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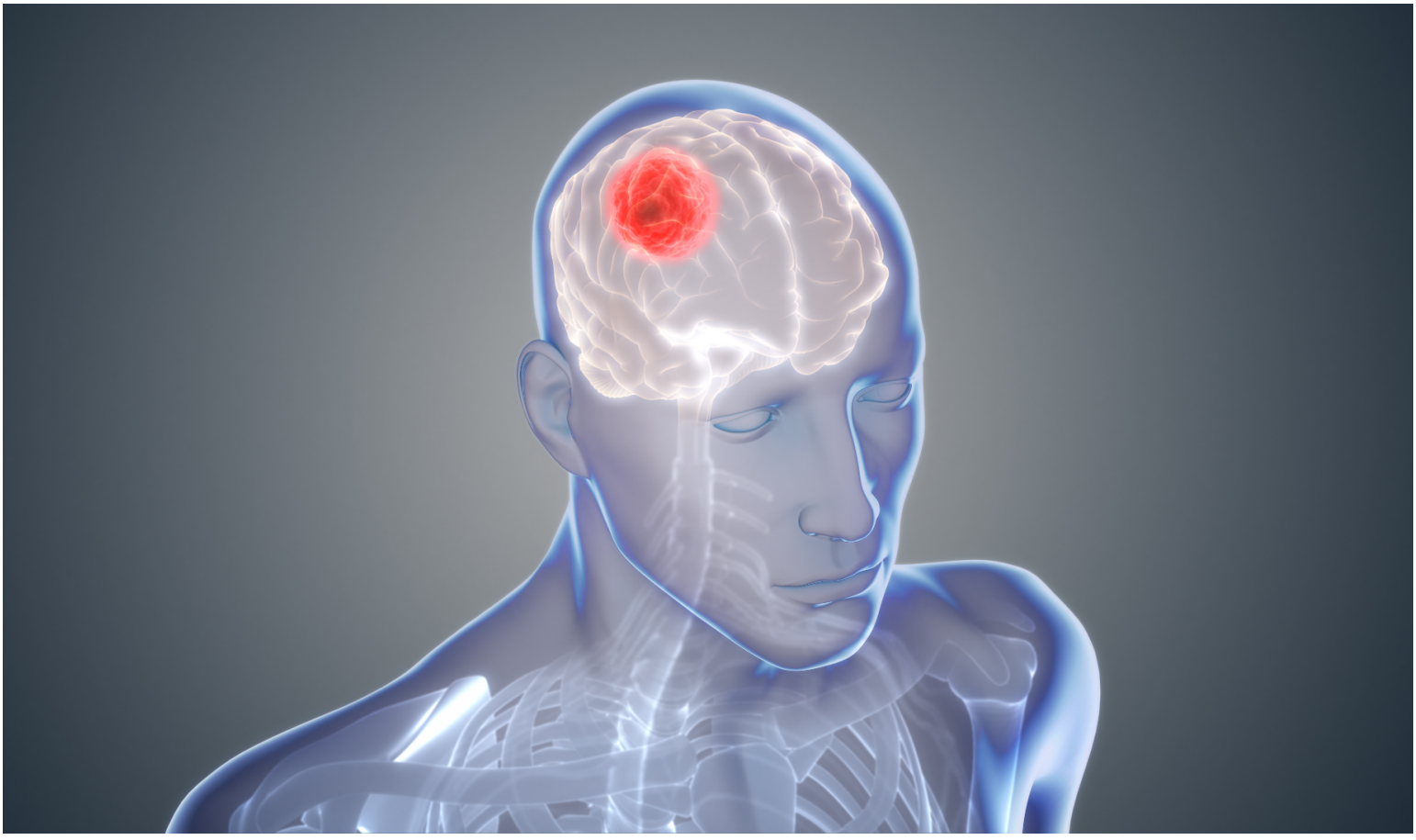
Genetically altered Receptor Tyrosine Kinase Signaling in Glioblastoma Multiforme

MOLECULAR MECHANISMS, CURRENT TARGETED THERAPIES AND FUTURE PERSPECTIVES



Bachelor thesis

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ABSTRACT

Glioblastoma (Glioblastoma Multiforme, GBM) is a WHO grade IV astrocytic tumor of the central nervous system (CNS) with a highly malignant phenotype. Standard therapy of newly diagnosed patients consists of surgical removal of the tumor, followed by a combination of chemotherapy and radiotherapy. Despite these aggressive therapies, newly diagnosed GBM patients have poor prognosis. Median survival is 15 months, with a 2-year survival of 26,5% and a 5-year survival of 3-5%. Consequently, research has been focused on investigating the genetic drivers and molecular mechanisms behind GBM pathogenesis, mainly by Next Generation Sequencing methods. As Receptor Tyrosine Kinase (RTK) signaling is found frequently genetically altered in GBM, numerous therapeutic agents that target this signaling cascade have been investigated. They include inhibitors of both the RTKs itself and inhibitors of both downstream effector arms: the Ras/Raf/MAPK pathway and the PI3K/AKT/mTOR pathway. This article provides an overview of the exciting progress toward developing new therapeutic agents for GBM therapy and their implications for clinical practice.

Keywords: Glioblastoma Multiforme • GBM • genetic profile • RTK signaling pathway • therapeutic inhibitors • targeted therapy

INTRODUCTION

Glioblastoma (Glioblastoma Multiforme, GBM) is a World Health Organisation (WHO) grade IV astrocytic tumor of the central nervous system (CNS), characterized by poorly differentiated astrocytes. GBM accounts for approximately 50% of all gliomas and possesses the most malignant phenotype (Ostrom et al. 2016; Bastien et al. 2015; Louis et al. 2007; Reni et al. 2017)

Clinically, GBMs can be classified into primary and secondary GBMs. Primary GBMs show no clinical evidence of a primary lesion (de novo synthesis) and are most common in elderly patients (median age 60 years). These GBMs progress rapidly and account for approximately 90-95% of the total GBMs. The remaining 5-10% of GBMs develops from a lower grade glioma by accumulation of mutations and are therefore classified as secondary GBMs. They are most common in young patients (median age 45 years) and show a longer history of disease progression. In the 2016 WHO Classification of Tumors of the Central Nervous System a new classification of GBMs was made, based on their genetic driver mutations. The IDH1 and IDH2 genes encode critical metabolic enzymes and are found mutated mainly in secondary GBMs (73-85%), whereas mutations in these genes are rare or absent in primary GBMs. Therefore, in the 2016 WHO classification, GBMs are subcategorized into IDH-wildtype GBMs (comparable to primary GBMs) and IDH-mutant GBMs (comparable to secondary GBMs). Nowadays, this classification is used most commonly (Bastien et al. 2015; Preusser et al. 2011; Szopa et al. 2017; Reni et al. 2017).

Although clinically and genetically different, both IDG-wildtype and IDH-mutant GBMs demonstrate similar histopathological and pathophysiological features; they are characterized by uncontrolled cell proliferation, diffuse invasiveness, resistance to apoptosis, angiogenesis and widespread genomic instability (Furnari et al. 2007; Ohgaki & Kleihues 2007).

