

Novel Therapeutic Venues for Glioblastoma: Novel Rising Preclinical Treatment Opportunities

Siegelin Markus David and Siegelin Yasemin
*Cell Biology, Columbia University, College of Physicians & Surgeons
United States of America*

1. Introduction

High grade gliomas, including anaplastic glioma WHO grade III and glioblastoma WHO IV (GBM), carry a dismal prognosis. Taking all nowadays-available therapeutics options, including radiation, chemotherapy and surgery, for GBM into consideration the prognosis after initial diagnosis is about 12 month. Despite this bad prognosis, researchers gained a tremendous insight into the molecular and genetic signatures of low and high grade gliomas. Several different subtypes of GBM were demonstrated with respect to their genetic background. These genetic alterations include *p53* mutation in secondary GBMs and EGFR amplification in primary GBMs, respectively. Very recently, great excitement was raised after the discovery of *IDH1* mutation in low-grade gliomas and secondary GBMs. This discovery is of great significance since it allows further categorizing of GBMs and is helpful in distinguishing low-grade gliomas from non-neoplastic adjacent brain tissue. Despite all this progress there is an urgent need for fresh additional therapeutic strategies. In addition to the identification of novel therapeutic regimens it is of utmost importance to gain an understanding about the molecular mechanisms on how GBMs manage to evade from almost any anti-cancer treatment regimen. In experimental models of glioblastoma there are a number of novel therapeutic regimens that exhibited promising results. These novel therapeutics include, but are not limited to: Apoptosis-based therapeutics (Tumor necrosis factor alpha related apoptosis inducing ligand, TRAIL), tyrosinkinase-inhibitors, Heat-shock-protein 90 (HSP90) inhibitors, polyphenols, novel drug combinations and intracranial application based strategies. This chapter will primarily review and focus on molecular mechanisms of resistance in GBM and rising new therapeutic venues for high-grade gliomas. High-grade gliomas are a group of primary heterogenous tumors of which glioblastoma World Health Organisation, WHO IV (GBM), is the most common one. Once the diagnosis of GBM is made, the average survival time is approximately 12-15 month (Hegi, Diserens et al., 2005). Treatment usually consists of temozolomide (commonly used chemotherapeutic drug for the treatment of GBM, TMZ), radiation (either alone or in combination with chemotherapeutics) and surgery (Hegi, Diserens et al., 2005). TMZ is a chemotherapeutic drug whose efficacy depends on the expression of the DNA repair protein MGMT (O6-methylguanine-DNA methyltransferase) in the glioblastoma tumor specimens (Krakstad&Chekenya). MGMT has been known to counteract

chemotherapy-induced DNA damage by repairing the structural integrity of O⁶-alkylated bases (Krakstad&Chekenya). The expression of MGMT is determined by its promoter methylation status. A hypermethylated promoter in a glioblastoma specimen indicates silenced expression of MGMT and suggests sensitivity of this patient's tumor to TMZ. Regarding the efficacy of TMZ, Stupp et al. showed that in patients suffering from GBM the combined administration of radiation and TMZ increased the median survival rate by 3 months as compared to radiotherapy alone (Hegi, Diserens et al., 2005). Furthermore, the 2 year survival rate in patients that received combined treatment (radiotherapy +TMZ) was increased from 10% to 26 % (Hegi, Diserens et al., 2005; Mercer, Tyler et al., 2009). The efficacy of combined TMZ and radiotherapy was largely dependent on the epigenetic silencing of the MGMT gene since patients with GBM harboring a methylated MGMT promoter that were treated with the therapeutic combination of radiotherapy and TMZ exhibited a median survival of approximately 22 months (Hegi, Diserens et al., 2005; Mercer, Tyler et al., 2009). Approximately 50 % of patients diagnosed with a GBM exhibit an unmethylated MGMT promoter. Unfortunately, this above mentioned patient group does not respond to TMZ treatment. Particularly for this group there is an evident need for fresh and novel treatment approaches. Overall, it can be concluded that new treatment approaches for this highly aggressive and deadly disease is warranted. In this chapter, novel-target specific and novel experimental approaches from recent preclinical developments will be illustrated.

2. Targeting novel genetic alterations in GBM

Genetically, GBM can be grouped into primary and secondary GBMs. Primary GBMs develop de novo and harbor EGFR (epidermal growth factor receptor) amplifications and alterations of PTEN (phosphatase and tensin homolog mutations), resulting in a profound activation of the PI3 - Kinase pathway that is well known to enhance apoptotic resistance, drive tumor cell proliferation and angiogenesis (Chakravarti, Zhai et al., 2004; Parsons, Jones et al., 2008). In contrast, secondary GBMs develop out of lower grade lesions and often reveal TP53 and IDH1 (isocitrate dehydrogenase 1, affected amino acid 132) mutations (von Deimling, Korshunov et al.; Parsons, Jones et al., 2008). The recent finding of IDH1 mutations in GBM and low-grade gliomas has raised great excitement in the scientific community. In 2008 the *IDH1* mutation was first identified in a comprehensive genomic analysis of 22 human GBM samples (Parsons, Jones et al., 2008). Interestingly, they found recurrent mutations in the active site of isocitrate dehydrogenase 1 (IDH1) in 12% of GBM patients (Parsons, Jones et al., 2008). They reported that these mutations were found in a large fraction of young patients and in patients with secondary GBMs (Parsons, Jones et al., 2008). Importantly, IDH1 mutations were shown to be associated with an increase in overall survival in patients (Parsons, Jones et al., 2008). The finding of IDH1 mutation in patients with GBM represents the beginning of a new molecular era. Researchers have already tried to exploit IDH1 as a therapeutic target with rather moderate effects. At this point it seems more likely that IDH1 will be a great asset for the practicing neuropathologist since recently a IDH1 mutation specific antibody was identified (Capper, Zentgraf et al., 2009) and successfully used to detect the mutation in gliomas with both high sensitivity and specificity, respectively. This has also diagnostic implications since with the aid of this mutation specific antibody researchers were able to distinguish diffuse astrocytoma from reactive astrocytes (astrocytosis), e.g. surrounding an infarct, (Camelo-Piragua, Jansen et al.)

which is a well-known diagnostic issue for the practicing neuropathologist. An established problem in glioma research is the fact that certain genetic alteration cannot be transported into a *vitro* setting. One of the most important examples is that glioma cells with confirmed EGFR amplification lose this genetic alteration when taken into culture (Piaskowski, Bienkowski et al.). The same issue arises when researchers were unsuccessfully trying to culture glioma cells harboring IDH1 mutations (Piaskowski, Bienkowski et al.). These results are in so far of high importance as our current culturing system of glioma cells seems not to have much in common with the relevant *in vivo* conditions, suggesting that based on these models it will be challenging to identify suitable treatment strategies. Nevertheless, researchers found that the IDH1 mutation at R132 might still be used as a therapeutic target. The mutated IDH1 results in a dependence on α -ketoglutarate that is produced from glutamine via the enzyme, glutaminase (Seltzer, Bennett et al.). They inhibited glutaminase both pharmacologically and genetically (siRNA approach) and found that cells harboring an IDH1 mutation grew slower than cells expressing wild-type IDH1 (Seltzer, Bennett et al.). Since there are no IDH1 mutated cell lines available, they transfected established glioma cells with a plasmid carrying IDH1 mutation and created stable IDH1-mutated clones (Seltzer, Bennett et al.). They concluded that the inhibition of glutaminase in IDH1 mutated cells might be a novel therapeutic strategy (Seltzer, Bennett et al.). In summary, the novel identification of IDH1 mutation might have both therapeutic and diagnostic implications.

