Present and potential future adjuvant issues in high-grade astrocytic glioma treatment

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Abstract

Despite major advances in the management of malignant gliomas of which glioblastomas represent the ultimate grade of malignancy, they remain characterized by dismal prognoses. Glioblastoma patients have a median survival expectancy of only 14 months on the current standard treatment of surgical resection to the extent feasible, followed by adjuvant radiotherapy plus temozolomide, given concomitantly with and after radiotherapy.

Malignant gliomas are associated with such dismal prognoses because glioma cells can actively migrate through the narrow extra-cellular spaces in the brain, often travelling relatively long distances, making them elusive targets for effective surgical management.

Clinical and experimental data have demonstrated that invasive malignant glioma cells show a decrease in their proliferation rates and a relative resistance to apoptosis (type I programmed cell death) as compared to the highly cellular centre of the tumor, and this may contribute to their resistance to conventional pro-apoptotic chemotherapy and radiotherapy. Resistance to apoptosis results from changes at the genomic, transcriptional and post-transcriptional level of proteins, protein kinases and their transcriptional factor effectors. The PTEN/PI3K/Akt/mTOR/NF-κB and the Ras/Raf/MEK/ERK signaling cascades play critical roles in the regulation of gene expression and prevention of apoptosis. Components of these pathways are mutated or aberrantly expressed in human cancer, notably glioblastomas. Monoclonal antibodies and low molecular-weight kinase inhibitors of these pathways are the most common classes of agents in targeted cancer treatment. However, most clinical trials of these agents as monotherapies have failed to demonstrate survival benefit.

Despite resistance to apoptosis being closely linked to tumorigenesis, tumor cells can still be induced to die by non-apoptotic mechanisms such as necrosis, senescence, autophagy (type II programmed cell death) and mitotic catastrophe. Temozolomide brings significant therapeutic benefits in glioblastoma treatment. Part of temozolomide cytotoxic activity is exerted through pro-autophagic processes and also through the induction of late apoptosis. Autophagy, type II programmed cell death, represents an alternative mechanism to overcome, at least partly, the dramatic resistance of many cancers to pro-apoptotic-related therapies.

Another way to potentially overcome apoptosis resistance is to decrease the migration of malignant glioma cells in the brain, which then should restore a level of sensitivity to pro-apoptotic drugs.

Recent series of studies have supported the concept that malignant gliomas might be seen as an orchestration of cross-talks between cancer cells, microenvironment, vasculature and cancer stem cells.

The present chapter focuses on (i) the major signaling pathways making glioblastomas resistant to apoptosis, (ii) the signaling pathways distinctly activated by pro-autophagic drugs as compared to pro-apoptotic ones, (iii) autophagic cell death as an alternative to combat malignant gliomas, (iv) the major scientific data already obtained by researchers to prove that temozolomide is actually a pro-autophagic and pro-apoptotic drug, (v) the molecular and cellular therapies and local drug delivery which could be used to complement conventional treatments, and a review of some of the currently ongoing clinical trials, (vi) the fact that reducing the levels of malignant glioma cell motility can restore pro-apoptotic drug sensitivity, (vii) the observation that inhibiting the sodium pump activity reduces both glioma cell proliferation and migration, (viii) the brain tumor stem cells as a target to complement conventional treatment.

Keywords: High-grade gliomas; apoptosis resistance; chemotherapy; temozolomide; tyrosine kinase inhibitors; cellular and molecular therapies; clinical trials.

Introduction

Malignant gliomas continue to remain incurable, and the aim of multidisciplinary treatment is to improve neurological deficits and to increase survival while maintaining the best possible quality of life [30, 67]. The standard treatment for high grade gliomas is surgery followed by radiotherapy and chemotherapy. Maximum surgical resection constitutes the mainstay of treatment for operable high-grade gliomas [40, 60]. The extent of tumor removal and the residual tumor volume correlate significantly with median tumor progression and survival time [40, 60]. In a study involving 416 patients with glioblastoma multiforme a significant survival advantage was associated with the resection of 98% or more of the tumor volume [57]. A recent analysis carried out by Chang and colleagues [13] on 565 patients with newly diagnosed high grade gliomas showed that tumor resection was attempted on most of the patients (75%), with most (87%) receiving radiotherapy, and only 54% chemotherapy. It seems that practice patterns varied significantly between academic and community settings [86]. Establishing further clinical guidelines together with a best knowledge of the ongoing clinical trials may help reduce variability in practice patterns and increase the number of patients included into promising trials. O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status, age, performance status, extent of resection, and Mini-Mental State Examination (MMSE) are suggested as eligibility or stratification factors for future trials in patients with newly diagnosed

glioblastoma [31]. Moreover, stratifying by MGMT promoter methylation status should be mandatory in all glioblastoma trials that use alkylating chemotherapy [31].

Despite the advances in the management of malignant gliomas, their prognosis remains poor. After the surgical resection and the adjuvant treatment of a glioma, the residual tumor cells peripheral to the highly cellular removed part of the lesion give rise to a recurrent tumor which, in more than 90% of the cases, develops immediately adjacent to the resection margin or within 2 cm of the resection cavity [61]. At recurrence, reoperation should be considered associated with a morbidity/mortality rate similar to at following initial surgery. It is evident that progressive neurological dysfunction may develop despite the absence of a mass effect or a recurrent bulk disease. This indicates that the infiltrative disease significantly contributes to the unsatisfactory course of events for glioma patients.

Natural resistance of migrating malignant glioma cells to apoptosis (radiotherapy and chemotherapy)

Clinical and experimental data demonstrate that invasive glioma cells show a decrease in their proliferation rates and a relative resistance to apoptosis as compared to the highly cellular center of the colony, and this may contribute to their resistance to conventional pro-apoptotic chemotherapy and radiotherapy [28, 61].

The natural resistance of glioblastomas to radiotherapy and chemotherapy is attributed, at least partly, to the PTEN (phosphatase and tensin homologue on chromosome ten)/Akt/PI3K (phosphatidylinositol 3-kinase)/mTOR (*the mammalian target of rapamycin*)/NF-κB pathway [54–56, 61, 64, 95, 100, 132] (Fig. 1). The activity of the PI3K/Akt pathway is often up-regulated in brain tumors due to excessive stimulation by growth factor receptors and Ras [85, 88]. PTEN tumor suppressor gene mutations which result in activation of the PI3K-dependent activation of Akt signaling [52, 88] are frequent in *de novo* glioblastomas [52]. Methylation of the PTEN promoter may represent an alternate mechanism by which PI3K signaling is increased in grade II and III gliomas as well as secondary glioblastomas [132]. The activation of the PI3K pathway is associated significantly with increasing tumor grade, lower levels of apoptosis and an adverse clinical outcome in the case of human gliomas [11]. Narita *et al.* [83] and Choe *et al.* [15] suggest that the PI3K/Akt pathway is a particularly interesting target in the case of glioblastomas with aberrant EGFR (*epidermal growth factor receptor*) expression, because the aberrant EGFR expression and abnormal PI3K/Akt signaling also modulate the levels of migration of tumor astrocytes [61]. A number of publications have already reported that an aberrantly activated PI3K/Akt pathway renders tumor cells