Standard therapy of newly diagnosed GBM patients consists of surgical removal of the tumor, followed by a combination of chemotherapy with Temozolomide (TMZ) and radiotherapy (RT). If there is disease progression bevacizumab, an inhibitor of Vascular Endothelial Growth Factor (VEGF), is commonly used. Despite these relatively aggressive therapies, newly diagnosed GBM patients have poor prognosis. Median survival is only 15 months, with a 2-year survival of 26,5% and a 5-year survival of 3-5%. Several factors attribute to this glooming life perspective for GBM patients. Firstly, due to the diffuse and highly invasive growth of GBM tumors, complete surgical removal is almost never possible and recurrence close to the primary site of the tumor occurs in 90% of the cases. Secondly, response to treatment with TMZ and RT is low; due to the inaccessibility and heterogeneity of the tumors, resistance to TMZ and RT is commonly obtained (Bleeker et al. 2012; Szopa et al. 2017; Stupp et al. 2005; Reni et al. 2017; Giese et al. 2003; Vitucci et al. 2013; Gao et al. 2013).

In other words, the need for novel therapies is high. Recent research has therefore been focused on identification of the mechanisms responsible for GBM disease formation. In the past decades, Next Generation Sequencing techniques have led to the discovery of several genetically altered molecular pathways in GBM, the most notable being the TP53 pathway, the RB1 pathway and the RTK signaling pathway (Parsons et al. 2008; McLendon et al. 2008; Vitucci et al. 2013; Verreault et al. 2016).

Receptor Tyrosine Kinases (RTKs) are cell surface receptors that regulate their downstream signaling via two important effector pathways: the Ras/Raf/MAPK pathway, which promotes cell proliferation and differentiation, and the P13K/AKT/mTOR pathway, which is important for regulating cell survival, apoptosis, cell cycle and protein translation. Studies have demonstrated that in the majority of GBMs there is an activation or overexpression of the positive regulators, and an inactivation or down-regulation of the negative regulators of the RTK signaling pathway, overall resulting in an up-regulation of this pathway. This manifests itself in a prolonged cell survival, increased migration and invasiveness and down-regulation of apoptosis in GBM. Therefore, the RTK signaling pathway provides with several new interesting targets for therapy, which can be found either at the top of the signaling pathway (RTKs), or downstream in either one of the effector

pathways (McLendon et al. 2008; Stommel et al. 2007; Szerlip et al. 2012; Gao et al. 2013; Vitucci et al. 2013; Omerovic et al. 2007).

The aim of this article is to present an overview of the exciting progress that has been made in investigating the RTK signaling pathway in primary GBM; its molecular mechanisms, genetic alterations and their prevalence in GBM will be discussed, along with their implications for clinical practice (see figure 1).

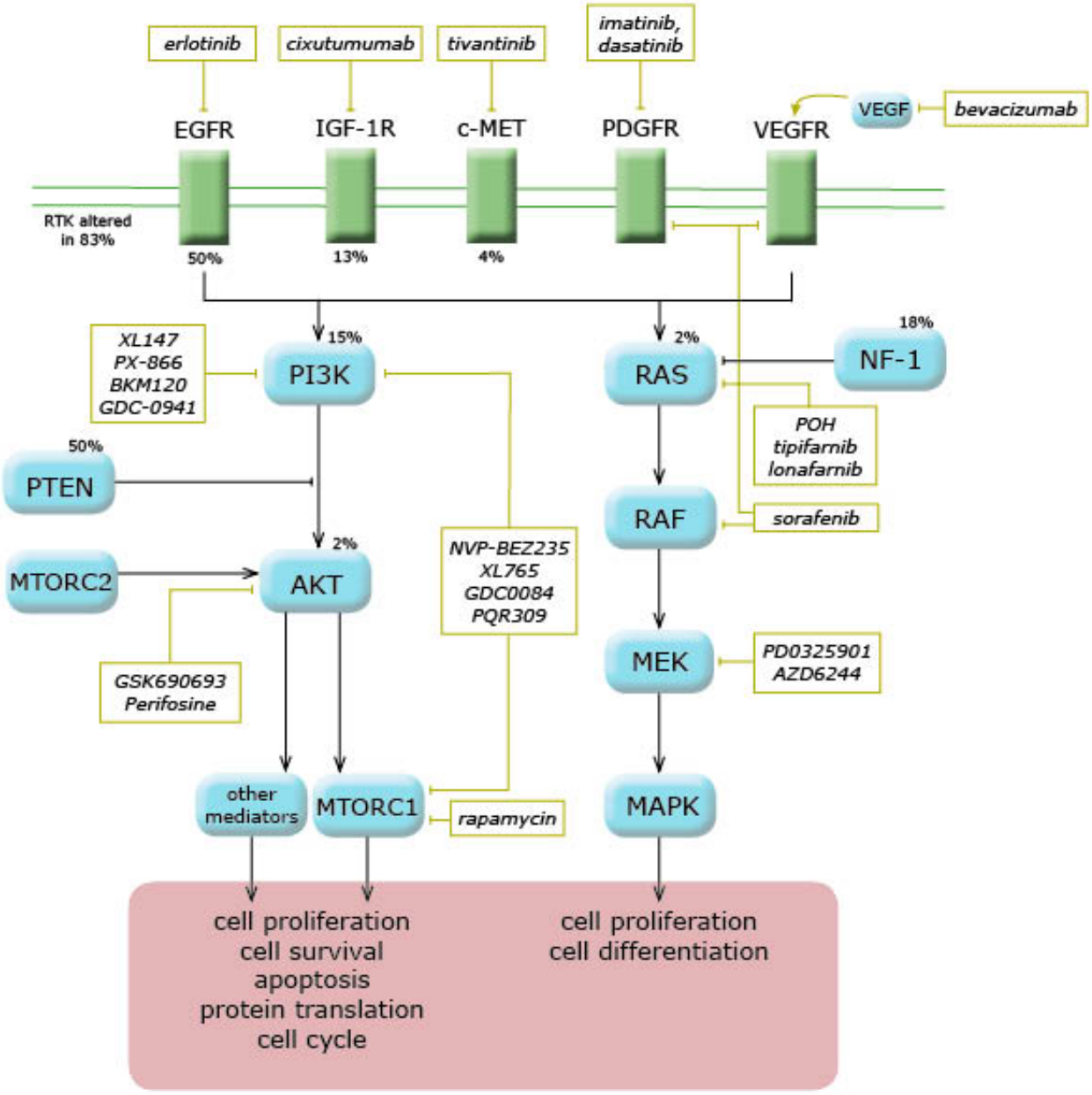

Figure 1: overview of RTK signaling pathway in GBM and possible therapeutic targets. Percentages are frequency of mutations/alterations/amplifications found in primary GBM. In italic are inhibitory compounds of the RTK signaling pathway that are currently being tested in clinical trials. Made by author (Gao et al. 2013; McLendon et al. 2008; Brennan et al. 2013; Furnari et al. 2007; Szopa et al. 2017)

RTKS AS TARGET FOR THERAPY

RTKs can be subcategorized into different receptors, among which Endothelial Growth Factor Receptor (EGFR), Vascular Endothelial Growth Factor Receptor (VEGFR), Platelet Derived Growth Factor Receptor (PDGFR), Hepatocyte Growth Factor Receptor (c-MET) and Insulin-like Growth Factor 1 Receptor (IGF-1R). In 83% of GBMs at least one RTK is altered, causing an up-regulation of the RTK signaling pathway. Targeting these receptors therefore provides an interesting strategy for inhibition of GMB cell growth (Gao et al. 2013).