3. Kinase inhibitors in GBM therapy

High grade gliomas organize a molecular network that provides them the ability to maintain massive growth and resistance towards cell death. From a mechanistic point these properties are closely linked to receptor- or intracellular kinases. With regards to receptor kinases, there are a few known receptor kinases that have gained particular importance, such as epidermal growth factor receptors (EGFR), platelet-derived growth factor receptors (PDGFR), vascular endothelial growth factor receptors (VEGFR) (Ren, Yang et al., 2007). Once their ligand has bound to the cognate receptor, an intracellular signal transduction cascade is initiated leading to the modulation of a number of important pathways, e.g. Ras/Raf/mitogen-activated protein (MAP)-kinase and phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathways (Ren, Yang et al., 2007) (Figure 1).

For gliomas and particularly glioblastomas, one of the most important pathway is the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, because primary glioblastomas often exhibit genetic alterations in this pathway, such as PTEN and EGFR. Regarding these genes it is also worthwhile to point out that PTEN (30%) and EGFR (37%) are amongst the most frequently altered genes in glioblastoma specimens (Krakstad&Chekenya). Chakravarti et al. have recently demonstrated that an active PI3K pathway was associated with a reduction in survival in patients suffering from glioma (Chakravarti, Zhai et al., 2004). Patients with a loss of PTEN revealed a higher level of active PI3K signaling and had a significant worse prognosis with regards to survival. Considering the absolute number in this recent study, glioblastoma patients with an activated PI3K pathway revealed a median survival of only 11 month, whereas patients that had suppressed activation levels of PI3K signaling exhibited a median survival of impressive 40 month. These observations suggest that patient with an active PI3K pathway require additional targeted treatment strategies. From preclinical experiments it has been known over two decades that the PI3K pathway plays an important

role in gliomas and that its inhibition is beneficial at least in preclinical settings (Ekstrand, James et al., 1991). Consequently, several PI3K were developed and nowadays are also implicated in clinical trials, e.g. XL765, erlotinib and gefitinib (www.clinicaltrials.gov). Unfortunately, these EGFR – inhibitors targeting a key step in molecular biology of glioma reveal rather moderate efficacy in patients (Krakstad&Chekenya; Sathornsumetee, Desjardins et al.; Yung, Vredenburgh et al.). Monotherapy with EGFR inhibitors, erlotinib and gefitinib, did not exhibit a dramatic increase in survival. Recently, a randomised, controlled phase II study by the European Organisation for Research and Treatment of

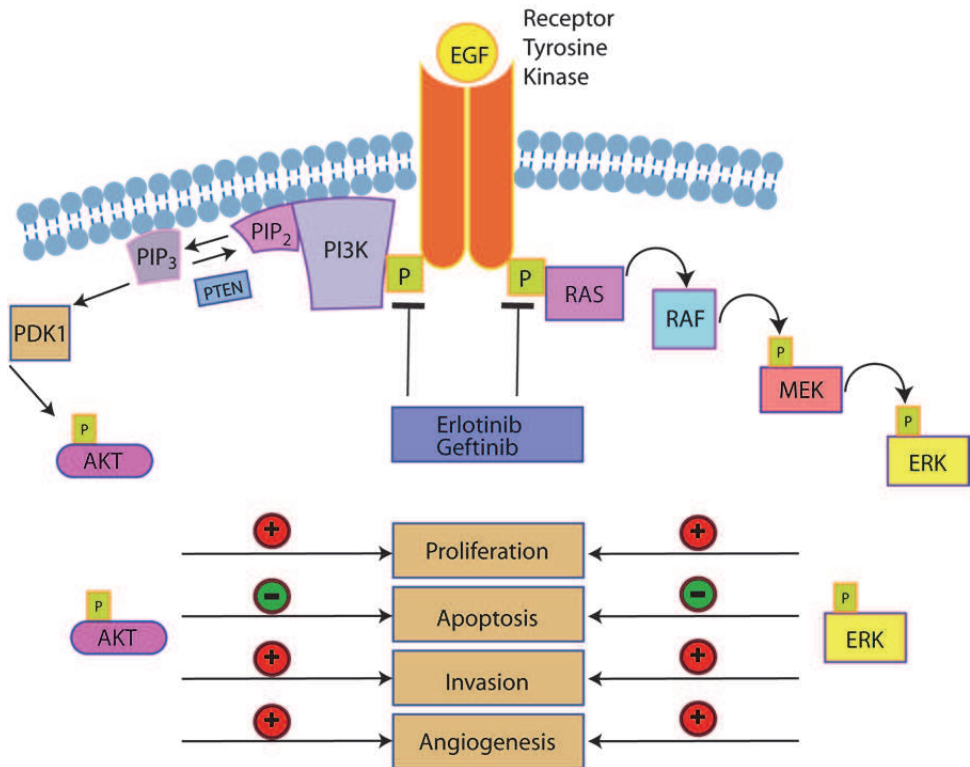


Fig. 1. Tyrosine kinase signaling. Glioblastomas often reveal genetic alterations, e.g. epidermal growth factor (EGFR). Once a ligand (EGFR) has bound to the receptor, PI3K binds to the cytosolic phosphorylated EGF-receptor. In turn, PI3K phosphorylates phosphoinositide-4,5 bisphosphate, PIP2, to phosphatidylinositol-3,4,5 triphosphate, PIP3, and PIP3 binds and phosphorylates phosphoinositide-dependent kinase 1 (PDK1). PDK1 activates protein kinase-B, AKT, by phosphorylation (p-AKT). PTEN is a tumor suppressor that inhibits PI3K activity by dephosphorylating PIP3 to PIP2 and thereby inhibiting PI3K signaling (Krakstad&Chekenya). Similarly to the Akt signaling, Ras binds to phosphorylated EGF-receptor and induces a phosphorylation cascade to give rise to phosphorylated ERK (p-ERK). P-ERK and P-AKT promote tumor cell proliferation, invasion and angiogenesis. They inhibit programmed cell death, apoptosis. Erlotinib and Gefitinib are tyrosine kinase inhibitors and interact with the EGFR to suppress its activity.