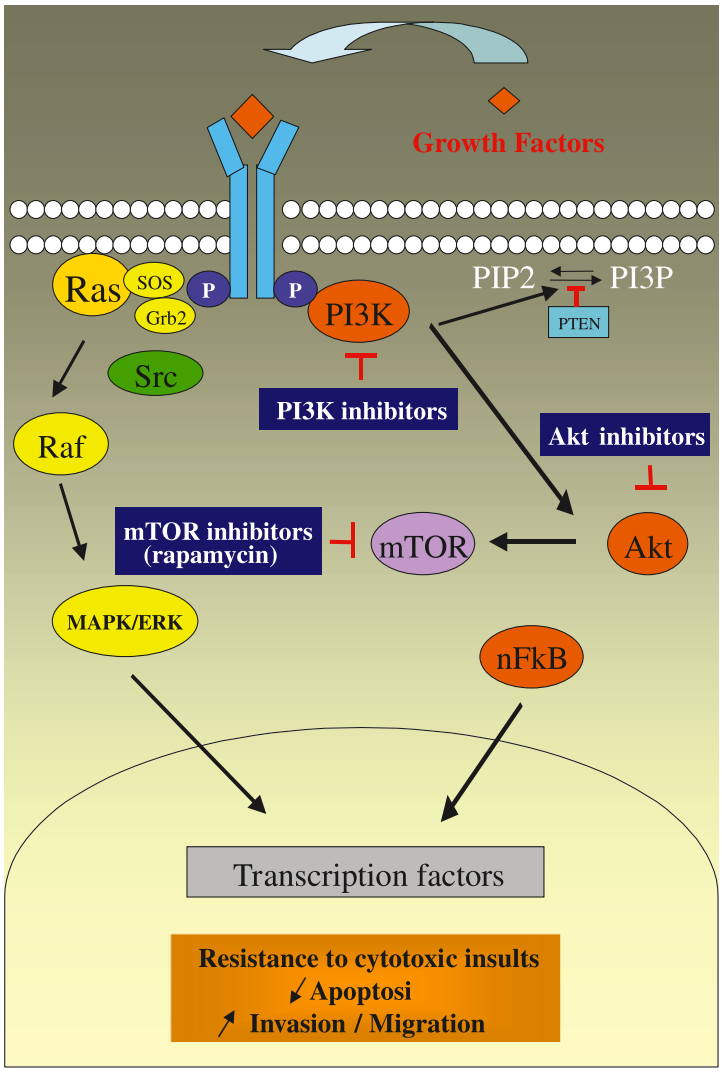

Fig. 1. Pathways involved in the natural resistance of glioblastomas to apoptosis (radio/chemotherapy). Pathways involved in cytotoxic insult resistance are presented in orange and the inhibitors that could be used to restore chemosensitivity to pro-apoptotic drugs are presented in blue. The binding of a ligand to EGFR causes receptor dimerization leading to tyrosine kinase activation. The resultant receptor autophosphorylation initiates signal-transduction cascades involved in cell proliferation and survival. Antibodies block the binding of ligands to the receptor, thereby inhibiting receptor phosphorylation and events downstream. Drugs have also been developed that inhibit the activity of the intracellular tyrosine kinase domain and so inhibit receptor autophosphorylation. Other drugs specifically block proteins of the downstream signal transduction involved in cell proliferation, migration and survival. Phosphatase and tensin homologue (PTEN) gene mutation or methylation promotes Akt activation

resistant to cytotoxic insults, notably to anti-cancer drugs [47, 114, 115]. Shingu *et al.* have shown that the inhibition of this pathway restores or even augments the effectiveness of chemotherapy on glioma cells [114, 115]. PI3K inhibitors could also be used to reduce the levels of tumor astrocyte migration, a feature that could restore a certain level of apoptosis to these cells [47]. Cell survival through Akt signaling also involves the NF-κB pathway because Akt signals to various cell-death regulators including IKK, which controls NF-κB activity. NF-κB itself plays a dramatic role in gliomagenesis [54, 61]. For example, the NF-κB signaling pathway is constitutively activated in a large proportion of glioblastomas [82] and this activation enables cancer cells to resist cytotoxic insults [1, 4]. The constitutive activation of Akt and NF-κB contributes significantly to the progression of diffuse gliomas, and the activation of Akt may lead to NF-κB activation in high-grade gliomas. As detailed below, cardenolide-induced inhibition of sodium pump activity leads to the deactivation of the NF-κB pathway.

Rapamycin inhibits the phosphorylation of the retinoblastoma protein (Rb), and rapamycin-treated glioblastoma cells are therefore not fully committed to entering the S-phase after their release from drug-induced G1 arrest [41, 112]. Constitutive Rb phosphorylation frequently occurs in glioblastomas due to mutation-induced p16 gene inactivation. mTOR can also control cell migration in glioblastomas [61].

Several strategies could be used potentially to overcome glioma cell resistance to cytotoxic insults: i) inhibition of the signaling pathways that are constitutively activated in a specific malignant glioma specimen, ii) use of inhibitors to reduce the levels of tumor astrocyte migration, a feature that could restore a certain level of apoptosis to these cells, iii) induction of autophagic cell death as opposed to apoptosis and iv) inhibition of sodium pump activity which disorganizes the actin cytoskeleton (reducing motility and proliferation) and induces autophagic processes in malignant glioma models. We further developed these new strategies in the treatment of high-grade gliomas.

Patterns of cell death

The therapeutic goal of cancer treatment has been to trigger tumor-selective cell death [126]. Although cell death can be achieved not only by apoptosis (type I programmed cell death) but also by necrosis, mitotic catastrophe and autophagy (type II programmed cell death), drugs inducing apoptosis remain the main chemotherapeutic agents in medical oncology [62, 90, 104].

Cell death can be divided into apoptotic cell death and non-apoptotic cell death [90]. The term apoptosis often has been used interchangeably with the term programmed cell death [90]. Apoptosis is the principal mechanism by which cells are physiologically eliminated. The original definition of apoptosis

Table 1. *Comparison of apoptosis, autophagy and necrosis*

	Apoptosis (Type I PCD)	Autophagy (Type II PCD)	Necrosis
Morphological changes			
Nucleus fragmentation	+	−	−
Chromatin condensation	+	−	−
Apoptotic bodies formation	+	−	−
Cytoplasmic vacuolation	−	+	+
Organelles degradation	−	+	+
Mitochondrial swelling	sometimes	late	+
Cytoplasmic swelling and Membrane breakdown	−	−	+
Biochemical features			
Caspase activity (caspase 3)	+	−	−
PARP	cleavage	−	activation
Lysosomal activity (cathepsin B)	−	+	−
Molecular pathways			
DAP	+	+	+
PI3-K, mTOR	−	+	
Bcl-2 proteins and cytochrome c	+	−	

DAP Death-Associated Proteins; *mTOR* mammalian target of rapamycin; *PARP* poly (ADP-ribose) polymerase; *PCD* Programmed Cell Death; *PI3-K* phosphatidylinositol 3-kinase.

as a form of cell death distinct from necrosis was based on morphological criteria (Table 1). Necrosis, traditionally been considered as an unregulated, passive, energy-independent form of cell death, has emerged as an alternate form of programmed cell death. Extensive failure of normal physiological pathways that are essential for maintaining cellular homeostasis, such as regulation of ion transport, energy production and pH balance can lead to necrosis. A classic example of necrotic conditions is ischemia that leads to a drastic depletion of oxygen, glucose, and other trophic factors and evokes massive necrotic death of endothelial cells and cells of surrounding tissues, such as in the center of large malignant tumors and which is the characteristic of glioblastomas. Because glioblastoma cells carry mutations that inactivate apoptotic pathways (Fig. 1) [61], necrosis could also represent an alternative pathway for tumor cells to be eliminated. Here after we describe autophagy, a mechanism for non-apoptotic programmed cell death that is distinct from apoptosis and necrosis by the criteria of morphology, biochemistry, and molecular pathways (Table 1) (Fig. 2) [64, 62].