EGFR-INHIBITORS

Of all RTKs, EGFR is altered most frequently, namely in 40-50% of the primary GBMs (Gao et al. 2013; Brennan et al. 2013). In half of these cases, deletion of exons 2-7 generates the mutant variant EGFRvIII which is constitutively active, even in absence of a ligand (Mellinghoff et al. 2005). Several inhibitors have been produced to target EGFR or its mutant EGFRvIII, the most notable of them being **Erlotinib**, a small molecule inhibitor. Erlotinib has been frequently studied in (pre-)clinical settings, to determine both its treatment efficacy in patients with GBM and the corresponding biological markers of its efficacy. However, despite promising preclinical results, in subsequent phase I/II studies no significant effect of single-agent Erlotinib on GBM patient survival was seen. Furthermore, EGFR amplification was not found correlated to Erlotinib response. Overall, all studies conclude that single-agent Erlotinib treatment is not sufficient to inhibit GBM tumor growth (Raizer et al. 2010; van den Bent et al. 2009; Gallego et al. 2014; Yung et al. 2010). Currently, a few clinical trials of single-agent Erlotinib are ongoing. However, most clinical trials investigate the biological activity of Erlotinib in combination with other targeted drugs (see table 1).

Agent	Targets	Clinical Development	Trial	ID status
Erlotinib	EGFR	Phase I	NCT00030498	Completed
Erlotinib	EGFR	Pilot	NCT01257594	Active, not recruiting
Erlotinib	EGFR	Phase II	NCT00337883	Completed
Erlotinib	EGFR	Phase I	NCT00227032	Terminated
Erlotinib, TMZ, Carmustine	EGFR	Phase II	NCT00086879	Completed
Erlotinib + RT	EGFR	Phase I/II	NCT00124657	Completed
Erlotinib + TMZ, RT	EGFR	Phase II	NCT00039494	Completed
Erlotinib + TMZ, RT	EGFR	Phase II	NCT00274833	Completed
Erlotinib + Bevacizumab	EGFR + VEGF	Phase II	NCT00671970	Completed
Erlotinib + Bevacizumab + TMZ	EGFR + VEGF	Phase II	NCT00525525	Completed
Erlotinib + Bevacizumab + TMZ, RT	EGFR + VEGF	Phase II	NCT00720356	Active, not recruiting
Erlotinib + Dasatinib	EGFR + PDGFR	Phase I	NCT00609999	Completed
Erlotinib + Sorafenib	EGFR + Raf	Phase I/II	NCT00335764	Completed
Erlotinib + Sirolimus	EGFR + mTOR	Phase I	NCT00509431	Completed
Erlotinib + Sirolimus	EGFR + mTOR	Phase II	NCT00672243	Completed
Erlotinib + Temsirolimus	EGFR + mTOR	Phase I/II	NCT00112736	Completed

Table 1: Ongoing clinical trials of EGFR inhibitors in GBMs (from https://clinicaltrials.gov)

OTHER RTK INHIBITORS

Although EGFR might be the most promising target for inhibition of GBM cell growth, for it is the most frequently altered RTK in GBM, other RTKs are a subject of interest as well. Two other RTKS that have been demonstrated to be altered in GBM, although to a lesser extent, are IGF-1R and c-MET. Therefore these receptors also provide an interesting target for therapy.

IGF-1R is found overexpressed in the majority of GBMs when compared with the healthy brain, hence several inhibitors of IGF-1R have been developed to suppress GBM cell growth. **Cixutumumab** (IMC-A12), a fully human monoclonal antibody, is the most frequently studied IGF-1R inhibitor. Cixutumumab demonstrated to inhibit GBM progression in two in vivo xenograft models. Interestingly, this anti-tumor effect was maintained through different mechanisms; in one of the models growth inhibition was maintained by direct inhibition of tumor cell proliferation and invasion, whereas in the other model reduction of tumor vascularization lead to tumor regression. Currently there are phase I/II clinical trials of Cixutumumab (both as single-agent and as multi-agent) ongoing in many types of cancer, but none of them in GBM or lower-grade gliomas (NCT00880282, NCT00503685, NCT00870870, NCT00831844 among others) (Yin et al. 2010; Maris et al. 2015; Zamykal et al. 2015).

Another RTK that could provide an interesting target for therapy is c-MET, which is activated through binding of its ligand Hypatocyte Growth Factor (HGF). Although c-MET is rarely found altered in GBM, the HGF/c-MET pathway often demonstrates over-activation in these tumors. This over-activation is attained by ligand-independent activation of c-MET, for increased levels of EGFR/EGFRvIII have demonstrated to be able to activate c-MET. Several small molecule inhibitors that target c-MET activation have been investigated, the most notable of them being **Tivantinib** (ARQ197), a highly specific inhibitor of the MET receptor. Tivantinib is currently in Phase I/II trials for several types of cancer (NCT01152645, NCT00612209, NCT00302172, NCT01656265 among others), although none of them in GBM (Huang et al. 2007; Jo et al. 2000; Awad et al. 2014).

The third RTK that has been investigated in anti-GBM therapy research is PDGFR. Although in a subset of GBM patients high levels of PDGFR mutation were found, anti-PDGFR therapy with **Imatinib**, a tyrosine kinase inhibitor of PDGFR among others, did not have a significant beneficial clinical response (Reardon et al. 2009). However, several clinical trials of single- or multi-agent Imatinib therapy in gliomas are currently ongoing (see table 2).

Table 2: Ongoing clinical trials of PDGFR inhibitors in GBMs (from https://clinicaltrials.gov)

Agent	Targets	Clinical Development	Trial	ID status
Imatinib	PDGFR	Phase II	NCT00039364	Completed
Imatinib	PDGFR	Phase I/II	NCT00010049	Completed
Imatinib	PDGFR	Phase II	NCT00171938	Terminated
Imatinib + TMZ	PDGFR	Phase I	NCT00354068	Completed
Imatinib + TMZ + hydroxyurea	PDGFR	Phase I	NCT00613132	Completed
Imatinib + hydroxyurea	PDGFR	Phase II	NCT00354913	Completed
Imatinib + hydroxyurea	PDGFR	Phase II	NCT00290771	Terminated

COMBINATION THERAPIES

Because of the modest efficacy of the single-agent trials, several multi-agent therapies have been proposed to increase the biological activity and ultimately overall survival in GBM patients. GBM cells might be able to interchange between different RTKs to activate the same downstream signaling pathway, thereby limiting the efficacy of single-agent therapies. Therefore, inhibiting multiple RTKs in one therapy might prove to be more efficient to block RTK signaling and thus GBM cell growth.

One of the multi-agent therapies that has been proposed is the combination of Erlotinib and **Dasatinib**, a small molecule inhibitor with effects on several tyrosine kinases such as SRC, KIT, PDGFR, EPHA2, and BCR-ABL. The combination therapy of Erlotinib and Dasatinib targets two RTKs at one, EGFR and PDGFR, and was therefore suggested to be more efficient when compared to single-agent therapy. Singe-agent Dasatinib therapy demonstrated promising results in preclinical settings, yet proved ineffective in recurrent GBM in a phase II clinical trial, therefore also suggesting a need for combination with other therapeutic agents. In a phase I trial, the combination of Erlotinib with Dasatinib proved to be well tolerated, although progression-free survival (PFS) at 6 months was only 2% (Lassman et al. 2015; Ahluwalia et al. 2010; Reardon et al. 2012; Schade et al. 2008). Currently, there is only one clinical trial of combination therapy of Dasatinib and Erlotinib ongoing in GBM (NCT00609999), although Dasatanib is running as single-agent therapy in several phase I/II clinical trials in GBM (NCT00869401, NCT00423735, and NCT00895960).