Cancer (EORTC) was unable to demonstrate even improvement in survival and radiographic responses in erlotinib treated patients (Krakstad&Chekenya). Notably, the EORTC recently showed that neither the expression of EGFR, EGFRvIII nor PTEN in glioblastoma patients (specimens) are correlated with the responses of patients to erlotinib. Furthermore the response rate was actually worse in patients harboring the EGFRvIII genotype. This finding is in so far surprising as lung cancer patients with a EGFRvIII genotype had clinical and radiographic improvements (Krakstad&Chekenya). Overall, the effects of EGFR inhibitors seem rather moderate for GBM treatment according to the currently available literature. Nevertheless, they represent one current example of molecular-target based cancer therapy that arose out of the discovery of an alteration of a genetic pathway in glioblastoma specimens. Additional trials, experimentally modifications and novel drugs might be useful for future directions.

4. Hsp90 antagonists for preclinical glioblastoma treatment

One promising tumor druggable target is Hsp90. Hsp90 has been shown to be either over-expressed or harbors an about 100 times higher ATPase activity in tumor specimens, including malignant gliomas (Kang, Plescia et al., 2009; Siegelin, Habel et al., 2009). Hsp 90 is an abundant protein that is located in the cytosol and several organelles, including the endoplasmatic reticulum and mitochondria (Kang, Plescia et al., 2007). In addition, it is also known to be secreted, stabilizing extracellular factors. Hsp90 is closely related to proliferation, apoptosis, angiogenesis and tumor cell migration and chaperones a number of cellular proteins, Akt, survivin, BRAF, p53, JAK2, STATs, etc. , to prevent their degradation and finally drive tumor progression. Since p53 and the Akt pathway are commonly altered in high grade gliomas, it is conceivable that Hsp90 antagonist, such as Geldanamycin derivatives like 17-AAG, might target GBMs. In line with this assumption, researchers have demonstrated that 17-AAG was able to inhibit growth of human established glioblastoma and glioma-stem cell like cells *in vitro* (Sauvageot, Weatherbee et al., 2009). Furthermore, 17-AAG was shown to synergize with radiation therapy (Sauvageot, Weatherbee et al., 2009), suggesting that 17-AAG might be an interesting candidate for a combination therapy. In addition these *in vitro* effects were successfully transferred into an *in vivo* model and 17-AAG administered systemically inhibited the growth of intracranial tumors and also synergized with radiation (Sauvageot, Weatherbee et al., 2009). Unfortunately, 17-AAG was not able to enhance the effects of temozolomide on glioblastoma cells *in vitro* or *in vivo*. Another recent report confirms the finding that inhibition of Hsp90 by 17-AAG does not synergize with TMZ. Nevertheless, this group found that 17-AAG at suboptimal concentrations (nanomolar range) enhanced the cytotoxicity of the DNA-crosslinking agents cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea (Ohba, Hirose et al.). Mechanistically, this synergism was a consequence of prolonged cell cycle arrest and degradation of anti-apoptotic proteins, such as Akt and survivin (Ohba, Hirose et al.). Of note, 17 - AAG was capable to synergize with cisplatin in a nude mice based animal model (xenografted U87 cells). In addition, recent data also suggests that 17-AAG might enhance the cytotoxic effects of Tumor necrosis factor related apoptosis inducing ligand (TRAIL) which from a mechanistic point was attributed to the suppression of the anti-apoptotic molecule, survivin, an established client protein of Hsp90 (Siegelin, Habel et al., 2009). One

possible obstacle in treatment of glioblastoma with Hsp90 antagonists might be that GBM cells may acquire resistance towards 17-AAG. This resistance was attributed to low activity of the mitochondrial enzyme NAD(P)H/quinone oxidoreductase 1 (NQO1) (Gaspar, Sharp et al., 2009). However, if this enzyme is the sole cause of resistance might be uncertain and remains to be determined in future analysis and there might be additional factors that contribute to the acquired resistance of 17-AAG. Another known issue with 17-AAG is the fact that it possesses unfavorable pharmacokinetics. Therefore, researchers are developing novel small-molecule compounds targeting Hsp90. One of these new molecules is the potent synthetic diarylisoazole amide resorcinol HSP90 inhibitor, NVP-AUY922 (Gaspar, Sharp et al.). NVP-AUY922 exhibited anti-proliferative activity in a broad range of human glioblastoma cell lines. Cell death (apoptosis) was also induced by NVP-AUY922, albeit after prolonged exposure of the drug to the cells. Furthermore, in a xenograft model NVP-AUY922 (50 mg/kg i.p x 3 days) caused growth inhibition and induced apoptosis, whereas 17-AAG used at maximum tolerated dose was less effective (Gaspar, Sharp et al.). The in vivo anti-tumor activity by NVP-AUY922 was accompanied by anti-proliferative, pro-apoptotic and anti-angiogenic effects (Gaspar, Sharp et al.). Another second generation novel Hsp90 inhibitor is NXD30001 that exhibited favorable pharmacokinetics as compared to 17-AAG (Zhu, Woolfenden et al.). This compound inhibited GBM growth. Of note, this group demonstrated that NXD30001 showed anti-cancer activity in an EGFR-driven genetically engineered mouse model and concluded that the Hsp90 inhibitor NXD30001 is a therapeutically multivalent molecule, representing a compelling rationale for its use in GBM treatment (Zhu, Woolfenden et al.). Another interesting viable, rising option is targeting Hsp90 in its different subcellular compartments. Recent reports have shown that Hsp90 localizes to mitochondria, nucleus and ER. In addition, Hsp90 is also secreted by tumor cells. Strikingly, recent reports have shown that Hsp90 readily accumulates in tumor mitochondria (Kang, Plescia et al., 2007; Kang, Plescia et al., 2009). Within tumor mitochondria, Hsp90, TRAP-1 and Cyclophilin-D are part of a complex that antagonizes the cell death-promoting factor, Cyclophilin-D (Kang, Plescia et al., 2007; Kang, Plescia et al., 2009). Based on the fact that Hsp90 is over-expressed in tumor mitochondria and is involved in antagonizing tumor cell death, Kang and colleagues developed several novel Hsp90-targeted drugs that were modeled on the basis of 17-AAG (Kang, Plescia et al., 2007; Kang, Plescia et al., 2009) (Figure 2). 17-AAG was modified and linked to mitochondrial target groups, such as Triphenylphosphoniumion (TPP), giving rise to a molecule called Gamitrinib-TPP. Since these drugs are expected to target the mitochondrial matrix protein, Cyclophilin-D, and are synthesized on the 17-AAG back bone, they were called, Geldanamycin - mitochondrial - matrix - inhibitors, gamitrinibs. So far, gamitrinibs were tested successfully in a number of cell lines, including breast, prostate and colon cancer (Kang, Siegelin et al.; Kang, Plescia et al., 2009). Recent results also suggest that these molecules are capable of cell death induction in primary and established glioblastoma cell lines (Siegelin, Dohi et al.). The mechanism of action by gamitrinib on glioblastoma cells seem to be independent on the genetic background as glioma cells harboring PTEN mutation, e.g. U87 and U251, or harboring mutated p53, e.g. LN229, responded equally to gamitrinib treatment. Once glioblastoma cells were treated with Gamitrinib-TPP, a sudden loss of mitochondrial membrane potential occurred, leading to a significant loss in cellular viability.