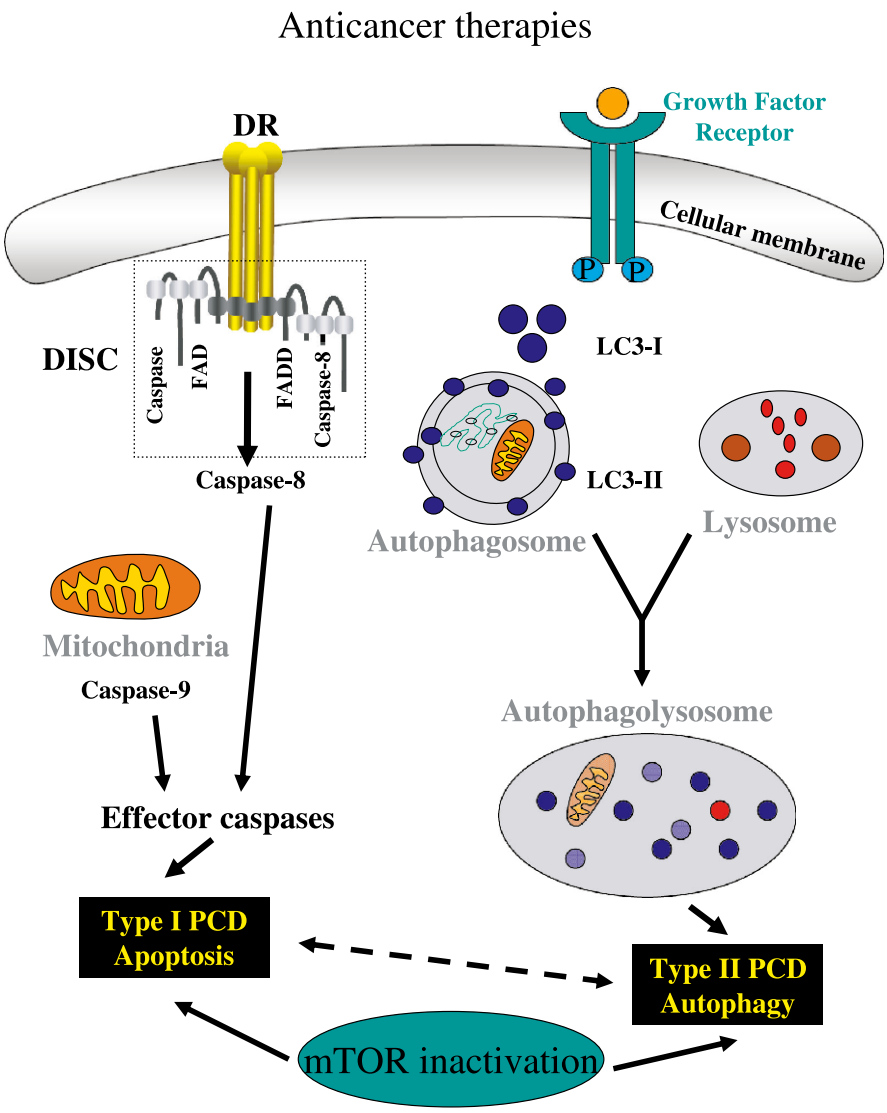

Fig. 2. The molecular regulation of autophagy and the link with apoptosis. In the presence of growth factors, growth factor receptor signaling activates cascades to the mammalian target of rapamycin (mTOR), resulting in the inhibition of autophagy or type II programmed cell death (PCD: right hand side of the figure). In contrast, inactivation of mTOR can induce both apoptosis and autophagy. The left hand side of the figure represents a simplified version of the two main signaling pathways of apoptosis. The intrinsic pathway includes the mitochondria. The extrinsic cell death pathway is mediated by death receptors (DR). After formation of the death receptor (DR)/DISC complex and recruitment of caspases, downstream signaling pathways lead to apoptosis

Autophagy: a potential Trojan horse for malignant gliomas

As recently reviewed by Okada and Mak [90] and ourselves [61, 62], resistance to apoptosis is closely linked to tumorigenesis, but tumor cells can still be induced to die by non-apoptotic mechanisms such as necrosis, senescence, autophagy and mitotic catastrophe. Autophagy is a novel concept in cancer research and malignant transformation is frequently associated with the suppression of autophagy [89].

As glioblastoma cells carry mutations that inactivate apoptotic pathways (Fig. 1) [61], non-apoptotic cell death could represent an alternative for apoptosis-resistant glioma cells to be destroyed [62, 64]. While apoptosis is a caspase-dependent process characterized by the condensation of cytoplasm and the preservation of organelles, essentially without any autophagic degradation, autophagic cell death is a caspase-independent process that exhibits extensive autophagic degradation of the Golgi apparatus, the polyribosomes and the endoplasmic reticulum (Table 1) (Fig. 2), with all these features preceding the destruction of the nucleus [53, 62]. Autophagy begins with the sequestration of cytoplasmic organelles into membrane vacuoles called autophagosomes which then fuse with lysosomes to form autolysosomes, in which materials are subsequently degraded and recycled (Figs. 2 and 3) [49, 53, 64].

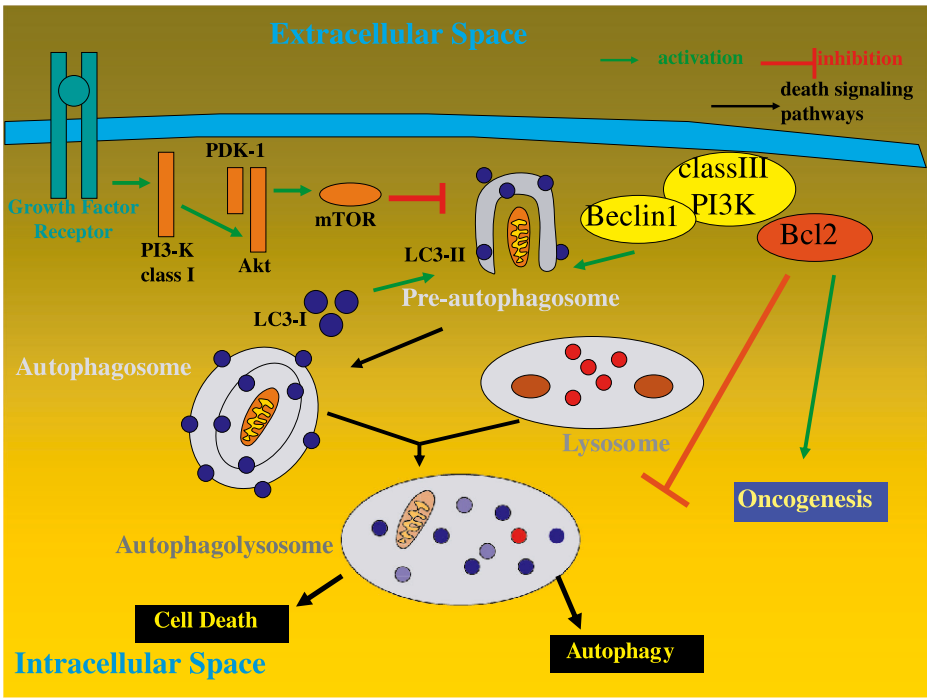

Fig. 3. Simplified view of the pathways of autophagy

The autophagic process in mammalian cells is regulated by homologues of the Apg and Aut autophagy-relevant family of yeast genes, now renamed Atg genes [103]. Several Atg proteins have been implicated in autophagosome formation (Fig. 3). Atg5, Atg7, Atg10 and Atg12 are required to form the autophagic vacuole (Fig. 3). PI3K, the enzyme synthesizing phosphatidylinositol-3-phosphate (PtdIns 3P) from PtdIns, is a major player in mammalian autophagic pathways. While class III PI3K is required in the early stages of autophagosome generation, class I PI3K activity has an inhibitory effect, mediated at least partially through mTOR (Fig. 3) [53, 64]. Thus class I PI3K, Akt and mTOR are components of the pathways that are involved both in apoptosis and autophagy resistance of cancer cells (Figs. 1 and 3). Beclin 1, a homologue of the yeast autophagy protein Atg6, which belongs to the class III PI3K complex, is required for vacuolar formation and transport (Fig. 3) [53]. In addition to interacting with class III PI3K, beclin 1 is able to bind Bcl-2 pro-apoptotic family members (Fig. 3) [94]. While beclin 1's interaction with class III PI3K stimulates autophagy and inhibits oncogenesis, its interaction with Bcl-2 inhibits autophagy and stimulates oncogenesis (Fig. 3) [94]. Autophagy is also induced by the cell death-associated protein kinase (DAPK) and the death-associated related protein kinase 1 (DRP1) [53, 64]. Recent research has also revealed that autophagy is activated by p53, the guardian of the genome, a critical tumor suppressor that is involved and mutated in more than 50% of cancers of all tissue origins [24, 44]. Crighton *et al.* have described a damage-regulated autophagy modulator gene (DRAM), a p53 target gene encoding a lysosomal protein that induces autophagy [17]. Like beclin 1, analysis of DRAM in a subset of epithelial tumors revealed frequent decreased expression [17]. Finally, MAP1LC3, the microtubule associated-protein 1 light chain 3, exists in two forms, MAP1LC3-I localized in the cytosol and its proteolytic derivative MAP1LC3-II localized in autophagosomal membranes (Fig. 3) [53]. During autophagy, MAP1LC3-I is cleaved and conjugated to phosphatidylethanolamine to form MAP1LC3-II which is essential for the formation of the autophagosome [53]. MAP1LC3-II thus can be used to estimate the abundance of autophagosomes before they are destroyed through fusion with lysosomes (Fig. 3) [53].

