) and 4EBP1, mTORC1 triggers the synthesis of proteins involved in cell survival, growth and proliferation [71–73]. Mutations of the *mTOR* gene are rare in GBM, but frequently deregulated upstream signaling drives mTORC1 activation. Inhibition of mTORC1 by rapamycin or other rapalogs has shown efficacy in a subset of cancers [74,75]. However, rapamycin and other rapalogs only inhibit mTORC1 and not mTORC2 [76]. This can lead to activation of AKT via an mTORC2-driven positive-feedback loop [74,77,78]. The novel generation of mTOR inhibitors are multitargeting agents, which are capable of inhibiting dual targets in the PI3K pathway or even more targets, to more completely block the feedback loop activation caused by inhibition of mTORC1. Dual mTORC1 and mTORC2 inhibitors that disrupt downstream signaling of mTORC1, and at the same time inhibit AKT activation by blocking mTORC2 activity, are interesting candidates for evaluation of treatment efficacy in GBM. AZD8055, a dual mTORC1 and mTORC2 inhibitor, is a highly potent, ATP-competitive and specific mTOR kinase inhibitor. *In vivo*, AZD8055 demonstrated potent single-agent antitumor activity against a range of subcutaneous xenografts, including U87 malignant glioma [79]. AZD8055 is currently being evaluated in a clinical trial in adults with recurrent glioma [202]. To date, no data have been presented in orthotopic brain tumor models, or to assess whether AZD8055 is able to cross the BBB. Similar to AZD8055, Palomid 529 is another dual mTORC1/mTORC2 inhibitor that markedly reduces the phosphorylation of AKT (S473-Akt) signaling by inhibition of both mTORC1 and mTORC2 activity. *In vivo* studies demonstrated that Palomid 529 reduced angiogenesis, vascular permeability and tumor growth [80]. Moreover, Palomid 529 was shown to enhance the antiproliferative effect of radiotherapy in GBM in an orthotopic model [81], as well as in prostate tumor models [82]. Another way to interrupt the mTORC2–PI3K positive-feedback loop is by combined inhibition of mTORC1 and PI3K. Particularly, the imidazo(4,5-c)quinoline derivative, NVP-BEZ235, selectively inhibits both PI3K and mTOR kinase activity by binding the ATP-binding cleft of these enzymes, thus, resulting in G1 arrest and autophagy in tumor cells. It displayed remarkable antitumor activity in U87MG GBM xenograft models with a dose-dependent effect, and it could further enhance the efficacy of temozolomide [83]. Further studies using U87 intracranial xenograft models also confirmed the antitumor potency of NVP-BEZ235 in the treatment of GBM [84]. NVP-BEZ235 has not been tested clinically against glioma, most likely because the company (Novartis, Basel, Switzerland) has prioritized NVP-BKM120 for development in treating glioma. BKM120 is a

pan-class 1 PI3K inhibitor, but has no inhibitory activity against mTOR [85]. This compound is assumed to penetrate the BBB [86].

Targeting the RAS–RAF–MEK–ERK pathway

The MAPK pathway is activated in the majority of GBMs through various mechanisms, such as via *EGFR* mutation or amplification (45%), *PDGFR* amplification (13%) or deletion of *NF1* (18%) (Figures 1 & 4) [27]. Upon activation, the growth factor receptors generate binding sites for adaptor proteins, such as GRB2, containing a SH2 domain. Next, GRB2 recruits SOS to the membrane, which, in turn, activates RAS through the replacement of inactive GDP with active GTP. As a result, RAS is able to recruit RAF kinases (A-RAF, B-RAF and C-RAF) to the plasma membrane, where they are activated. RAF is able to phosphorylate and thereby activate MEK1 and MEK2, which, in turn, activate ERK1 and ERK2. Activation of ERK leads to activation of a variety of nuclear and cytoplasmic substrates associated with gene regulation, cell cycle progression, differentiation and cell division [27,87,88]. Due to its important role in cell proliferation and survival, the MAPK pathway is frequently altered in a variety of tumors. K-RAS, one of the three *RAS* genes, is often mutated in leukemia, colon cancer, pancreatic cancer and lung cancer. Although human GBMs rarely show *RAS* mutations (2%), almost all malignant human gliomas show elevated levels of activated RAS as a result of other upstream molecular alterations.