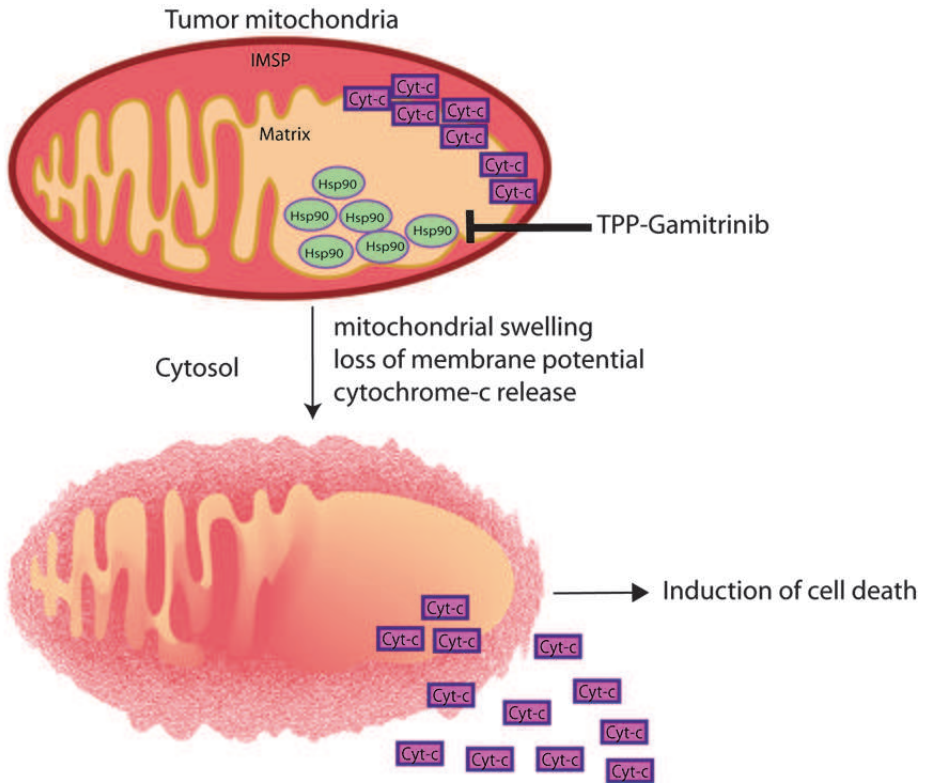


Fig. 2. Mechanism of action by TPP-Gamitrinib. TPP-Gamitrinib inhibits the mitochondrial pool of Hsp90 that is specifically over-expressed in tumor mitochondria and located in the mitochondrial matrix. Upon inhibition of Hsp90, mitochondrial swelling with loss of mitochondrial membrane potential is initiated. This leads to release of cytochrome-c from inter-membranous space (IMSP) into the cytosol. Within the cytosol, cytochrome-c induces induction of apoptosis by formation of the apoptosome and subsequently activation of caspases.

Mechanistically, this cell death had features of apoptosis and to some extent vacuole formation with evidence of autophagy. Inhibition of autophagy pharmacologically or genetically (siRNA mediated suppression of *atg5*) resulted in enhanced loss of cellular viability (Siegelin, Dohi et al.). Usually, mitochondrial mediated cell death is dependent on the bcl-2 family of proteins. The bcl-2 family of proteins comprises both pro-apoptotic and anti-apoptotic proteins, respectively. Finally, these proteins mediate the outer membrane permeability of the mitochondria. The bax and bak protein are known to enhance the outer membrane permeability, leading to a release of cytochrome-c from the inter-membranous mitochondrial space with the formation of the apoptosome with profound activation of caspases and induction of apoptosis (Kang, Plescia et al., 2009). Gamitrinibs do not require bax/bak protein for sufficient cell death induction (Kang, Plescia et al., 2009). In addition, gamitrinib-mediated cell death is also not inhibited by adenoviral-mediated bcl-2 over-

expression (Kang, Plescia et al., 2009). Since many tumor cells, including glioblastomas, mediate therapeutic resistance by the over-expression of anti-apoptotic bcl-2 family proteins, e.g. bcl-2, Mcl-1, bcl-xL, the fact that gamitrinibs are not dependent on these molecules is a true advantage of this new class of molecules when compared for instance to established chemotherapeutic drugs. Since gamitrinibs exert anti-cancer and anti-glioma activity, additional studies are required to answer whether these reagents might synergize with either radiation and/or chemotherapy, particularly Temozolomide.

In summary, there is mounting evidence from preclinical studies that inhibition of Hsp90 by small-molecules inhibitors might represent a viable and suitable therapeutic strategy for the treatment of GBM. However, up to date no clinical trial has been launched for any Hsp90 antagonists to treat high-grade and low-grade gliomas in patients.

5. Polyphenols as a novel treatment options for gliomas

Recent research suggest a potential therapeutic value of polyphenols for cancer treatment. Polyphenols are natural compounds that can be found in a number of vegetables and fruits, green tea, roots, spices and red wine (Szliszka&Krol). According to their chemical structure, polyphenols can be subdivided by their chemical structure, consisting of flavonols, flavones, flavanols, isoflavones, flavonoligans and stilbenes (Szliszka&Krol). The main representatives of flavonols are quercetin, kaempferol and myricetin. Braganhol et al. showed that quercetin has anti-proliferative effects on U138 glioma cells (Braganhol, Zamin et al., 2006). Quercetin led to a G2 cell-cycle arrest accompanied by a reduction of mitotic rate (Braganhol, Zamin et al., 2006). In addition, this group was able to show that ischemic damage to hippocampal slice cultures was attenuated by quercetin (Braganhol, Zamin et al., 2006). These results are in so far exciting as quercetin obviously exerts anti-cancer effects on one of the most highly therapeutic resistant tumor, glioblastoma, while at the same concentrations it exhibits neuro-protection to oxygen-sensitive hippocampal neuronal cells. Other researchers have also confirmed the observation that quercetin might have anti-glioma activity. Kim et al. found that quercetin induced cell death in human glioma cells (Kim, Choi et al., 2008). Mechanistically, they found that quercetin inhibited the ERK and Akt pathway and that ectopic expression of constitutively active forms of ERK and Akt protected against quercetin mediated cell death in glioma (Kim, Choi et al., 2008). Furthermore, the inhibitor of apoptosis protein, survivin, was suppressed in a concentration dependent manner by quercetin (Kim, Choi et al., 2008). Since primary glioblastomas often exhibit loss of PTEN and/or amplification of EGFR, leading to a profound activation of the Akt pathway and possible resulting in an increased expression of survivin as survivin has been shown to be a downstream target of the Akt pathway, quercetin may be a novel welcome contribution in glioma therapy. However, additional *in vivo* studies are warranted to confirm these *in vitro* observations. In this regard, it has to be demonstrated that quercetin might inhibit the growth of xenografted human glioma cells in an orthotopic model of glioblastoma which would also indicate whether quercetin can cross the blood-brain barrier. Another interesting setting would be the combination of quercetin with established treatment modalities for glioblastoma, such as temozolomide or radiation therapy. In line with this hypothesis a recent report showed that quercetin acts in synergy with temozolomide in a astrocytoma cell line (Jakubowicz-Gil, Langner et al.) . However, so far it has not been validated whether also radiation therapy might synergize with quercetin. In