A second possible multi-agent therapy was suggested based on an interesting set of studies to the crosstalk between EGFR/EGFRvIII and c-MET; blockage of EGFRvIII with an anti-EGFR antibody (**panitumumab**) led to a rapid reversion to the c-MET/HGF pathway, confirming that GBM cells can rapidly interchange between RTKs that signal through the same downstream pathway. Co-treatment with an anti-HGF antibody (**rilotumumab**) blocked the reversion from EGFRvIII signaling to c-MET signaling and conversely, HGF was shown to be able to activate EGFR ligand, thereby activating downstream EGFR signaling. Overall, the crosstalk between EGFR/EGFRvIII and c-MET signaling provides GBM cells with an independence on either RTKs for their necessary downstream signaling, which also explains why single-agent Erlotinib treatment was not sufficient for inhibiting GBM cell growth (Pillay et al. 2009; Reznik et al. 2008).

Furthermore, combinations of RTK inhibitors with therapeutic agents against downstream effectors are also being studied (see Table 1). These therapies will be discussed later on.

RAS/RAF/MAPK PATHWAY AS TARGET FOR THERAPY

Ras proteins have been a subject of interest in research ever since their first discovery over 50 years ago. Ever since, it has been shown that Ras proteins are important molecular switches, located at the top of numerous cellular cascades and therefore they form the primary regulator of the activation or inactivation of these cascades. Ras proteins are membrane-bound GTPases that transmit extracellular signals from RTKs to their downstream pathways. Ras protein activation is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs stimulate the dissociation of GDP from Ras, allowing the binding of GTP and therefore activating the Ras protein. GAPs stimulate the hydrolysis of GTP to GDP, resulting in an inactive Ras protein. Oncogenic mutation of the Ras gene interferes with the interaction between GAPs and Ras, resulting in inability of GAPs to inactivate Ras and thus in constitutively activate Ras proteins. However, Ras mutations have only sporadically been found in gliomas suggesting an alternative source of their over-activation. Jeuken et al. found that rather than activating mutations, copy number gains of Ras/Raf and/or upstream growth factor (receptors) were responsible for the activation of the Ras pathway in human gliomas. Furthermore, mutations in the gene encoding Neurofibromin 1 (NF1) have been found in a subset of GBM patients. NF1 is one of the GAPs that promote hydrolysis of GTP to GDP. Loss of NF1 therefore enhances Ras activation, which promotes numerous cell regulatory functions; cell survival, proliferation, differentiation, apoptosis, cytoskeletal organisation and membrane trafficking (Omerovic et al. 2007; da Fonseca et al. 2008; Davies et al. 2002; Basto et al. 2005; Jeuken et al. 2007; See et al. 2012; Malumbres & Barbacid 2003).

FTASE-INHIBITORS

Membrane anchorage of Ras via association with RTKs is essential for the transmission of signals to its downstream pathway. This membrane Ras anchor is activated via post-translational modifications, catalysed by farnesyltransferase (FTase). Farnesylation of Ras thus stimulates its downstream signaling, while unfarnestylated Ras is unable to transmit signals. FTase-inhibitors are therefore a recent target of interest to interfere with the Ras signaling of GBM cells (da Fonseca et al. 2016).

PERILLYL ALCOHOL

One of the suggested FTase-inhibitors is the natural component POH (perillyl alcohol). Although the anti-carcinoma mechanism of POH is not yet known, it has been suggested that POH limits cancer activity via Ras and Ras-related proteins (da Fonseca et al. 2008). POH has been shown to inhibit cell proliferation, decrease cell viability and induce cell death by apoptosis through Ras-mediated pathways, both in vitro as in vivo, in a GBM patient (da Fonseca et al. 2008). A phase I clinical trial revealed no significant advantages of oral administration of POH in patients with advanced malignancies for whom no standard therapy was available. A following phase II clinical trial also showed no beneficial effects of oral administration of POH in advanced treatment-refractory breast carcinoma. Both clinical trials showed toxicities, predominantly nausea, gastrointestinal distress, and fatigue (Bailey et al. 2004; Bailey et al. 2008). Intranasal administration of POH in patients with malignant glioma proved to be an interesting alternative; patients demonstrated good tolerance and absorbance, and tumor size was decreased in some patients. Moreover, apoptosis was increased in a recurrent tumor (da Fonseca et al. 2008). In following Phase II clinical trials, Da Fonseca et al. showed that intranasal administration of POH increased the survival of patients with primary and secondary GBM, especially those with tumors located deep in the brain. Toxicity was low to almost non-

existent. After 4 years of treatment with POH, 19% of GBM patients was still in remission. Overall, Da Fonseca et al. conclude that long-term POH inhalation is a safe and effective strategy for recurrent malignant glioma (da Fonseca et al. 2011; da Fonseca et al. 2013). Despite these promising results, there is still a need for more effective therapies with even better tolerance and lower toxicity. To achieve this goal, more knowledge needs to be obtained about the exact anti-carcinogenic mechanism of POH and why POH affects cancer cells, but not healthy cells. To answer this question, Da Fonseca et al. evaluated the effect of POH on the lipid bilayer, for it has been shown that many drugs work by affecting the plasma membrane. They demonstrated that POH could modify the dipole potential, thereby influencing the proteins of proteins located in the lipid bilayer, such as Ras. Based on these findings, they suggest that in the near future POH can be used as a carrier for drugs targeting regulators of cell proliferation (da Fonseca et al. 2016).

TIPIFARNIB

Another FTase inhibitor that has been studied is tipifarnib, a small molecule inhibitor that demonstrated a single-agent anti-tumor response in various malignancies. Phase I trials of tipifarnib in recurrent GBM showed promising results; FTase activity was significantly decreased and toxicities were small, both in single-agent therapy as in combination with RT (Cohen-Jonathan Moyal et al. 2007; Cloughesy et al. 2005). In following phase II trials, treatment with tipifarnib in patients with recurrent GBM was still reasonably tolerated, but biological efficacy was modest; 11,9% of GBM recurrent GBM patients had PFS of more than 6 months (Lustig et al. 2008; Cloughesy et al. 2006).

LONAFARNIB

A third FTase inhibitor that demonstrated promising anti-tumor efficacy is lonafarnib. When added to therapy with standard cytotoxic agents in preclinical glioma models, lonafarnib significantly inhibited tumor cell growth (Chaponis et al. 2011). In phase I/Ib clinical trials, combination treatment of lonafarnib with TMZ demonstrated a 6-month PFS of 42% in patients treated with the maximum tolerated dose (MTD) of lonafarnib (Yust-Katz et al. 2013; Desjardins et al. 2011).

Thus, all three FTase inhibitors demonstrated moderate to promising results in clinical trials, although results are still preliminary and more research needs to be done. An overview of ongoing clinical trials with FTase inhibitors is provided in table 3.