MEK inhibitors

Inhibition of MEK is an effective strategy to prevent the subsequent downstream signaling of the RAS pathway, and consequently induces tumor regression and/or stasis. A recent study by See *et al.* demonstrated that PD0325901 and AZD6244, as single agents, suppressed the growth of *NF1*-deficient and MEK inhibitor-sensitive glioma cells both *in vitro* and *in vivo* [89]. Their findings indicate that a subset of *NF1*-deficient GBMs may be responsive to MEK inhibitors. Moreover, they found that *NF1*-deficient glioma cells that are intrinsically resistant to MEK inhibition were sensitized by the addition of the dual PI3K/mTOR inhibitor PI-103. Many commonly used MEK inhibitors are benzohydroxamate derivatives, sharing many similarities in chemical structure. These inhibitors result in MEK-specific inhibition by binding to the hydrophobic pocket, adjacent to the ATP binding site of the MEK protein, which keeps the kinase in a catalytically inactive state. This allosteric mechanism contributes to the high selectivity for MEK without affecting other protein kinases that have structurally similar ATP-binding pockets. Therefore, MEK inhibitors are usually highly specific and non-ATP-competitive inhibitors. PD-0325901 was the first clinically tested MEK inhibitor. *In vivo* results demonstrated that PD-0325901 potently inhibits growth of human tumor xenografts bearing activating mutations of B-Raf, concomitant with suppression of ERK1/2 phosphorylation [90]. Interestingly, during Phase I and II clinical trials in advanced cancers, antitumor activity was seen when treated with 4–30 mg twice-daily doses of PD-0325901 [91,92]. However, beside the more common side effects like rash, diarrhea and fatigue, the drug also caused ocular and CNS toxicities at doses above 15 mg, and Pfizer (NY, USA) has suspended its further evaluation. Notably, a similar ocular toxicity has been observed with the MEK1 inhibitor AZD6244 (selumetinib), albeit to a lesser extent than PD-0325901. Whether these CNS toxicities are a direct consequence of MEK inhibition in the brain or caused by off-target drug effects is still unclear; both are possible regarding the structural similarities of the MEK inhibitors tested so far. Clearly, these CNS toxicities suggest that MEK inhibitors, such as PD-0325901, are able to enter the CNS, which would qualify these as candidates for testing in GBM. However, MEK inhibitors are predominantly evaluated against non-CNS tumors and the selection of novel candidates is narrowed to those having a low BBB permeability to avoid CNS toxicities. It should be noted that this strategy holds the risk that a complete class of targeted agents may become useless for treating GBM. The central role of an activated RAS pathway in GBM argues in favor of using MEK inhibitors, although it is obvious that finding the optimal dose level will be a challenging task.

Rb pathway & CDK inhibiton

Deregulation of the G1/S checkpoint is very common in GBM. Cyclin-dependent kinases (CDKs) are serine/threonine protein kinases whose activity depends on binding and activation by cyclin partners, and they are required for cell cycle progression. CDK4 and CDK6, which are both under control of $P16^{INK4a}$ and $P15^{INK4b}$, bind to cyclin D and phosphorylate Rb, causing subsequent release of the transcription factor E2F and synthesis of proteins that are needed in the S phase. The most

common alteration of the Rb pathway in GBM (52% of cases) is a homozygous deletion of parts of the *CDKN2* locus that code for $P16^{INK4a}$ and $P15^{INK4b}$. Other alterations include amplification and overexpression of *CDK4* (15–20%) and homozygous deletion/mutation of the *RB1* gene (~10%) (Figure 1 & 5). Deletion of *CDKN2A* (or amplification of *CDK4*), *CDKN2B* and *CDKN2C* leads to loss of cell cycle control and increased cell proliferation. Codeletion of *CDKN2A* and *CDKN2C* serves as a strong predictor of sensitivity to a selective inhibitor of CDK4/6 [93]. Amplification of *CDK6* and individual D-type cyclins, and homozygous deletion of *CDKN2C* encoding $P18^{INK4c}$ are less common [27].

■ CDK inhibitor PD-0332991

CDK4 is a logical target, taking into consideration that the loss of *CDKN2A/B* or amplification of *CDK4* is a frequent event in GBM. PD-0332991 is an orally bioavailable CDK inhibitor, which selectively inhibits CDK4 and CDK6. Antiproliferative activity has been demonstrated in luminal breast cancer, myeloma and GBM cell lines [94,95]. As expected, *RB1*-deficient tumors were resistant to PD-0332991. Michaud *et al.* demonstrated that PD-0332991 was effective in suppressing the growth of intracranial U87MG tumors, including those that recurred after initial therapy with temozolomide [94]. The combination of PD-0332991 and radiation therapy resulted in significantly increased survival compared with either therapy alone. Based on these results, it was argued that this compound can efficiently cross the BBB [94]. It should be noted, however, that the BBB in U87MG tumors is very leaky [96].