mTOR seems to be involved in autophagic cell death as its inactivation can induce autophagy (Figs. 2 and 3) [62]. mTOR is a downstream effector of the PI3K/Akt signaling pathway (Fig. 1) and it is also a central modulator of cell proliferation in malignant gliomas [41, 124]. In fact rapid tumor proliferation (that can result from low apoptotic levels) may contribute to the clinical resistance of glioblastomas to radiotherapy, and disruption of mTOR signaling by rapamycin appears to return a certain level of sensitivity to resistant glioblastoma cells (Fig. 1) [22]. Takeuchi *et al.* showed that rapamycin induced autophagy but not apoptosis in rapamycin-sensitive malignant glioma U87-MG and T98G cells by inhibiting the function of mTOR [124]. In contrast, in rapamycin-

resistant U373-MG cells, the inhibitory effect of rapamycin is minor [124]. A number of potential common targets in apoptosis and autophagy resistance pathways i.e. mTOR, PI3K and Akt have been identified (Figs. 1 and 3). Inhibitors of such targets might be able to increase the level of sensitivity of migrating apoptosis-resistant cancer cells to both pro-apoptotic and pro-autophagic drugs (Fig. 1). Thus, novel successes in the fight against devastating cancers including glioblastoma might be achieved by the combination of pro-autophagic drugs such as temozolomide with mTOR, PI3K or Akt inhibitors as adjuvant chemotherapies and as illustrated by several ongoing clinical trials.

Recent results show for the first time that brain tumor stem cells are susceptible to adenovirus-mediated cell death via autophagy *in vitro* and *in vivo* [45], a topic that we developed after.

As traditional clinical end points prove more difficult to apply in evaluation of molecularly targeted therapies, a great need exists to define and validate surrogate markers of effect and benefit [110]. mTOR expression could be evaluated and high tumor mTOR protein levels might indicate suitability for a pro-autophagic inhibitor strategy. Most trials using mTOR inhibitors do not measure mTOR levels. Instead the commonly studied biomarkers are usually downstream effectors such as the phosphorylation of ribosomal p70 S6 kinase which is considered to be a good indicator of the activated Akt/mTOR pathway as well as rapamycin sensitivity [87]. Analysis of DRAM expression, a damage-regulated autophagy modulator gene encoding a lysosomal protein that induces autophagy could potentially also be used as a surrogate marker before the administration of a pro-autophagic drug. In addition, the analysis of MAP1LC3-II that estimates the abundance of autophagosomes before they are destroyed could be used to follow the pro-autophagic effects of a new compound. Similar analyses could also be performed to determine the activation status of other potential biomarkers (PI3K, Akt, NF-κB) in tumor tissues. It seems that whether activation of the PI3K pathway confers sensitivity or resistance to therapy depends on the therapy used, as well as secondary gene events [130]. Thus although genome-wide and proteomic profiling of tumors may orient the therapeutic choice, understanding the genotype-response relationships in human tumors will be important for the effective use of compounds targeting the PI3K pathway in the clinic. Evaluation of these potential biomarkers in preclinical studies can be used to help select lead pro-autophagic compounds and better define patients who will benefit from this treatment.

Therapeutic benefits of temozlomide

A source of real hope in glioma chemotherapy is offered by temozolomide, a second-generation imidazotetrazine alkylating agent [33, 67, 122]. Temozolomide is a small lipophilic molecule which can be administered orally and which

crosses the blood–brain barrier effectively. Moreover, temozolomide is less toxic to the hematopoietic progenitor cells than conventional chemotherapeutic agents and does not require any hepatic metabolism for activation [49]. An international clinical trial [122] has shown that the addition of temozolomide to radiotherapy increases the survival of patients suffering from newly diagnosed glioblastomas. Indeed, addition of temozolomide to radiotherapy improves median survival in newly diagnosed glioblastoma patients from 12.1 to 14.6 months and it increases 2-year survival from 10.4% to 26.5% [122].

Part of temozolomide's cytotoxic activity is exerted through pro-autophagic processes, at least in glioblastoma cells, due to the formation of O^6-methylguanine in DNA which mispairs with thymine during the next cycle of DNA replication [48, 49]. Glioma cells thus respond to temozolomide by undergoing G2/M arrest but will ultimately die from autophagy [48, 49]. While apoptosis is labeled as type I caspase-dependent programmed cell death, the type II caspase-independent process describes autophagic cell death. Knowing that O6-alkylguanine-DNA alkyltransferase (AGT) is a DNA repair enzyme that limits the efficacy of temozolomide in glioblastoma cells [48] showed that inhibition of AGT by O6-benzylguanine can render previously resistant glioblastoma cells sensitive to temozolomide. Hegi *et al.* and Chinot *et al.* showed that patients who had glioblastomas that contained a methylated MGMT (O^6-methylguanine-DNA methyltransferase) promoter benefited from temozolomide, while those who did not were less responsive [14, 37]. However, results contradicting these findings are present in the literature and the clinical and genetic context framing MGMT methylation is poorly characterized. Recent observations suggest that MGMT methylation is part of the genetic signature of glioblastomas that develop from lower-grade gliomas [21].

Part of temozolomide's cytotoxic activity is however also due to the induction of late apoptosis. Indeed Roos *et al.* [106] showed that malignant glioma cells undergo apoptosis following treatment with the methylating agents N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and temozolomide. O(6)MeG-triggered apoptosis in gliomas is a late response (occurring >120 h after treatment) that requires extensive cell proliferation. Overall, the data reported by Roos *et al.* [106] demonstrate that cell death induced by temozolomide in gliomas is due to apoptosis and that determinants of sensitivity of gliomas to temozolomide are MGMT, p53, proliferation rate and double-strand break repair. Given that the response to temozolomide is at least partly associated with MGMT promoter methylation status, MGMT methylation analysis by means of RT-PCR techniques could be used to predict tumor sensitivity to the drug [37, 31]. However, this surrogate marker of response is not really related directly to the pro-autophagic phenomenon.

The data reported by Kanzawa *et al.* [49] and Roos *et al.* [106] are not contradictory because autophagy and apoptosis may be triggered by common

upstream signals, and sometimes this results in combined autophagy and apoptosis [62, 72] (Fig. 2). In other instances, the cell switches between the two responses in a mutually exclusive manner [72]. On a molecular level, this means that the apoptotic and autophagic response machineries share common pathways that either link or polarize the cellular responses (Fig. 2) [72]. We will show below that inhibiting sodium pump activity in human apoptosis-resistant glioblastoma cells can induce marked processes of autophagy.

Recent data have also emerged showing that temozolomide could be considered as a potential radiosensitizer [12].

Local therapies for glioblastomas

Recurrence near the tumor resection site may be due to changes in the extracellular matrix coincident with scar tissue. Moreover, the extracellular matrix that supports a migratory phenotype retards the growth rate of the cell population. In this sense, control of the disease by local therapy applied to the resection cavity during surgery may reduce the rate of local failure and increase the time for local progression. These agents function inside the tumor cells with microscopic and submicroscopic precision. Molecular therapies such as neural stem cells, immunotherapies, biodegradable polymers and convection-enhanced drug delivery could complement existing surgical, radiological, and chemotherapeutic approaches to the treatment of gliomas. However, local chemotherapy protocols using degradable polymers applied to the resection cavity depend on the diffusion of the drugs delivered to the brain parenchyma, which presumably lies within millimeters of the site of implantation [131].

The only FDA-approved drug delivery system consists of carmustine (BCNU)-impregnated polymers in the form of wafers (Gliadel®) [131]. These wafers are implanted into the tumor cavity during surgery and slowly release the active drug. As compared to empty polymers, this treatment significantly improves the survival of patients with newly diagnosed glioblastomas with both groups receiving radiotherapy after surgery [131]. This result seems similar to the benefit derived from systemic adjuvant nitrosoureas [121]. However, so far no studies have compared a systemic chemotherapy strategy with this local delivery.