summary, quercetin seems to be an interesting future drug candidate for gliomas. Kaempferol is another member of the above-mentioned group and has been shown to have activity against primary and established glioblastoma cells. In this context, it has been recently shown that kaempferol effectively sensitized glioblastoma cells to the cytotoxic effects of TRAIL (Siegelin, Reuss et al., 2008). While kaempferol and TRAIL by itself had almost no effect on cell death induction or specific induction of apoptosis, the combination of both had a dramatic effect on apoptosis induction with strong activation of the effector- and initiator caspases. This tremendous synergy effect was attributed at least in part to the suppression of the inhibitor of apoptosis protein, Survivin. Survivin inhibits the apoptotic cascade at the core of the machinery, namely the effector caspases. Kaempferol led to a dramatic suppression of Survivin by a mechanism most likely involving the proteasome and the Akt pathway. Out of the flavanols, Epigallocatechin-3-gallate has been shown to have anti-glioma activity both as a single reagent as well as in combination with TRAIL (Siegelin, Habel et al., 2008) or even more relevant to current therapeutic approaches, temozolomide (Chen, Wang et al.). The combination of EGCG and Temozolomide was more effective than single reagents both *in vitro* and *in vivo* (Chen, Wang et al.). Mechanistically, the anti-glioma effect of EGCG and Temozolomide was mediated through CHOP (CCAAT/enhancer binding protein homologous protein/GADD153), a stress-response transcriptional factor which was confirmed by specific siRNA mediated knock-down experiments *in vitro* (Chen, Wang et al.). Furthermore, in an intracranial model either utilizing U251 glioblastoma cells (*p53 mutant*) or U87 glioblastoma cells (*p53 wild-type*) EGCG did not exert survival improvement, whereas the combination of EGCG and Temozolomide significantly prolonged animal survival when compared to temozolomide alone (Chen, Wang et al.). Although EGCG did not increase animal survival by itself, it demonstrates efficacy in a combination regimen with temozolomide, suggesting that EGCG is capable of effectively crossing the blood-brain-barrier in orthotopic mouse glioblastoma models independent of *p53* mutational status (Chen, Wang et al.). It has also to be emphasized that EGCG and likewise temozolomide were administered orally, suggesting that even the oral route might be a suitable treatment strategy to combat glioblastoma. Although the glioblastoma xenografts were not completely eradicated by the combined strategy, these results are nevertheless promising given the fact that the treatment was orally well tolerated and that there are only a very limited number of treatment options for GBM. In this regard, it would be interesting whether EGCG would also synergize with radiation therapy in the above-mentioned GBM xenograft models. Moreover, as U87 cells harbor a methylated MGMT promoter, one might also consider a triple therapy consisting of EGCG, temozolomide and radiation. Because glioblastomas diffusely infiltrate the adjacent brain parenchyma, it is of highest significance to inhibit tumor cell migration with regards to glioma therapy. EGCG was also capable of inhibiting migration in glioma cells by suppression of Metalloproteinase-2 (MMP-2) secretion. This finding further establishes a potential role for EGCG as an anti-glioma reagent. In summary, flavonoids might represent an interesting novel therapeutic venue for glioblastoma. Figure 3 provides a quick overview of the pathways and molecules that are inhibited by polyphenols in glioblastoma cells. Polyphenols inhibit apoptotic and proliferation pathways (c-FLIP, inhibitors of apoptosis proteins, Akt and ERK pathway). In addition they even exert anti-migratory activity by MMP-2 suppression.

Particularly exciting is the fact that these molecules are abundant in food and that their anti-glioma activity seems to be tumor specific as hippocampal neurons were even protected by polyphenol treatment after ischemic injury. Therefore, researchers often link these molecules to chemoprevention. Moreover, the fact that these molecules are able to synergize with TMZ (EGCG) and TRAIL (Quercetin) suggests even that these molecules might have a significant value in combinatorial drug treatments. Up to date, no clinical trial with polyphenol for treatment of glioblastoma has been initiated.

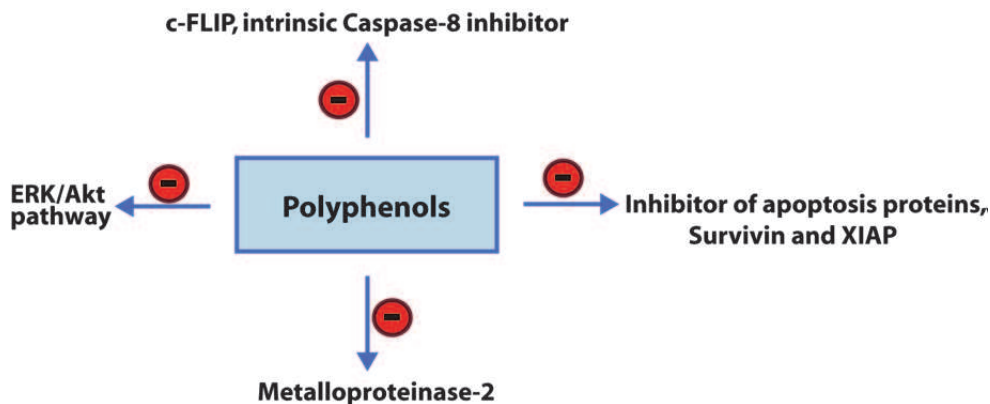


Fig. 3. Anti-glioma activity of polyphenols. Recent data suggests that polyphenols inhibit apoptotic pathways, Erk/ Akt pathway, c-FLIP and the inhibitor of apoptosis proteins. They also inhibit glioma migration by suppression of Metalloproteinase-2.

6. The exploitation of the extrinsic apoptotic pathway by tumor necrosis factor alpha related apoptosis inducing ligand, TRAIL, in glioblastoma

TRAIL is a promising death receptor ligand that upon interaction with its receptors induces programmed cell death, called apoptosis. TRAIL interacts with its membrane-bound receptors (DR4/DR5), leading to the activation of caspase-8 in the tumor cells. Caspase 8 can either directly activate caspase-3 to induce apoptotic cell death (extrinsic pathway) or it can cleave the protein BID, leading to release of cytochrome -c from the mitochondria with subsequent formation of the apoptosome and activation of caspase-9 (Figure 4). In turn, caspase-9 activates caspase - 3 (intrinsic pathway). Depending on the individual cell type, there are so called type-I cells that signal straight from caspase-8 to caspase-3 for the induction of apoptosis (Figure 4). In contrast, type-II cells, which encompass the majority of glioblastoma tumor cells, require signal amplification by activation of the intrinsic pathway. This has further important implications since glioblastomas over-express a number of anti-apoptotic factors that exert their activity through inhibition of the release of cytochrome - c from the mitochondria into the cytosol.