Two completed Phase I trials showed that PD-0332991 is generally well tolerated and neutropenia was the sole significant toxicity at maximum tolerated dose (125 mg once daily) [97,98]. A Phase II clinical study to test PD-0332991 in patients with recurrent Rb-positive GBM is currently ongoing [203].

Future strategies for targeted therapy

■ Combined inhibition of multiple pathways

As outlined above, at least three core signaling pathways (RAS–RAF–MEK–ERK,

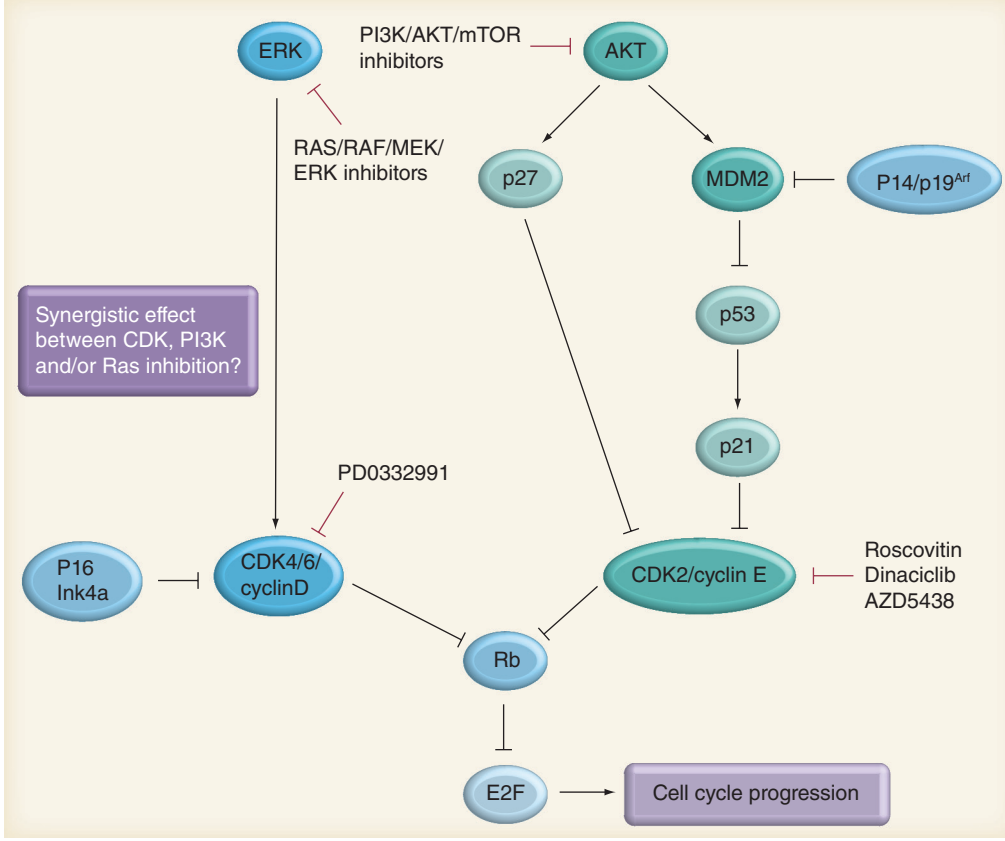

Figure 5. Core pathways involved in glioblastoma multiforme. The Rb pathway and a listing of some example drugs that have been developed to inhibit these pathways.

PI3K–AKT–mTOR and CDKN2–CDK4/6–RB1) are jointly activated in the majority of GBMs through different mechanisms, and targeting just one of these components may be insufficient to achieve a meaningful effect on tumor progression. In addition, crosstalk between different molecules of two or more pathways increases the plasticity of tumor-survival signaling and reduces oncogene addiction [56].

As depicted in Figure 3, inhibition of EGFR will not be able to suppress the activation of PI3K and RAS pathways in case other oncogenic alterations in parallel (e.g., other RTKs) and/or downstream components (e.g., PI3K activation) have occurred. Similarly, as shown with mTOR inhibitors, treatment with an inhibitor of a single pathway may also not sufficiently block parallel signaling pathways to reach a significant antiproliferative effect. For example, Di Nicolantonio *et al.* have shown that a number of human cancer cell lines carrying alterations in the PI3K pathway responded to everolimus, but only when there was no concomitant KRAS mutation [99].