Table 3: Ongoing clinical trials of RAS inhibitors in GBMs (from https://clinicaltrials.gov)

Agent	Targets	Clinical Development	Trial	ID status
Perillyl alcohol	FTase	Phase I/II	NCT02704858	Recruiting
Lonafarnib + TMZ	FTase	Phase I	NCT00102648	Active, not recruiting
Lonafarnib + TMZ	FTase	Phase II	NCT00038493	Terminated
Lonafarnib + TMZ	FTase	Phase I	NCT00612651	Completed
Tipifarnib	FTase	Phase II	NCT00005859	Completed
Tipifarnib + RT	FTase	Phase II	NCT00058097	Completed
Tipifarnib + RT	FTase	Phase II	NCT00209989	Completed
Tipifarnib + TMZ	FTase	Phase I/II	NCT00050986	Completed
Tipifarnib + TMZ, RT	FTase	Phase I	NCT02227901	Completed
Tipifarnib + TMZ, RT	FTase	Phase I	NCT00049387	Completed
Tipifarnib + Sorafenib	FTase + Raf	Phase I/II	NCT00335764	Completed

RAF INHIBITORS

Although the localization of Ras at the top of the signaling cascade makes it a suitable target for inhibiting RTK signaling, several downstream components of the Ras/Raf/MAPK pathway are interesting targets for GBM therapy as well. Raf is one of the first components to get activated by Ras and therefore several Raf-inhibitors have been developed to possibly inhibit Ras/Raf/MAPK signaling. A recent subject of interest in this area is **Sorafenib**, due to its multisite actions against Raf kinases and its anti-angiogenic effects via VEGFR and PEDGFR (Gao et al. 2013). Sorafenib has been shown to sensitize different kinds of cancer cells to chemo- and radiation therapy. However, this effect has not been found in GBM cell lines, although sorafenib did inhibit proliferation in the cell lines in a MAPK-independent manner (Riedel et al. 2016). Sorafenib was tested in combination with TMZ and RT in a phase I clinical trial in patients with newly diagnosed GBM, but results were not very promising; toxicities were high, response rates were minimal and PFS was not significantly improved (Hottinger et al. 2014). Despite these disappointing results, phase II clinical trials of sorafenib in combination with TMZ and RT were conducted in patients with recurrent GBM. Although the drug combination was reasonably well tolerated, biological efficacy was minimal (Zustovich et al. 2011; Hainsworth et al. 2010). To further investigate the possible biological effects of sorafenib in GBM, several combination therapies were studied in phase II clinical trials. Combination with temserolimus, a mTOR-inhibitor, was highly toxic and minimal biological activity was seen; no patients remained progression free at 6 months (Lee et al. 2012). Combination with bevacizumab, a VEGF inhibitor, did not improve the outcome of recurrent GBM patients versus the single-agent bevacizumab treated patients (Galanis et al. 2013) and combination with Erlotinib, an EGFR-inhibitor, also did not significantly improve outcome for recurrent GBM, PFS at 6 months was 14% (Peereboom et al. 2013). Several clinical trials of Sorafenib, both single- and multi-agent, are currently running (see table 4).

Table 4: Ongoing clinical trials of Raf inhibitors in GBMs (from https://clinicaltrials.gov)

Agent	Targets	Clinical Development	Trial	ID status
Sorafenib	Raf	Phase I	NCT00884416	Completed
Sorafenib	Raf	Phase I	NCT00093613	Completed
Sorafenib + TMZ, RT	Raf	Phase I	NCT00734526	Completed
Sorafenib + Bevacizumab	Raf + VEGF	Phase II	NCT00621686	Active, not recruiting
Sorafenib + Erlotinib	Raf + EGFR	Phase II	NCT00445588	Completed
Sorafenib + everolimus	Raf	Phase I/II	NCT01434602	Recruiting
Sorafenib + temserolimus	Raf + mTOR	Phase I/II	NCT00329719	Active, not recruiting
Sorafenib + Erlotinib, tipifarnib, temserolimus	Raf + EFGR, FTase, mTOR	Phase I/II	NCT00335764	Completed

MEK-INHIBITORS

Another compound of the Ras pathway that provides a possible target for inhibition of this pathway is MEK, one of the downstream effectors. Acute Myeloid Leukemia (AML), driven by NF1 loss, and other tumors with activating mutations of Ras have shown to be sensitive to MEK-inhibitors. For NF1 loss can also be found in a subset of GBM patients, MEK-inhibitors might be a promising novel therapy for them. In a preclinical setting, MEK-inhibitors **PD0325901** and **AZD6244** decreased the level of phospho-ERK, a downstream effector of MEK, regardless of NF1 status in GBM cell lines. However, only a subset of NF1 deficient GBM cells demonstrated growth inhibition after MEK-inhibitor treatment. Treatment with the dual PI3K/mTOR inhibitor PI-103 increased the sensitivity of the MEK-inhibitor resistant subset to MEK-inhibitor therapy (See et al. 2012). Furthermore, PD0325901 as single-agent inhibited NF1 deficient GBM cell growth both in vitro as in vivo (See et al. 2012; Shannon et al. 2017).
Taken together, downstream inhibition of the Ras/Raf/MAPK pathway via MEK-inhibitors is an interesting novel target for inhibition of tumor cell growth in a subset of GBM patients. Phase I/II clinical trials of PD0325901 and AZD6244 as single-agent or combination therapy are currently running in patients with several types of cancers (NCT02022982, NCT02039336, NCT02510001, NCT01146756, NCT02583542 and NCT01011933 among others), though none of them in GBM patients.

P13K/AKT/MTOR PATHWAY AS TARGET FOR THERAPY

The second main effector pathway of RTKs is the P13K/AKT/mTOR pathway, which regulates biological functions such as cell proliferation, cell survival, apoptosis, motility and angiogenesis. RTK activation by binding to its ligand activates conformational changes in the RTK, thereby promoting the interaction between the RTK and Phosphoinositide 3-kinase (PI3K). Activated PI3K subsequently initiates the activation of AKT, by anchoring it to the cell membrane. Complete activation is achieved by phosphorylation and interaction with several kinases, among which mTORC2. Activated AKT can then phosphorylate downstream effectors, among which mTORC1, to mediate the biological functions of the PI3K/AKT/mTOR pathway.
Hyper-activation of the PI3K/AKT/mTOR pathway is found in 80% of GBMs and attributes to rapid growth, tumor progression and to resistance to radiation therapy through suppression of apoptosis. Several mechanisms have been shown to contribute to the increased activation of the PI3K/AKT/mTOR pathway in carcinomas. Up to 50% of GBMs contain mutations and deletions of Phosphatase and Tensin Homolog (PTEN) tumor suppressor gene, a compound that blocks the translocation of AKT to the cell membrane, therefore inhibiting its activation. Loss of PTEN therefore increases activity of AKT. Furthermore, it has been shown that the PI3K/AKT/mTOR pathway can also be activated in a PTEN-independent manner, namely by the mutant EGFRvIII, by up-regulated signaling of other RTKs, or by mutations or deletions in PI3K or AKT. Thus, the PI3K/AKT/mTOR pathway provides several interesting targets for GBM therapy (Zhao et al. 2017; Gao et al. 2013; Salphati et al. 2012; Chakravarti et al. 2004; Choe et al. 2003; Bastien et al. 2015)

PI3K INHIBITION

Although PI3K mutations are present in only around 15% of GBMs, over activation of PI3K is commonly observed, mainly by indirect activation due to up-regulated signaling from RTKs. PI3K inhibition might therefore be an efficient target in GBM therapy (Gao et al. 2013).