One promising surgical technique for the delivery of drugs directly into the brain parenchyma involves a convection-enhanced delivery system (CED) [38]. CED uses positive pressure infusion to generate a pressure gradient that optimises the distribution of macromolecules within the tumor and the surrounding tissue [108]. This system is notable in a small number of treatments of recurrent high-grade gliomas. A first system uses interleukin-(IL)13-PE38QQR, a recombinant toxin composed of the enzymatically-active portion of Pseudomonas Exotoxin A conjugated with human IL13 binding selectively to IL13 alpha2 receptors overexpressed by malignant gliomas [43]. This re-

combinant cytotoxin (0.5 μg/mL) administered via CED before standard radiochemotherapy seems to be well tolerated in adults with newly diagnosed malignant gliomas [127]. Another system consists of a toxin conjugated with the EGFR binding ligand transforming growth factor-alpha (TGF-α) [107]. The binding of the ligand to the receptor (overexpressed or constitutively activated in malignant gliomas, Fig. 1) permits the internalisation of the recombinant toxin, and this results in a selective and potent cytotoxicity at nanomolar concentrations. In this context, the TransMID study uses transferrin-CRM107, a conjugate of human transferrin and diphteria toxin [129]. These are currently undergoing Phase III study. Recent data reveal that target tissue anatomy and catheter position are critical parameters in optimizing drug delivery [108].

Ongoing clinical trials for glioblastomas

Using as a reference the internet site http://www.clinicaltrials.gov/ct/search;jsessionid=854243DC237D32C8BED356A7ADB0F791?term=glioblastoma – a service developed by the National Library of Medicine for the US National Institute of Health – we obtained information on about 392 clinical glioblastoma management trials currently under way (12th February 2008) for resistant progressive glioblastomas or newly diagnosed ones. These studies include 102 clinical trials using temozolomide and 83 studies of combined therapy based on the hypothesis that combining more than one chemotherapy drug with radiotherapy may kill more tumor cells. We describe here the compounds used in the so-called target therapy to inhibit growth factor receptors and tyrosine kinases. Many of these drugs were generally well tolerated in clinical trials; however, as single-agents, the results have been disappointing. As emphasized by Omuro and Delattre [91], the oncogenetic process in such tumors is driven by several signaling pathways that are differentially activated or silenced with both parallel and converging complex interactions. Therefore, it has been difficult to identify prevalent targets that act as key promoters of oncogenesis and that can be successfully addressed by novel agents. Several drugs have been tested, including epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (gefitinib and erlotinib), mammalian target of rapamycin (mTOR) inhibitors (temsirolimus and everolimus), and vascular endothelial growth factor receptor (VEGFR), protein kinase C-beta, and other angiogenesis pathways inhibitors (vatalanib, bevacizumab, and enzastaurin). Most of the present clinical trials evaluate conventional drugs in combination with target therapy which may improve their clinical activity. Ongoing investigations evaluating temozolomide in combination with other systemic agents, and additional agents (e.g., motexafin gadolinium, mammalian target of rapamycin inhibitors, farnesyltransferase inhibitors) seem to show promising activity in combination with radiotherapy [12].

Growth factor receptor inhibitors

Growth factor receptors such as EGFR and PDGFR, whose abnormal functioning leads to the accelerated clinical progression of malignant gliomas, have already been specifically targeted (Fig. 1). Highly specific small molecule inhibitors of these tyrosine kinase receptors have been developed [96, 97, 101, 105]. Gefitinib (Iressa) and erlotinib (Tarceva) are orally active selective EGFR inhibitors which were clinical tested with respect to a number of tumors, including malignant gliomas [96, 97, 105]. The accelerated approval of gefitinib for non-small lung cancer has been revoked by the Food and Drug Administration due to the lack of efficacy in published randomised phase III studies. In the same manner, in the absence of objective responses, some limited antitumor activity was suggested for the treatment of glioblastomas with gefitinib [105]. Objective responses were seen in phase I and phase II trials with erlotinib associated or not to TMZ for recurrent glioblastoma [97]. However, only 10–20% of patients respond to EGFR kinase inhibitors. On the basis of the sequencing of genomic tumor DNA and immunhistochemistry analysis it seems that the coexpression of EGFRvIII (EGFR deletion mutant variant III) and PTEN by glioblastoma cells is strongly associated with responsiveness to EGFR kinase inhibitors [76]. Data also suggest that the downstream inhibition of the PI3K pathway, perhaps at the level of the mammalian serine/threonine protein kinase target of rapamycin (mTOR) (as detailed below), could be combined with EGFR kinase inhibitors to promote responsiveness in patients with PTEN-deficient tumors [32].

PI3K/Akt, mTOR and NF-κB inhibitors

The clinical struggle against malignant gliomas should also include inhibitors against signaling pathways controlled by PI3K/Akt, mTOR and NF-κB (Fig. 1). Indeed, reducing the signaling abilities of PI3K, Akt, mTOR and NF-κB would not only reduce the growth levels of malignant gliomas, but should also reduce the migration levels of individual glioma cells migrating into the brain parenchyma [7]. This reduction in the migratory abilities of individual migrating glioma cells should restore pro-apoptotic drug sensitivity in these individual migrating glioma cells that are naturally resistant to apoptosis (and so to current chemotherapies) due to the constitutive activation of one or another of the PI3K/Akt, the mTOR or the NF-κB signaling pathways [88]. The fact remains that all these pathways are not activated at the same time in any single glioma. It remains unclear how best to integrate recent discoveries regarding glioma molecular biology into clinical practice [59]. Particular inhibitor(s) should therefore only be chosen if the target(s) is (are) present in the tumor tissue, but this is only possible if individual patients are submitted to the molecular profiling of their tumors. The stratification of cases has not yet been carried out in the

majority of trials conducted by the National Brain Tumor Consortia funded by the National Cancer Institute, the NABTC and the NABTT (New Approaches to Brain Tumor Therapy). This aim should be at least partly satisfied in the near future by integrating into clinical practice the data provided by molecular profiling as in the search for 1p19q deletion to identify glioma patients who are to benefit from intensive adjuvant chemotherapy.

Rapamycin, a macrocyclic lactone, is a highly specific inhibitor of mTOR [7], and mTOR is a direct target of the PI3K/Akt signaling pathway in mitogen-stimulated cells (Fig. 1) [7, 74]. Bjornsti and Houghton recently reviewed the TOR pathway as a target for cancer therapy [7]. As emphasized by Sekulié *et al.* [112], rapamycin is a potent immunosuppressive drug and investigational agent, the major mechanism of action of which involves the inhibition of cell proliferation mediated by blocking cells moving from the G1 to the S phase of the cell cycle. In fact, rapamycin inhibits the phosphorylation of the retinoblastoma protein, and rapamycin-treated cells are therefore not fully committed to entering the S-phase after their release from drug-induced G1 arrest [112]. Constitutive Rb phosphorylation frequently occurs in glioblastomas due to mutation-induced p16 gene inactivation and, as expected, clinical trials with mTOR inhibitors (including, for example, CCI-779) were proceeding on patients with recurrent glioblastomas [26]. This phase II trial of temsirolimus (CCI-779) in 65 recurrent glioblastoma patients has shown radiographic improvement in 36% of them, and was associated with significantly longer median time to progression [26]. Based on preclinical evidence that phosphatase and tensin homolog deleted on Chromosome 10 (PTEN) loss sensitizes tumors to the inhibition of mammalian target of rapamycin (mTOR) [16] conducted a proof-of-concept Phase I neoadjuvant trial of rapamycin in patients with recurrent glioblastoma, whose tumors lacked expression of the tumor suppressor PTEN.

Matrix metalloproteinase (MMP) inhibitors (MMPI)

Specific anti-migratory compounds should be added to conventional radio- and/or chemotherapy. MMP are zinc-dependent endopeptidases that degrade some components of the extracellular matrix. A review of MMP and the development of MMPI can be found [39]. MMP degrade the basement membrane and the extracellular matrix, thus facilitating tumor growth, invasion, and spread. MMP expression is enhanced in most cancers, including gliomas. Of all the known MMPI in clinical development, marimastat, metastat, and prinomastat have been, or are being, tested in trials against gliomas [35, 39]. Combined with temozolomide the MMPI marimastat has given the best results to date in phase II trials, increasing the rate of 6-month progression-free survival in cases of recurrent glioblastomas and anaplastic gliomas [35]. A more recent study revealed that even though this regimen was more efficacious than a comparator

of historical controls in recurrent anaplastic gliomas, the regimen was roughly equivalent to single-agent temozolomide and was associated with additional toxicity. The sub-analysis suggests pharmacokinetic and drug–drug interactions which may positively impact responses to marimastat [34].