TRAIL binds to DR4/5 (DR:death receptor), TRAIL R1/R2 (TRAIL R: TRAIL receptor), and subsequently leads to an activation process of caspase-8 (through involvement of the adaptor protein FADD). Activation of caspase-8 is inhibited by the intrinsic caspase-8 inhibitor, c-FLIP. C-FLIP itself consists of several isoforms, of which the long and short isoform have gained considerable importance. Caspase-8 activates Caspase-3 to mediate

apoptotic cell death (type I pathway). Caspase-8 may also cleave BID, resulting in a release of cytochrome-c from the mitochondria and formation of a complex, called apoptosome (Cytochrome-c, Apaf-1, Procaspase-9 and dATP (not shown)). Within the apoptosome Caspase-9 is activated and cleaves Caspase-3 to engage apoptotic cell death (type-II pathway). The release of cytochrome-c can be inhibited by the bcl-2 family of proteins, e.g. Bcl-2, Bcl-Xl and Mcl-1. Effector caspase activation (Caspase-3) is intrinsically inhibited by the Inhibitor of apoptosis proteins, IAP. Examples of IAPs are XIAP and Survivin.

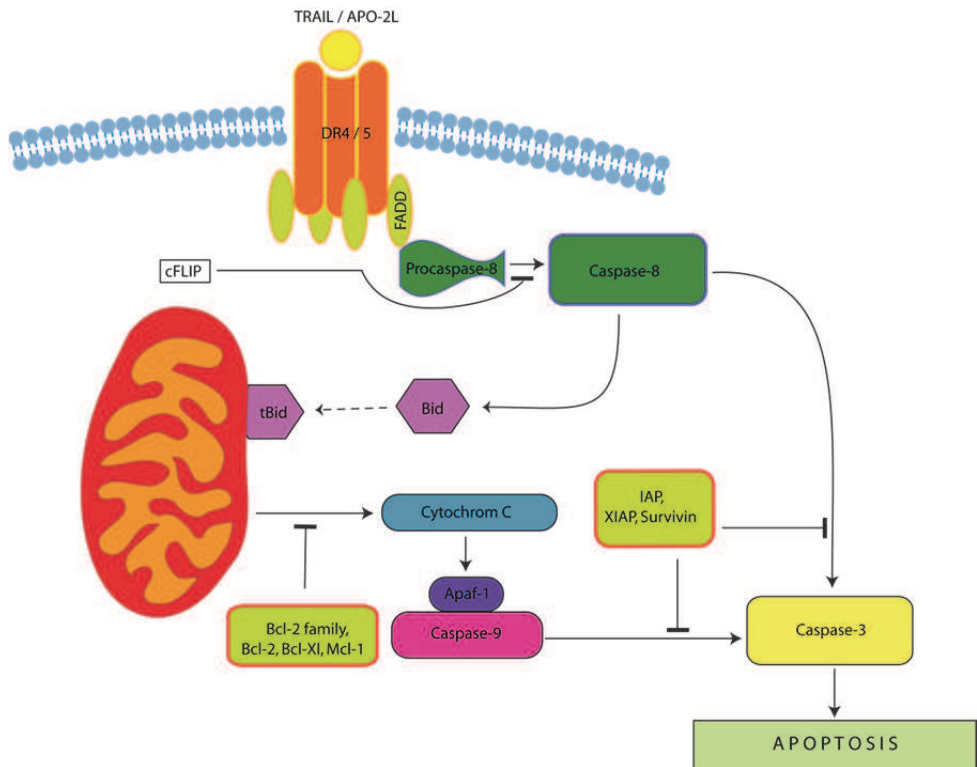


Fig. 4. Signaling pathway of TRAIL.

TRAIL was initially described and cloned based on its homology to CD95 in 1995. In 1999, two independent research groups identified TRAIL as a powerful and highly specific anti-cancer molecule. Walczak et al. created a “trimeric TRAIL” by introducing a leucine-zipper backbone into the TRAIL molecule (leucine zipper TRAIL, LZ-TRAIL) (Ashkenazi, Pai et al., 1999; Walczak, Miller et al., 1999). This led to a tremendous intrinsic activity towards cancer cells without any significant toxicity against non-neoplastic cells. In sharp contrast to CD95, which upon systemic administration to rodents led to fulminant hepatotoxicity, LZ-TRAIL was shown to be non-toxic to hepatocytes (Walczak, Miller et al., 1999). They also demonstrated that LZ-TRAIL was effective in a skin-xenograft model using human mammary adenocarcinoma cell line MDA-231 that was sensitive to LZ-TRAIL *in vitro* (Walczak, Miller et al., 1999). Histologically, TRAIL induced an apoptotic like cell death as

demonstrated by H.E. staining. In the same year, Ashkenazi and colleagues reported about another form of TRAIL that might be used for therapy (Ashkenazi, Pai et al., 1999). They presented a version of TRAIL that is devoid of tags, spans from amino acid 114 to 281 and is produced in bacteria (Ashkenazi, Pai et al., 1999). In addition, they found that TRAIL (114-281) did not kill normal cells and also injection in monkeys did not cause toxicity (Ashkenazi, Pai et al., 1999). In contrast, TRAIL had either cytotoxic or cytostatic effects on a number of tumor cell lines, including lung, breast, colon and also glioma (Ashkenazi, Pai et al., 1999). Strikingly, the *in vivo* administration in xenograft models was well tolerated and prolonged animal survival without detectable toxicity in non-cancerous tissues (Ashkenazi, Pai et al., 1999). The above-mentioned reports have several things in common. They clearly demonstrate that TRAIL has tumor specificity and that its effects are really dramatic in sensitive tumors. The major differences in these reports are the TRAIL formulations being employed. Walczak et al. used a version of TRAIL with a tag, whereas Ashkenazi used a tag-free version of TRAIL. Recently, the tag-free version of TRAIL (Dulanermin) has also entered several clinical trials (www.clinicaltrials.gov). One recent phase Ib, open-label, multicenter trial study in which Dulanermin was used has been completed. In this study, Dulanermin was administered in combination with rituximab in patients with low-grade lymphomas (www.clinicaltrials.gov). In addition, there are two trials involving either locally advanced or metastatic colorectal cancer, in which Dulanermin is combined with bevacizumab and the FOLFOX regimen (www.clinicaltrials.gov).