BKM120

Several PI3K inhibitors have been studied in (pre-)clinical trials. **BKM120** (Buparlisib), an orally bioavailable inhibitor of all PI3K isoforms, showed promising results in the first phase I dose-escalation study. Patients with advanced solid tumors (mostly breast and colorectal cancers) and alterations in PI3K and/or PTEN showed preliminary activity in response to BKM120, with low toxicity (Rodon et al. 2014). In a phase Ib clinical trial however, the combination of BKM120 with high-dose chemotherapy in patients with PTEN deficient tumors was not tolerable, although some anti-tumor activity was detected (Smyth et al. 2017). Furthermore, in phase I/II trials in other types of carcinoma (endometrium, breast), BKM120 had toxic effects and did not show a beneficial anti-tumor effect when compared to chemotherapy alone (Heudel et al. 2017; Martin et al. 2016). Nevertheless, BKM120 is currently the most frequently-used PI3K inhibitor used in clinical trials, because its relative mild toxicity compared to other agents (Zhao et al. 2017). Currently, phase I/II trials of BKM120 in combination with chemotherapy or other targeted therapies are ongoing in patients with recurrent or newly diagnosed GBM (NCT01339052, NCT01473901, NCT01349660, see Table 6).

PX-866

Another PI3K-inhibitor that has been extensively studied is **PX-866** (Sonolisib), a biologically stable semisynthetic viridin. PX-866 demonstrated single-agent anti-tumor activity and increased the anti-tumor efficacy of radiation in several cancer cell lines (Ihle et al. 2004). In phase I trials in patients with advanced solid tumors, PX-866 was well tolerated and correlated with prolonged stable disease (Hong et al. 2012; Bowles et al. 2013). In a following phase II trial in GBM patients, PX-866 was still reasonably well tolerated but did not meet the predefined efficacy endpoints. However, part of the patients did show a prolonged stable disease while treated with PX-866. No responsible biomarker for these clinical outcomes could be identified (Pitz et al. 2015). PX-866 is currently being studied in a phase II clinical trial in GBM (NCT01259869, see table 6).

XL147

A third potent PI3K-inhibitor is **XL147** (Pilaralisib, SAR245408). Phase I/II clinical trials in various cancers have demonstrated an acceptable safety profile with low toxicity. Moreover, in patients with Chronic Lymphocytic Leukemia (CLL) preclinical single-agent activity was demonstrated (Brown et al. 2015; Tolaney et al. 2015; Matulonis et al. 2015). No clinical trials of single-agent XL147 have been conducted in GBM, only a multi-agent phase I trial of XL147 in combination with XL765 (Voxtalisib, a dual PI3K/mTOR inhibitor) was completed. Results suggest these compounds possess a moderate capacity to suppress GBM growth (NCT01240460, see table 6).

GDC-0941

The last PI3K inhibitor that is currently being studied in a clinical setting is **GDC-0941** (Pictilisib), a class I PI3K inhibitor with low mTOR inhibition properties. Preclinical studies of this compound in xenograft GBM models suggested that GDC-0941 was insufficient to pass the Blood Brain Barrier (BBB) and thus was not able to reach the distant part of GBM tumors. An in vivo study showed that combination of GDC-0941, Irinotecan (a chemotherapeutic agent used primarily in colon cancer), Sunitinib (a small molecule RTK-inhibitor) and TMZ did not prolong survival in GBM xenografts in mice, thereby confirming the inability of GDC-0941 to pass the BBB. Nevertheless, a phase IIb clinical trial is ongoing to study the role of several PI3K-inhibitors (among which GDC-0904) in combination with pembrolizumab (a monoclonal antibody) in patients with recurrent GBM (NCT02430363, see table 5).

Table 5: Ongoing clinical trials of PI3K inhibitors in GBMs (from https://clinicaltrials.gov)

Agent	Targets	Clinical Development	Trial	ID status
BKM120	PI3K	Phase II	NCT01339052	Active, not recruiting
BKM120 + TMZ, RT	PI3K	Phase I	NCT01473901	Active, not recruiting
BKM120 + Bevacizumab	PI3K + VEGF	Phase I/II	NCT01349660	Active, not recruiting
PX-866	PI3K	Phase II	NCT01259869	Completed
XL147 + XL765	PI3K + mTOR	Phase I	NCT01240460	Completed
GDC0941	PI3K	Phase I/II	NCT02430363	Enrolling by invitation

AKT INHIBITION

A second promising target for inhibition in the PI3K pathway is AKT. PI3K pathway activity is often determined by identifying the phosphorylation status of AKT. Indeed, in 84% of GBM cell lines activity of AKT, and thus over-activation of the PI3K pathway, has been detected. Therefore, although amplification of AKT can only be found in 2% of GBMs, AKT-inhibition is a promising target for inhibition of GBM cell growth (Bastien et al. 2015; McLendon et al. 2008; Furnari et al. 2007).

GSK690693

One of the several AKT-inhibitors that has recently been studied is **GSK690693**, a small molecular inhibitor of AKT. Daily administration of GSK690693 inhibited growth of human breast, ovarian and prostate xenografts and showed anti-proliferative effects in cancer tissue culture. However, this compound inhibits other kinases with an equal potential as it inhibits AKT, therefore it's not known if its biological activity is primarily due to inhibition of AKT (Rhodes et al. 2008). In stage I preclinical testing, GSK690693 did not show significant anti-tumor effects in in vivo xenografts panels, suggesting that the use of GSK690693 as a single agent against carcinomas might not be of great value without additional optimization (Carol et al. 2010).

PERIFOSINE

Another AKT inhibitor that has been of great interest in oncology research is **Perifosine**. Perifosine inhibits the translocation of AKT to the cell membrane, thereby preventing its phosphorylation. Perifosine demonstrated promising results in GBM xenografts models, as it significantly inhibited tumor growth rates and had anti-proliferative effects in combination with TMZ (Gao et al. 2013). However, in a subsequent phase II trial Perifosine showed minimal anti-tumor activity as a single-agent (NCT00590954). Currently, there is an ongoing phase II clinical trial to study the effect of Perifosine in patients with recurrent or progressive malignant glioma (NCT00590954) and also the combination of Perifosine with temserolimus, an mTOR-inhibitor, is being studied (NCT01051557, NCT02238496, see table 6).

Table 6: Ongoing clinical trials of AKT inhibitors in GBMs (from https://clinicaltrials.gov)

Agent	Targets	Clinical Development	Trial	ID status
Perifosine	PI3K	Phase II	NCT00590954	Active, not recruiting
Perifosine + temserolimus	PI3K + mTOR	Phase I/II	NCT01051557	Active, not recruiting
Perifosine + temserolimus	PI3K + mTOR	Phase II	NCT02238496	Active, not recruiting

MTOR INHIBITION

The third interesting target for PI3K pathway inhibition can be found downstream of the signaling cascade. mTOR recently gained a lot of interest mainly because of its dual function; it can associate with two different compounds: rictor and raptor. The thus formed mTOR-rictor (mTORC2) and mTOR-raptor (mTORC1) complex can function both upstream as a regulator and downstream as an effector of AKT, thereby giving mTOR a crucial role in the regulation of cell survival and proliferation by AKT (Gao et al. 2013).