Angiogenesis targeting

Malignant gliomas are remarkably angiogenic, and vascular endothelial growth factor (VEGF) is the dominant pro-angiogenic factor. Few trials use drugs to try to inhibit the formation of new blood vessels in glioblastomas and this type of approach has recently been reviewed by [98, 58]. Results have been published on phase II trials with thalidomide – a putative inhibitor of angiogenesis – in the treatment of adults with previously irradiated, recurrent high-grade gliomas [80]. Using the rabbit corneal micropocket assay it has already been shown that thalidomide inhibits basic fibroblast-growth-factor-induced angiogenesis and decreases the proliferation of endothelial cells in cultures without any modification to the proliferation of glioma cell lines *in vitro*. However, if thalidomide is generally well tolerated, it may display antitumor activity in a minority of patients only [80]. The clinical activity of a combination of thalidomide and temozolomide is currently being explored in a phase I/II trial in the case ofpatients with newly diagnosed glioblatomas (http://www.clinicaltrials.gov/ct/search;jsessionid=854243DC237D32C8BED356A7ADB0F791?term=glioblastoma).

Strategies of inhibition of vascular endothelial growth factor (VEGF), which increases vascular permeability and stimulates endothelial proliferation and migration and commonly overexpressed in glioblastomas, have been developed. The most positive results with targeted therapy remain to date the high response rates with bevacizumab and irinotecan in a phase II trial for recurrent malignant gliomas [91, 118]. The monoclonal antibody bevacizumab targets VEGF, the paracrine stimulator of angiogenesis. An update on survival from the original phase II trial of bevacizumab and irinotecan in recurrent malignant gliomas was presented at the ASCO annual meeting 2008 [128]. The overall response rates for both grade III and IV were 59% (grade III 61%, grade IV 57%), the 6-month period free survival and overall survival for grade III were 59% and 79% and for grade IV 43% and 74% respectively. For the grade IV patients, the 2yr overall survival is 15% [128]. Therefore, the combination of bevacizumab and irinotecan provides a clinically meaningful treatment option for patients with recurrent malignant gliomas. However, combination of anti-angiogenic drugs with more potent chemotherapy will probably be necessary.

An array of additional clinical trials evaluating anti-angiogenic strategies are underway for both recurrent and newly diagnosed malignant glioma patients [101].

Cellular and vaccination therapies

The discovery of dendritic cells, the most potent antigen presenting cells to initiate specific immune responses and the possibility of producing them *ex vivo* have given rise to new protocols of active immunotherapy against gliomas [20, 46]. Phase I clinical trials [10, 51, 134, 136] have shown that vaccination using patients' peripheral blood dendritic cells pulsed with tumor lysates, cell fusions, RNA, and/or peptides can elicit antitumor immune responses against CNS neoplasms. Dendritic cell vaccination elicits T-cell-mediated antitumor activity and intratumoral CD4+ and CD8+ T-cell infiltration [10]. Although the currently available clinical data are too limited to arrive at any conclusions concerning its effectiveness, the advantages of dendritic cell-based immunotherapy and its documented safety and feasibility have stimulated further development and testing. The data suggest that coupled with the low transforming growth factor $\beta 2$ expression within the glioblastomas, the absence of bulky, actively progressing tumor may identify a subgroup of patients to target as potential responders in future clinical investigations of dendritic cell-based vaccines [70].

Gene therapy

Researchers have been exploring many candidate genes, developing improved viral and non-viral vectors, trying different methods to deliver genes, and combining gene therapies with other modalities such as immunotherapy. Although the antitumor effect of these gene therapies looked very promising on animal models, the effect on human patients has been disappointing. The recent review by Kanzawa *et al.* focuses on current therapeutic genes/vectors/delivery systems/targeting strategies in order to introduce updated trends and, hopefully, to indicate a prospective gene therapy for malignant gliomas [50].

Gene therapy has been disappointing, but major improvements in gene delivery technology could renew an interest in it. RNA interference (RNAi) has the potential to knock down oncogenes. However, the therapeutic potential of RNAi will not be realised until the rate-limiting delivery step has been dealt with [93].

Reducing malignant glioma cell motility in order to restore pro-apoptotic drug sensitivity

For motility, cells must acquire spatial asymmetry enabling them to turn intracellular generated forces into net cell body translocation [61]. One characteristic of this asymmetry is a polarized morphology i.e. a clear distinction between the front and rear of cells. An early event in this polarization is a change in filamentous F-actin distribution from azimuthal symmetry around the cell rim to a concentration in a particular region [61]. Additional molecular rearrange-

ments consist of the redistribution of chemosensory signaling receptors, integrins and other adhesion receptors, and the redistribution of integrin-cytoskeleton linkages [61]. Another important consequence of polarization is that the extension of active membrane processes, including both lamellipodia and filopodia, takes place primarily around the cell front, so that directional turning is generally accomplished gradually, with cell locomotion taking on the character of a persistent random walk.

Lamellipodia are broad flat sheet-like structures, whereas filopodia are thin cylindrical needle-like projections. These structures contain an abundance of actin and actin-associated proteins [61]. The extension of both the lamellipodia and the filopodia in response to migratory stimuli is almost universally found in conjunction with local actin polymerization [61]. Along with a bias towards membrane extensions at the cell front, attachments tend to form preferentially at the leading edge of lamellipodia and filopodia [61]. Rapid migration also requires efficient mechanisms to release adhesion at the rear [61]. While the actin cytoskeleton provides the driving force at the front, the microtubule network assumes a regulatory function in coordinating rear retraction [61].

As indicated above one potential way of overcoming apoptosis resistance is by decreasing the migration of migrating glioma cells, which results in a net increase in the level of sensitivity of these cells to pro-apoptotic drugs [61]. We have recently shown that when compared to temozolomide alone cimetidine – an anti-inflammatory agent whose action against migrating epithelial cancer cells has now been proved – when added to temozolomide is superior *in vivo* in extending the survival of nude mice with human glioblastoma cells orthotopically xenografted into their brains [63]. The benefit from cimetidine is partly due to an antiadhesive and therefore antimigratory effect on glioma cells [63]. We have consequently reviewed the various mechanisms of action potentially associated with the therapeutic effects of cimetidine in the case of experimental glioblastomas [68], and hope that our observations will encourage the clinical investigation of cimetidine with respect to the management of highly malignant gliomas.

We also showed that when compared to temozolomide alone, the therapeutic benefits obtained *in vivo* in experimental models of human orthotopic glioblastomas were significantly more pronounced when combining temozolomide and a siRNA directed against galectin-1 [69], which is a potent modulator of glioma cell migration [9]. Similar data were obtained when we combined temozolomide with the targeting of sigma1 receptors in glioblastoma cells [75].

The sodium pump constitutes a new target to combat malignant gliomas

The growth of a glioma requires the destruction of the normal brain parenchyma by the glioma cells [117]. This is achieved through the cellular release of

glutamate into the peritumoral space [71] in the absence of functional Na^+-dependent glutamate transporters in glioma cells, resulting in the consequent accumulation of excitoxic glutamate in the extra-cellular glia space [123]. Signaling through glutamate receptors is also involved in glioblastoma cell proliferation [3]. Glioma cells are "self-propelled" [117] and are able to adjust their shape and volume rapidly as they invade the brain parenchyma. Essential to this process is the activity of chloride channels, anion transport mechanisms [99] and aquaporins [36]. The sodium pump is another ion transporter which in addition to exchanging cations is also directly involved in the migration of cancer cells in general [6, 23, 78, 79] and of glioma cells in particular [66, 113]. Moreover, the activity of NaK can be modulated by glutamate and its receptors [81].