Following the discovery of TRAIL as an anti-cancer drug by Walczak et al. and Ashkenazi et al., Roth and colleagues found that TRAIL might also be effective for the treatment of Glioblastoma multiforme WHO grade IV (Roth, Isenmann et al., 1999). They nicely showed that TRAIL was also effective in the treatment for an orthotopic model of glioblastoma in nude mice. Only two repetitive doses of 2 μ g of TRAIL were necessary to achieve long-term survival in these animals (>100 days) as compared to animals treated with vehicle solution (36 days) (Roth, Isenmann et al., 1999). Histologically, TRAIL induced an apoptotic like cell death as shown by TUNEL (terminal transferase-catalyzed *in situ* end-labeling) staining (Roth, Isenmann et al., 1999). Importantly, no obvious acute or delayed neuronal toxicity was detected upon treatment with TRAIL. Although these results are very exciting and promising particularly with regards to the dismal prognosis of GBM, the significant limitation of this study is that TRAIL was administered intracranially and therefore it was not clear whether TRAIL could cross the blood-brain barrier. Two years later in 2001, a group of scientists found that Apo2L/TRAIL given systemically was an effective treatment of orthotopic glioblastoma xenografts (mice) utilizing U87 glioma cells (Pollack, Erff et al., 2001). Specifically, they found that at a dosage of 30 mg/kg of TRAIL administered by intraperitoneal infusion (pump system) resulted in a long-term survival (more than 120 days) of all animals (Pollack, Erff et al., 2001). These results suggested that TRAIL might be an effective drug for astrocytic brain cancers and that obviously TRAIL was able to penetrate the blood-brain barrier without any significant side effects in the animals. Of course, these results are promising but also still harbor significant caveats. The main pitfall is the requirement of constant administration of TRAIL which would be practical difficult in humans. Another important aspect is whether the dosage used in the animal studies can be translated into humans without buying serious and unwanted side effects. Nevertheless, the two reports by Pollack and Roth clearly demonstrate that TRAIL is a potential treatment strategy for malignant gliomas. Given the unfavorable pharmacokinetics of TRAIL and

presence of the blood-brain barrier in the setting of malignant glioma, a new exciting strategy was recently developed to specifically transport TRAIL to the tumor side. This excellent idea entails the usage of neural stem cells. In the year 2000, a group of researchers demonstrated that neural stem cells expressing a foreign gene for the exploitation of therapy revealed a strong tropism to glioblastoma cells *in vivo* (Aboody, Brown et al., 2000; Ehtesham, Kabos et al., 2002). Importantly, neural stem cells were shown to hunt down the infiltrating glioma cells while retaining the expression of a foreign gene (Aboody, Brown et al., 2000). When injected in the contra-lateral hemisphere neural stem cells were able to migrate into the glioma and even more relevant the administration of neural stem cells outside the central nervous system resulted in effective accumulation of neural stem cells in glioma tumors (Aboody, Brown et al., 2000). To test therapeutic efficacy, this group transduced neural stem cells with cytosine deaminase and then achieved significant reduction in tumor growth in an experimental model of glioma (Aboody, Brown et al., 2000). Two years later, Aboody et al. engineered neural stem cells expressing TRAIL and assessed the efficacy of neural stem cells secreting TRAIL in an orthotopic glioma model utilizing U343MG glioma cells. It has to be pointed out that the U343MG cells are relatively sensitive to the apoptotic effects of TRAIL as compared to other glioma cells lines such as LN229 or U87 cells that would require higher TRAIL dosages or a sensitizing reagent. The established gliomas (7 days after implantation) were treated with intra-tumoral injections of neural stem cells expressing TRAIL, neural stem cells expressing a control gene or saline. While saline and neural stem cells expressing gene had the same effect, the neural stem cells secreting TRAIL reduced the size of the tumors impressively (Aboody, Brown et al., 2000). One controversial aspect is of course the fact that this group did not utilize some sort of imaging to definitely confirm the establishment of the tumors. However, according to personal and also to other research groups experience orthotopic glioma models have a nearly 100% engrafting rate.

A main obstacle of TRAIL-based therapies is that a number of glioblastoma cells are resistant to the apoptotic effects of TRAIL. This issue becomes even worse considering the lacking markers on diagnostic tissue predicting TRAIL sensitivity. As described already TRAIL signals via distinct death receptors. In glioma recent data suggests that a descent number of high-grade gliomas exhibit silenced expression of death receptor 4, which is clearly explained through an epigenetic mechanism (Elias, Siegelin et al., 2009). Remarkable is the fact that treatment with a broad demethylating reagent restores expression of death receptor 4, suggesting that this resistance mechanism is reversible and targetable. However, it has been known already for quite some time that the actual expression of TRAIL receptors is per se not an indicator for TRAIL sensitivity (Wagner, Punnoose et al., 2007). In this context, it has been revealed that the expression of peptidyl O-glycosyltransferase GALNT14 determined the susceptibility of various cancer cells to TRAIL. However, this correlation was mainly detected in pancreatic, lung and skin cancer, suggesting that GALNT14 might be a predictor with obvious limitations, indicating that further markers to predict sensitivity are still required to be identified. Given the present issues with resistance it is of utmost importance to overcome this resistance which can be achieved by antagonizing certain anti-apoptotic molecules, such as c-FLIP, anti-apoptotic proteins of the bcl-2 family and inhibitors of apoptosis proteins, such as XIAP and Survivin. To this end, Fulda and colleagues combined TRAIL with a Smac-mimetic peptide that antagonizes XIAP (Fulda, Wick et al., 2002). This combination therapy was shown to eradicate orthotopic glioblastoma xenografts, leading to a long-term survival in animals treated with this drug combination

(Fulda, Wick et al., 2002). Despite this tremendous anti-glioma activity the limitation of this study was that TRAIL was applied loco regional. Nevertheless, this demonstration of drug efficacy was quite remarkable. Since GBMs usually do not metastasize and have a tendency to recur at their initial presentation spot, locoregional treatment approaches are feasible for malignant gliomas.

Many TRAIL-based drug combinations for the treatment of GBM have been employed successfully since 1999. Since the mainstay treatment for GBM consists of radiation and/or chemotherapy with temozolomide, combining these treatment modalities with TRAIL is a main issue. So far, these combinations in the setting of an experimental glioma model include, but are not limited to, TRAIL+Temozolomide (Saito, Bringas et al., 2004), TRAIL+radiation therapy (Nagane, Cavenee et al., 2007), TRAIL+Bortezomib (Jane, Premkumar et al.; Koschny, Holland et al., 2007), TRAIL+ABT737 (Tagscherer, Fassl et al., 2008), TRAIL+Quercetin (Siegelin, Reuss et al., 2009), TRAIL+Kaempferol (Siegelin, Reuss et al., 2008), TRAIL+Celecoxib (Gaiser, Becker et al., 2008), TRAIL + PI-103 (a PI-3 Kinase Inhibitor) (Bagci-Onder, Wakimoto et al.), TRAIL + Troglitazone (Akasaki, Liu et al., 2006; Schultze, Bock et al., 2006), TRAIL + 17-AAG (Siegelin, Habel et al., 2009) and TRAIL + Gamitrinibs (Siegelin, Dohi et al.). Most of these combinations work by enhancing either the extrinsic or intrinsic apoptotic pathway, respectively. As an example the extrinsic apoptotic pathway is controlled by the TRAIL-receptors, Caspase-8 and the c-FLIP. C-FLIP can be present in several isoforms, e.g. a long c-FLIP (L) and a short isoform c-FLIP (S). It is well established as an inhibitor of caspase-8, thereby inhibiting either direct activation of apoptosis through caspase -3/-7 or indirect through cleavage of BID and engagement of the intrinsic apoptotic pathway through cytochrome-c release, formation of the apoptosome and activation of caspase-9. Temozolomide has been shown to modulate the expression of death receptors (Saito, Bringas et al., 2004), and the proteasomal inhibitor, Bortezomib, decreased c-FLIP protein levels in primary glioma cells (Koschny, Holland et al., 2007). The intrinsic pathway is largely controlled by the Bcl-2 family of proteins, e.g. Bcl-XL, Mcl-1 and bcl-2. However, the intrinsic pathway is also indirectly influenced by the inhibitor of apoptosis protein family, such as XIAP and survivin. ABT-737 is an inhibitor of the bcl-2 family of proteins. ABT-737 is a molecule that requires high levels of bcl-2 and low levels of Mcl-1 in order to exert efficacy. As glioblastoma cells are known to over-express the anti-apoptotic bcl-2 proteins, ABT-737 was capable to sensitize glioblastoma cells to TRAIL-mediated apoptosis. Recently, the therapeutic efficacy of a novel PI3-kinase/mTOR inhibitor, PI-103, was evaluated in the combination with TRAIL. The importance of the mTOR and PI-3 Kinase pathway in gliomas has been well known and many GBM and tumor stem cells in gliomas rely on the activation of these two pathways giving them resistance to apoptotic stimuli by the enhancement of certain anti-apoptotic molecules regulating either the extrinsic or intrinsic apoptotic pathways, such as c-FLIP, IAPs or bcl-2 family of proteins. In an orthotopic glioblastoma animal model they showed that neural stem cell derived TRAIL in combination with PI-103 was more effective in tumor growth inhibition than either single treatments alone (Bagci-Onder, Wakimoto et al.). 17-AAG and flavonoids led to a down-regulation of survivin protein and thereby overcame TRAIL-resistance in glioma cells.