RAPAMYCIN

Several mTOR inhibitors have been developed over the past years; the most extensively used ones are **Rapamycin** (Sirolimus), a mTORC1 inhibitor, and its structural analogues everolimus and temserolimus. Rapamycin demonstrated promising results in a phase I clinical trial, where it was associated with a substantial reduction of tumor cell proliferation that correlated with mTOR inhibition in several patients with recurrent GBM (Cloughesy et al. 2008). In several phase II trials, these promising results could not be reproduced. Rapamycin and its analogues did show limited radiographic activity, suggesting biological activity, but no significant responses in PFS and overall survival (OS) could be detected, both in single-agent therapy as in combination with other targeted therapies (Galanis et al. 2005; Chang et al. 2005; Teri N. Kreisl et al. 2009; Reardon et al. 2010; Chong et al. 2015; Wen et al. 2014). The lack of clinical response may in part be due to incomplete inhibition of mTOR, for the previously described compounds only target mTORC1. Inactivation of mTORC1 can activate mTORC2 and AKT, thereby creating resistance to the inhibitor itself. Simultaneous inhibition of mTORC1 and mTORC2 might therefore be more effective (Bastien et al. 2015). Currently, phase I clinical trials with dual mTORC1/C2 inhibitors, both as single- and multi-agent therapy, in GBM are ongoing (NCT02142803 and NCT02619864).

COMBINATION THERAPIES

One of the multi-agent therapies that is currently being studied is the combination of mTOR-inhibitor Sirolimus with EGFR-inhibitor Erlotinib. In a phase I study, low toxicity and a small response to treatment with Erlotinib and Sirolimus was found in a small group of pre-treated recurrent GBM patients (Chheda et al. 2015). A phase II trial is currently ongoing (NCT00672243, see table 8).
A second multi-agent therapy that is suggested to be promising for novel therapies in GBM patients is dual PI3K/mTOR inhibition, because of the previously described negative feedback loop between mTOR, AKT and PI3K (Zhao et al. 2017). NVP-BEZ235, XL765, GDC-0084 and PQR309 are all dual inhibitors that are currently being tested in clinical trials (see table 7).

NVP-BEZ235 is a PI3K and mTOR inhibitor that works by binding to the ATP domain of these enzymes, thereby blocking their activation. NVP-BEZ235 has been found to reduce tumorigenic properties in GBM stem-like cells (GSCSs), confirming the hypothesis that the PI3K/mTOR pathway is crucial for the maintenance of the tumorigenic capacity of GBMs and therefore that blocking this pathway might be an effective therapy in GBM (Sunayama, Matsuda, et al. 2010; Sunayama, Sato, et al. 2010). In a preclinical trial, treatment of NVP-BEZ235 in combination with TMZ significantly inhibited tumor cell growth rates and prolonged median survival in GBM xenografts models in mice (Yu et al. 2015). However in a following phase Ib study, the combination of NVP-BEZ235 with everolimus was poorly tolerated in patients with advanced solid tumors (including GBM), and clinical efficacy was low (Wise-Draper et al. 2017).

XL765, **GDC-0084** and **PQR309** are all BBB-penetrating PI3K/mTOR inhibitors, which have demonstrated to inhibit tumor cell growth in xenografts models of different types of cancer. XL765 was tested in a phase I trial in advanced gliomas and showed tolerable toxicities and moderate levels of PI3K/mTOR pathway inhibition (NCT00704080). GDC-0084 was also tested in a phase I clinical trial in recurrent high-grade glioma, but no results have been posted (NCT01547546). PQR309 is currently being tested in a phase II clinical trial in GBM patients (NCT02850744) (Wen et al. 2015; Zhao et al. 2017).
Several other novel dual PI3K/mTOR inhibitors are currently being tested in phase I/II clinical trials. However, none of the advanced solid tumors tested is from patients with GBM (Zhao et al. 2017).

Table 7: Ongoing clinical trials of mTOR inhibitors in GBMs (from https://clinicaltrials.gov)

Agent	Targets	Clinical Development	Trial	ID status
Sirolimus	mTORC1	Phase I/II	NCT00047073	Completed
Sirolimus + Erlotinib	mTORC1 + EGFR	Phase II	NCT00672243	Completed
Everolimus	mTORC1	Phase II	NCT00515086	Terminated
Everolimus + TMZ, RT	mTORC1	Phase I/II	NCT00553150	Active, not recruiting
Everolimus + TMZ	mTORC1	Phase I	NCT00387400	Completed
Everolimus + TMZ, RT	mTORC1	Phase I/II	NCT01062399	Active, not recruiting
Everolimus + sorafenib	mTORC1 + Raf	Phase I/II	NCT01434602	Recruiting
Temsirolimus	mTORC1	Phase I/II	NCT00022724	Completed
Temsirolimus	mTORC1	Phase II	NCT00016328	Completed
Temsirolimus + TMZ, RT	mTORC1	Phase II	NCT01019434	Completed
Temsirolimus + TMZ, RT	mTORC1	Phase I	NCT00316849	Completed
Temsirolimus + Bevacizumab	mTORC1 + VEGF	Phase II	NCT00800917	Completed
Temsirolimus + Erlotinib	mTORC1 + EGFR	Phase I	NCT00509431	Completed
Temsirolimus + Erlotinib	mTORC1 + EGFR	Phase I/II	NCT00112736	Completed
Temserolimus + Perifosine	mTORC1 + PI3K	Phase I/II	NCT01051557	Active, not recruiting
Temserolimus + Perifosine	mTORC1 + PI3K	Phase II	NCT02238496	Active, not recruiting
Temsirolimus + sorafenib	mTORC1 + Raf	Phase I/II	NCT00329719	Active, not recruiting
Temsirolimus + sorafenib	mTORC1 + Raf	Phase I/II	NCT00335764	Completed
NVP-BEZ235	mTOR	Phase I/II	NCT02430363	Enrolling by invitation
XL765 + TMZ, RT	mTOR	Phase I	NCT00704080	Completed
XL765 + XL147	mTOR + PI3K	Phase I	NCT01240460	Completed
GDC0084	mTOR	Phase I	NCT01547546	Completed
PQR309	mTOR	Phase II	NCT02850744	Active, not recruiting

DISCUSSION AND CONCLUSION

The quest for novel effective therapies for GBM patients is still a challenging and difficult road. Although a lot of progression has been made the past decades, especially with the genetic and molecular characterization of GBM through Next Generation Sequencing methods, the clinical results of newly proposed anti-GBM therapeutic agents are moderate at best.