The sodium pump

In mammalian cells, active sodium transport and its derived functions (e.g., plasma membrane potential) are dictated by the activity of the sodium pump, whose regulation is essential for maintaining cell volume and composition, as well as other vital cell functions [116]. Because the plasma membrane is highly permeable to water, it is the concentration of ions across this membrane that is, in the short term, critical for maintaining an adequate cell volume. The plasma membrane sodium pump is important in this process because it provides the driving force for active sodium and potassium transport into and out of the cell, with water following isosmotically [116]. Increases in sodium permeability require concomitant increments in sodium pump-mediated outward sodium transport to prevent a disproportionate increase in the intracellular sodium concentration/osmotic pressure and, consequently, cell swelling (Fig. 4A).

The sodium pump consists of equimolar ratios of two main subunits, the catalytic α and regulatory β polypeptides, each of which exists as several isoforms [79]. To date, four different α and three distinct β isoforms have been identified in mammalian cells [8, 79, 133]. The α subunit contains the binding sites for the above cations, ATP and cardiotonic steroid inhibitors [8, 79, 133]. While the β subunit is essential for the normal activity of the enzyme, it also appears to be involved in the occlusion of K^+ and modulation of the enzyme's K^+ and Na^+ affinity [79, 133]. In addition to pumping ions (Na^+ and K^+) across the plasma membrane (Fig. 4A), the sodium pump functions as a receptor for cardiotonic steroids [8, 79, 133] including ouabain, digitoxin and digoxin and a novel cardenolide UNBS1450 [66, 78, 79] (Fig. 4).

During apoptosis, there is compelling evidence indicating an early increase in intracellular sodium followed by a decrease in both intracellular K^+ and Na^+ suggesting a regulatory role for these cations during both the initial signaling, and the execution phase of apoptosis [133]. Recent studies have shown that the

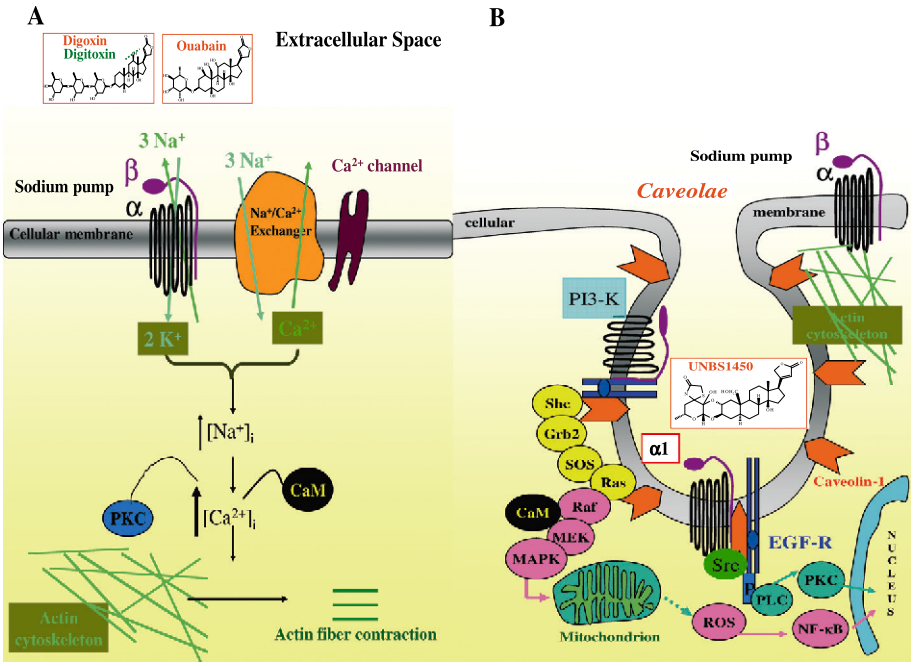

Fig. 4. The sodium pump functions as a receptor for cardiotonic steroid inhibitors. In addition to pumping ions (Na^+ and K^+) across the plasma membrane utilizing ATP as the driving force (A), the sodium pump in caveolae is engaged in assembly of multiple protein complexes that transmit signals to different intracellular compartments (B). (A) For every three Na^+ ions pumped out of the cell, two K^+ ions are pumped in. The partial inhibition of the sodium pump by the cardenolide ouabain causes a modest change in $[Na^+]_i$ and $[K^+]_i$, and a significant change in $[Ca^{2+}]_i$ via the Na^+/Ca^{2+}-exchanger. (B) The sodium pump signalosome closely interacts with major components of gliomagenesis: EGFR, caveolin-1, PI3K, Src and Ras. Upon ouabain binding, the Na^+/K^+-ATPase $\alpha 1$ subunit in caveolae associates with several proteins, such as caveolin-1 through two caveolin-binding motifs and Src via multiple domains. Activated Src secondarily trans-activates EGFR which in turn recruits the adapter protein Shc to relay signals to the Ras-Raf-MAPKs cascade. In contrast UNBS1450 at 10 nM, a concentration at which it demonstrates potent anti-proliferative and anti-migratory activity, does not elicit increases in $[Ca^{2+}]_i$ or $[Na^+]_i$ in cells but the compound decreases ($[ATP]_i$). *EGFR* Epithelial growth factor receptor; *PKC* protein kinase C; *PI3K* phosphoinositide 3′kinase; *Grb2* growth factor receptor bound protein 2; *Sos* son of sevenless; *Shc* src homology collagen-like protein; *PLC* phospholipase C; *MAPK* mitogen-activated protein kinase; *Ros* reactive oxygen species; *MEK* MAPK-ERK activating kinase

sodium pump is involved in controlling perturbations of Na^+ and K^+ homeostasis during apoptosis, and that anti-apoptotic Bcl-2 and Bcl-XL molecules influence these ionic fluxes.

Cardiotonic steroids: ligands of the sodium pump

Cardenolides and bufadienolides are the two chemical sub-classes that constitute the cardiotonic steroids [79]. The sodium pump is characteristically inhibited by cardiac glycosides. By binding to sodium pump, cardiotonic steroids elicit the activation of the so-called "Na^+/K^+-ATPase signalosome" [79] (Fig. 4B).

Cardenolides, such as digitalis (digitoxin and digoxin) have been used for the treatment of congestive heart failure for more than 200 years [79]. Digoxin is still used to treat approximately 1.7 million patients in the United States for heart failure and/or atrial fibrillation despite the development of newer pharmacological agents such as angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor antagonists and β-blockers [79]. Besides its classical use in cardiac diseases, the use of digitalis in oncology has already been proposed [119]. As mentioned above the constitutive activation of the NF-κB pathway in cancer cells leads them to become chemoresistant [1, 4]. Different types of cardenolides are able to suppress the constitutive activation of NF-κB [73, 77, 78] to induce apoptosis [42, 78] and to overcome multi-drug resistance [125]. Several cardenolides have been shown to display *in vitro* anti-tumor activities against various types of cancer cells [42, 73, 77, 78, 119] including glioma cells [66]. However, none have ever reached clinical application because either their therapeutic index (ouabain, digitoxin) or their anti-tumoral activity (digoxin) were too low.

UNBS1450 is a novel cardenolide hemi-synthesized from 2″-oxovoruscharine [125], with a toxicity profile similar to digoxin but with significantly more marked anti-tumor activity [78]. It displays potent anti-tumor activity in human non-small cell lung cancers, especially in those cancers in which the sodium pump α1 subunit is over-expressed [78]. UNBS1450 induces non-apoptotic cell death in apoptosis-resistant cancer cells including lysosomal membrane permeabilization-related death in non-small cell lung cancer cells and autophagic cell death in glioblastoma cells [66].

The sodium pump is involved in cancer cell proliferation, migration and death

There are in fact two pools of the sodium pump within the plasma membrane with two distinct functions. One constitutes the energy transducing pool of the enzyme broadly distributed in the plasma membrane (Fig. 4A). The other is the signal transducing pool of the enzyme restricted to the caveolae which is independent of changes in intra-cellular Na^+ and K^+ concentrations [133] and requires the initial association of the sodium pump with tyrosine kinase Src [120] (Fig. 4B). The Na^+/K^+-ATPase signalosome closely interacts with major components of gliomagenesis, including for example, the interaction of EGFR

with caveolin-1 [133], the binding of PI3K to NaK [6] and the involvement of Src, Ras and EGFR in the signal-transducing function of NaK [120] (Fig. 4B).