Although several studies with PI3K and RAS inhibitors, given as a single agent, have demonstrated promising tumor growth inhibitory potencies by *in vitro* or *in vivo* models using established GBM cell lines, such as U87-MG, it should be taken into account that these GBM cells have been cultured for many generations. When grown *in vivo* they form homogeneous noninvasive lesions with a relative stable genome, unlike the highly heterogeneous GBMs that are typically found in patients. This discrepancy may be a plausible explanation for their poor predictive value on the usefulness of these agents against GBM in the clinic.

The considerations above argue in favor of targeting multiple pathways simultaneously, by analogy with the polypharmacy commonly applied in antiretroviral therapy. Ideally, this would include targeting all three core signaling pathways simultaneously. Although it will be challenging to design combination therapies that result in sufficient inhibition of these three core pathways simultaneously with acceptable toxicities, this concept would have the intrinsic potential to be beneficial for a substantial fraction of GBM patients. To date, just a few studies on combinations of targeted agents have been reported. Clinical trials combining EGFR and mTOR inhibitors reported considerable toxicities and the potential of drug–drug interactions, highlighting some of the issues that may be encountered [100,101]. However, whereas cytotoxic drugs in oncology are traditionally dosed at the maximum tolerated dose level, this 'more equals better' strategy is most likely suboptimal for targeted agents. Taking into consideration the basic principles of pharmacokinetic–pharmacodynamic relationships, the optimal dose should be determined by verifying target inhibition, since higher dose levels may not contribute to improved efficacy, but may increase toxicities due to off-target effects. Implementing methods to verify target inhibition in tumor tissue will be crucial to the further development of combination therapy with targeted agents, not just in gliomas but in all cancers.

■ Targeted therapy combined with drug efflux transporters inhibitors

The important roles of ABCB1 and ABCG2 in drug resistance, and in limiting the brain penetration of therapeutic drugs, are well established. However, surprisingly little attention has been paid to this fact when designing clinical trials with targeted agents in GBM. Erlotinib, lapatinib and most other newly developed kinase inhibitors are substrates of ABCB1 and/or ABCG2 and, as a consequence, their usefulness in the treatment of GBM growth may be compromised by an inadequate brain penetration. The reality is that most targeted agents are initially developed for the treatment of major tumor types, such as lung and breast cancer, in which good BBB penetration is irrelevant or considered undesirable (e.g., MEK inhibitors). Consequently, however, agents from this panel that are being considered for further evaluation in GBM may not be the best BBB-permeable drugs.

Elacridar (GF120918) and tariquidar are both dual ABCB1 and ABCG2 inhibitors that were developed in the 1990s to improve the treatment of ABCB1-mediated multidrug resistant tumors. Due to the lack of success in this area, this concept is not currently receiving much attention. These same agents, however, have the potential to enhance the brain penetration of targeted therapies by blocking the efflux of drugs by these two transporters at the BBB, and perhaps also at the blood–tumor barrier. Coadministration of elacridar with a number of anticancer drugs has been proven to be an effective strategy to enhance the brain accumulation of these drugs, including a range of potentially effective targeted therapeutics [49–52,102–109]. Therefore, the use of elacridar might represent a feasible strategy to improve the brain entry of potentially effective targeted therapeutics for GBMs.

Conclusion & future perspective

The TCGA project and other collaborative research efforts have revealed how the oncogenetic processes of GBM are driven by multiple deregulated core signaling pathways and will provide new avenues for more effective targeted therapies in the treatment of GBM. Because the crosstalk between these molecular pathways fuels the plasticity of these processes, targeting a single, prevalent target that promotes and dominates GBM proliferation will, at best, provide only very short-lived effects. Consequently, the next generation of targeted therapies should focus on multitargeting agents or combinations of single-targeting agents against these core pathways.

Importantly, when selecting the most appropriate candidates of targeted therapeutics, the brain penetration of such candidates and, in particular, their interactions with the drug efflux transporters ABCB1 and ABCG2 should be taken into consideration. No matter how potent an agent is in inhibiting or activating its target, it has to reach that target at a therapeutic level, which is more difficult to achieve in the brain than in other tissues. Ideally, substances should be designed to have a low affinity for drug efflux transporters. Alternatively, coadminstration of targeted agents together with inhibitors of these drug efflux transporters (e.g., elacridar) may be helpful and should also be considered.

The progress that has been made in the treatment of GBM during recent years has been very modest. Therapies that are based on targeting core signaling pathways underlying the processes of malignant transformation is an emerging therapeutic strategy that holds great potential and receives a lot of attention. However, if we continue testing such agents against GBM, one-by-one and without considering whether the candidate drugs are able to cross the BBB sufficiently, it is likely that again little progress will have been made in 5–10 years from now.

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