Several therapeutic agents that demonstrated promising clinical results in multiple kinds of carcinoma did not prove to be efficient in patients with GBM. Like with all diseases of the brain, one of the suggested challenges of GBM is the localization of the tumors; orally or subcutaneous administered drugs have to pass the BBB before they can reach the tumor. The BBB is a physical and biological barrier, which protects the brain from pathogens and toxins from the blood. In the healthy brain, penetration of the BBB is strictly regulated and depends mainly on the size, liposolubility and charge of molecules. The BBB penetration has been described for several of the previously described therapeutic agents. Small molecules, such as Tipifarnib and BKM120, are presumably able to cross the BBB, while more sizable molecules, such as Perifosine, are probably too big to cross. Other factors might contribute to BBB penetration as well. For instance, Agarwal et al. demonstrated that the brain distribution of Sorafenib was restricted, due to the active efflux transport of Sorafenib at the BBB mediated by breast cancer resistance protein (BCRP). Nevertheless, the importance of the BBB penetration of therapeutic agents in GBM is somewhat doubted, for in glioblastoma the BBB is altered. This alteration creates an increased permeability of the BBB and the thus histologically and/or biologically altered BBB is commonly referred to as the Blood-Tumor Barrier (BTB). For this reason it is sometimes hypothesized that the BBB penetration of therapeutic agents does not matter, for the BBB in GBM patients is altered in such a way that most therapeutic agents can already pass (Cloughesy et al. 2006; Zhao et al. 2017; Gao et al. 2013; Dréan et al. 2016; Agarwal et al. 2011).

The second challenge that arises when trying to block GBM cell growth is the ability of the tumor cells to rapidly interchange between different RTKs, like previously discussed. Several cross talks between different RTKs have been investigated (EGFR-PDGFR and EGFR-c-MET), but more knowledge is needed to create effective combination therapies to effectively block a specific signaling cascade.

Furthermore, inhibiting just one signaling cascade might not be sufficient to inhibit GBM tumor cell growth due to the heterogeneity of GBM tumors. Clonal selection might play an important role in their growth and development, providing them with an independence of single pathways. Inhibiting the growth of one clone by blocking its essential molecular pathway, propagates the growth of another clone that has a different genetic profile and is therefore independent of that pathway (Bastien et al. 2015). In other words, blocking several pathways at once seems more effective.

Despite the numerous challenges and the disappointing clinical results so far, there is good reason to believe that a genomically and molecularly driven approach of GBM therapy will ultimately be fruitful. An important development to reach higher efficacy of GBM therapies, is the use of personalized medicine. As previously described, numerous components can be altered in GBM and therefore be responsible for disease progression. Targeting just one of them might therefore be only effective in a limited subset of all GBM patients. Thus for each therapy it is important to identify molecular biomarkers that can predict the sensitivity of GBM patients to that certain therapy. This has already been done for the standard therapy of TMZ, sensitivity of which can be determined by MGMT methylation. O^6-methylguanine-DNA methyltransferase (MGMT) is a repair enzyme that protects cells from alkylating agents (such as TMZ), by preventing accumulation of DNA damage. MGMT promoter methylation inhibits MGMT activity and is therefore associated with a better response to TMZ in GBM patients. Screening of GBM patients for MGMT methylations prior to TMZ treatment is therefore a good strategy to prevent patients from undergoing the heavy TMZ

treatment without the desirable beneficial effects (Zawlik et al. 2009). Likewise, the presence of EGFRvIII and/or PTEN deletions has proven to be a biomarker for EGFR-inhibitor sensitivity. Mellinghoff et al. found a strong positive correlation between the expression of these markers and the response to Erlotinib in GBM patients, proposing an easy way to select patients for treatment with Erlotinib (Mellinghoff et al. 2005). A following phase I study in glioma patients confirmed the positive correlation between EGFR expression and responsiveness to Erlotinib treatment in GBM, although none of these patients carried the EGFRvIII mutant (Haas-Kogan et al. 2005). Screening for EGFRvIII and PTEN deletions is therefore recommended to define the subset of GBM patients for which EGFR-inhibition will likely have a beneficial effect. Lastly, the same thing goes for Tipifarnib. This agent is suggested to have higher efficacy in GBM patients with high concentrations of farnestylated proteins in their tumors. To investigate this possibility, tissue interrogations on GBM tumor samples of patients treated with Tipifarnib need to be done. This way a subgroup of patients that might be more sensitive to Tipifarnib treatment can be defined (Cloughesy et al. 2006). Overall, the different genetic backgrounds of GBM patients might explain the modest efficacy of previously described agents in clinical trials; no studies discriminate patients based on their genetic profiles and therefore the overall efficacy of the tested treatment might be low. It is thus recommended to define the molecular biomarkers for all previously described agents so that for each GBM patient, an individual treatment strategy with highest efficacy can be composed.

This review focused on the genetic alterations in the RTK signaling pathway in GBM. However, the RTK signaling pathway can also be altered in non-genetic ways, leading to an over-activation of this signaling cascade and therefore in GBM tumor progression. The most important non-genetic alteration in the RTK signaling pathway in cancer is the up-regulation of angiogenesis through VEGF/VEGFR signaling, which leads to high vascularization, one of the hallmarks of cancer. In malignant gliomas, VEGF/VEGFR signaling has frequently been found up-regulated. Therefore, inhibiting angiogenesis via VEGF/VEGFR inhibitors might prove to be an efficient way to decrease GBM cell growth. **Bevacizumab** is a monoclonal antibody against VEGF that demonstrated significant biological activity in patients with recurrent GBM, both alone and in combination with chemotherapy. Therefore, the U.S. Food & Drug administration granted approval in 2009 to Bevacizumab as a salvage therapy after recurrence of GBM. However, randomized phase III clinical trials in patients with newly diagnosed GBM did not show a prolonged overall survival when Bevacizumab was added to standard therapy of TMZ and RT. Currently, Bevacizumab is studied in many clinical trials, both alone as in combination with TZM, RT or other targeted drugs (NCT01526837, NCT02157103, NCT02761070, NCT01349660 among others). The combination therapy of drugs against genetically altered compounds together with anti-angiogenic drugs such as Bevacizumab seems promising (Sathornsumetee et al. 2010; Friedman et al. 2009; Reardon et al. 2011; Teri N Kreisl et al. 2009; Gilbert et al. 2014; Chinot et al. 2014).

Apart from targeting both genetic and non-genetic alterations in the RTK signaling pathway, it is important to keep in mind that several other pathways might also play an important role in GBM disease progression. The cell cycle-regulating retinoblastoma (RB) tumor suppressor pathway and the p53 pathway are both also found frequently altered in GBM and might therefore provide an alternative strategy for therapy. Mutations in TP53 tumor suppressor gene, that encodes for cell cycle and differentiation regulating p53 protein, were initially found mainly in secondary GBMs (65% versus 28% in primary GBMs), but recent data has reported TP53 mutations in many primary GBMs. This suggests the p53 pathway is in interesting target for therapy in GBM. Secondly, the RB1 pathway is also an interesting target in GBM therapy, for inactivation of the RB1 pathway has been observed both in primary as in secondary GBMs. Mutations of the RB1 gene itself are not commonly found (11%), but genes encoding its upstream regulators are frequently altered. Despite these promising alterations in GBM, no useful prognostic biomarkers of the RB1 pathway have been defined (Szopa et al. 2017).

In conclusion, there is an urgent need to further deepen our knowledge about the genomic and molecular profile of GBM. Multi-agent therapies that inhibit several signaling cascades simultaneously need to be investigated further, to specify their biological activity, BBB penetration and tolerance in GBM patients. Defining specific biomarkers corresponding to these targeted-therapies will make it possible to increase efficacy and tolerability of treatment, overall resulting in better prognosis of GBM patients.

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