Recently, we discovered a novel mechanism to sensitize tumor cells, including glioma cells, to the cytotoxic effects of TRAIL. Tumor cells have been shown to organize a tumor-cell specific chaperone network within their mitochondria. This network consists of at least Hsp90, the mitochondrial Hsp-90 homologue, TRAP-1 and Cyclophilin-D. These proteins form a complex to antagonize the pro-apoptotic function of Cyclophilin-D (Kang, Plescia et

al., 2007). Antagonizing this chaperone network might be therefore interesting for the treatment of tumors. To this end, a novel molecule that encompasses 17-AAG linked with a mitochondrial targeting sequence, called Triphenylphosphoniumion (TPP), was developed (Kang, Plescia et al., 2009). This molecule (G-TPP) was part of the family of so called Gamitrinibs, Geldanamycin-mitochondrial-matrix-inhibitors (Kang, Plescia et al., 2009). Treatment of glioma cells with G-TPP led to the induction of a mitochondrial unfolded protein response (UPR) and to the initiation of a specific transcriptional program (Siegelin, Dohi et al.). This transcriptional induction involved the up-regulation of CHOP and CEBP/beta and concomitant to a strong suppression of NF-kB activity (Siegelin, Dohi et al.). The suppression of NF-kB activity was partially responsible for the tremendous sensitization of apoptosis-resistant glioblastoma cells to TRAIL *in vitro*. Furthermore, it was demonstrated that this combination of gamitrinib and TRAIL was highly efficient in *an vivo* setting of glioblastoma. To this end, a well-known orthotopic glioblastoma model was employed. In this model, U87 glioblastoma cells (p53 wt/PTEN mutant) were transduced with a viral luciferase construct giving rise to U87-luc cells. U87-luc cells were stereotactically injected into the right striatum of nude mice. Established tumors were then treated with vehicle solution, TRAIL, Gamitrinib and the combination of TRAIL and Gamitrinibs. Remarkably, only the combination treatment exhibited significant effects on bioluminescence intensities, suggesting a significant induction of cell death and inhibition of proliferation as indicated by Ki-67 and TUNEL-staining, respectively. Given all these novel and pretty promising experimental findings on TRAIL and glioblastoma, clinical trials involving this substance either as a single reagent or in combination is warranted. Since many of the combination partners of TRAIL are already in clinical use, the threshold of approval for a combined administration of TRAIL with established therapeutics should be reasonable.

7. Conclusion

Although glioblastoma (average reported incident of 6-7/100.000 new cases (Krakstad&Chekenya)) is a relatively rare disease and might be considered as an orphan disease, viable, effective treatment strategies need to be identified. Hence, regardless of its rareness humans die from this deadly disease. Regarding to its prevalence, pharmaceutical companies might not have a reasonable financial interest to invest into novel drug discoveries for glioblastoma. That being said, non-profit organizations such as universities are in charge to identify and characterize established and novel pathways in glioblastoma. A very recent example of a significant discovery in glioma biology was the identification of IDH1 mutation in low-grade and high-grade gliomas. This finding was made independently by several non-profit organizations showing that IDH1 is mutated particularly in secondary glioblastomas. Cell lines ectopically expressing this mutation showed slower tumor growth *in vitro* and *in vivo*, and patients harboring this mutation had a longer median survival. In addition, we reviewed some of the most recent novel research on glioblastoma therapy emphasizing mostly on preclinical developments. Specifically, we focused here on EGFR inhibitors, Hsp90 antagonists, polyphenols and the activation of apoptosis by TRAIL. The EGFR inhibitors are a recent example for the practical pathway of drug discovery. First, a genetic alteration was found. Second, the role of the genetic alterations was studied regarding its importance for tumor growth in experimental, preclinical models and in patients, respectively. Third, based on these data pharmaceutical companies developed

drugs (Erlotinib and Gefitinib) to specifically target these genetic alterations. Unfortunately, a recent phase II trial in GBM patients with erlotinib revealed no therapeutic benefit. Therefore, additional treatment approaches targeting multiple pathways should be exploited. In this line, targeting the Hsp90 by 17-AAG might be another suitable approach for the treatment of GBM as Hsp90 binds a number of important tumor growth driving molecules in GBM and 17-AAG revealed anti-glioma effects in several preclinical models of GBM. In addition, recent evidence suggested that targeting of the mitochondrial Hsp90 pool by a drug-modified 17-AAG showed efficacy on glioblastoma cells. Finally, 17-AAG has been successfully exploited in other tumor entities in patients. Polyphenols are also a promising group of molecules for the treatment of GBM, which is at least suggested by recent experimental data. Finally, induction of apoptosis by TRAIL might be a welcome contribution to glioblastoma therapy as its preclinical activity suggests dramatic, specific anti-glioma activity, particularly when administered in combination with other reagents. Although at this point glioblastoma remains incurable, even these reported small progresses will finally lead to the identification of novel, fresh effective drug combinations in the future.

8. References

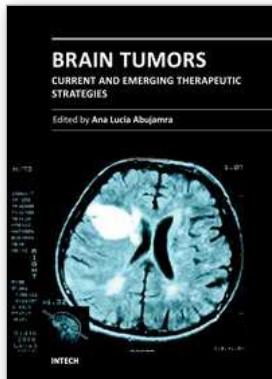
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University Campus STeP Ri
Slavka Krautzeka 83/A
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Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821