The $\beta 2$ isoform of the sodium pump is in fact a homologue of the adhesion molecule on glia (AMOG) which is a recognition element for cell adhesion that subsequently links cell adhesion and ion transport [111, 113]. The AMOG/$\beta 2$ and the $\alpha 1$ subunits of the sodium pump come together to form functional sodium pumps [111]. AMOG is down-regulated in human and mouse gliomas [113]. We have identified the sodium pump as a major element in the migration of tumor cells [78, 79] including glioblastoma cells [66] arising from its close interaction with caveolin-1 and its role in the organization of the actin cytoskeleton in the caveolae. We have found that the $\alpha 1$ subunit of the sodium pump is located at the lamellipodia of the human glioblastoma cell line U373-MG, where it co-localizes with caveolin-1 [66]. Caveolae functions rely on caveolin-1, their major protein, which drives the formation of plasma membrane caveolae and anchors them to the actin cytoskeleton (Fig. 4B). In addition, caveolin-1 modulates cell interaction with the extra-cellular matrix, and brings together and regulates the interaction of different signaling molecules, with significant roles in cell movement [84]. Importantly, caveolin-1 depletion results in the loss of focal adhesion sites and overall cell adhesion [84]. Furthermore, we have shown by means of quantitative RT-PCR that the levels of $\alpha 1$ subunit mRNA are higher in glioblastomas (7/10 human glioblastomas analyzed) than in normal brain tissue (0/4 normal brain tissue samples) [66]. Such measurements also revealed high levels of the $\alpha 1$ subunit mRNA in human glioblastoma U373-MG cells [66].

We have shown that various types of cancer including glioblastomas [66], non-small cell lung cancers [78] and melanomas (manuscript in preparation) over-express the a1 subunit of the sodium pump when compared to the normal tissues from which they arise.

UNBS1450 is more potent in inhibiting the proliferation of U373-MG glioblastoma cells than that of normal cells [66], a feature that can be explained, at least in part, by the fact that (i) U373-MG glioblastoma cells express higher levels of sodium pump $\alpha 1$ subunits than normal cells, (ii) UNBS1450 decreases the intra-cellular ATP concentration ($[ATP]_i$) more markedly in U373-MG glioblastoma cells than in normal cells and (iii) $[ATP]_i$ is higher in normal cells than in U373-MG cancer cells [66].

Our results show that UNBS1450-mediated anti-proliferative and anti-migratory effects on human glioblastoma cells occur as a result of the disorganization of the actin cytoskeleton [66]. It should be borne in mind that the actin cytoskeleton is involved in many cellular processes that are essential for cell growth, differentiation, division, membrane organization and motility [5]. Moreover, the association of actin filaments with the plasma membrane provides mechanical stability, maintains cell shape and adhesion, and regulates

dynamic surface protrusions such as lamellipodia and filopodia, which are fundamental determinants of motility and migratory potential of cells [19].

Our strategy has been to target the $\alpha 1$ subunit of the sodium pump in those glioblastomas that over-express this subunit with novel, potent and selective cardenolides. In so doing using UNBS1450, it has been possible to markedly impair (at least in experimental models of human glioblastomas) both glioblastoma cell proliferation and migration (through a disorganization of the actin cytoskeleton), with marked features of autophagy as the terminal outcome. In essence, the targeting of the sodium pump $\alpha 1$ subunit in glioblastoma cells appears to impair both their proliferation and migration, even if they are resistant to apoptosis.

UNBS1450 has undergone regulatory preclinical evaluation (toxicology, safety pharmacology, drug metabolism and pharmacokinetic development studies are ongoing) and has reached Phase I clinical trials in Belgium and the Netherlands.

Brain tumor stem cells a potential target to combat malignant glioma

Cancer stem cells are thought to be crucial for tumorigenesis [109]. The brain tumor stem cell hypothesis proposes the existence of multipotent glioma cells of origin that are characterized by the expression of stem cell markers, by the capacity for self-renewal, multilineage differentiation, re-establishment of tumor after transplantation and resistance to radiotherapy and chemotherapy [18, 127, 109]. Therefore, the eradication of brain tumor stem cells is essential for long-term brain tumor remission after treatment. Gilbertson and Rich recently reviewed data showing that stem cells of glioblastoma are found in intimate contact with the aberrant tumour vasculature [29]. These cancer stem cells can secrete diffusible factors such as VEGF, which recruit aberrant tumor vasculature to the niche. In turn, tumor vasculature and other glioma cells secrete factors that maintain aberrant cancer stem cell self-renewal. Recent results show for the first time that brain tumor stem cells are susceptible to adenovirus-mediated cell death via autophagy *in vitro* and *in vivo* [45]. Because adenoviral proteins can completely overcome the molecular machinery of the infected cell, the authors hypothesized that Delta-24-RGD, an oncolytic adenovirus with enhanced tropism to glioma cells and selective replication in cancers cells with an abnormal Rb pathway [25], may act as a potent therapeutic agent to target brain tumor stem cells and prevent them from developing resistance to standard adjuvant therapy. The authors showed that Delta-24-RGD induced the formation of acidic vesicular organelles associated with an increase in the membrane-bound MAP1LC3-II protein, a modification that is essential for the formation of autophagosomes, without an increase in the

levels of beclin1 (Atg6) in several glioma treated stem cell lines (Fig. 3). By contrast, the protein levels of Atg5, a key molecule in the conversion of LC3-I to LC3-II and therefore required for autophagosome formation and autophagic cell death, were dramatically increased. Immunofluorescence analyses of the brains of nude mice bearing xenograft tumors and treated with intratumoral injections of Delta-24-RGD identified high levels of expression of the proautophagic protein Atg5 in the tumor area adjacent to the necrosis. No other area in the tumor or any area of untreated tumors was positive for Atg5. Therefore, the *in vivo* assessment of adenovirus-induced autophagy by means of Atg5 may be a useful way to monitor oncolytic adenovirus efficacy in future clinical trials [45]. More recently, the same group has shown that Delta-24-RGD in combination with RAD001 (everolimus, an mTOR inhibitor) induced enhanced antiglioma effect via autophagic cell death [2].

Conclusions

The treatment of glioblastomas requires a multidisciplinary approach that takes the presently incurable nature of the disease into consideration. Treatments are multimodal and include surgery, radiotherapy and chemotherapy. Current recommendations are that patients with glioblastomas should undergo maximum surgical resection followed by concurrent radiation and chemotherapy with the novel alkylating drug temozolomide, and this in its turn to be followed by additional adjuvant temozolomide for a period of up to 6 months. However, glioblastomas almost invariably recur near their initial sites. Disease progression usually occurs within 6 months and rapidly leads to death. A number of signalling pathways can be constitutively activated in migrating glioma cells, thus rendering these cells resistant to pro-apoptotic insults such as conventional chemotherapies.

However, a number of strategies are emerging to overcome, at least partly, the resistance of migrating glioma cells to apoptosis. These involve (i) inhibition of the molecular pathways involved in apoptosis resistance that are overexpressed in glioma cells, (ii) induction of autophagy in glioblastoma cells as well as in glioma stem cells and (iii) reducing malignant glioma cell migration; a process which in turn seems to restore a certain level of sensitivity to pro-apoptotic cytotoxics in migration-restricted glioma cells.

Control of glioblastomas by topical therapy applied to the resection cavity during surgery may reduce the rate of local failure and increase the time for local tumor progression. New local agents including targeted pro-autophagic therapies as well as advances in delivery systems including CED are likely to play a role in the future trial for migrating glioma cells.

Until recently, the new treatments for malignant gliomas have focused largely on the intrinsic properties of glioma cells with the targeted therapy.

Given the disappointing results from these clinical trials, it seems clear that novel biomarker-guided clinical trial designs are needed. One such trial design uses a pharmacodynamic marker to monitor the efficacy of a given therapeutic agent. A recent series of studies have supported the concept that malignant gliomas might to be seen as an orchestration of cross-talk between cancer cells, micro-environment, vasculature and cancer stem cells. Successful treatment of invasive brain tumors will depend on blending cocktails of targeted agents that are tailored for an individual patient.

It is hoped that novel therapies derived from a cellular and molecular understanding of glial tumorigenesis and of the interaction between these cancers and their micro-environment, and advances in non-invasive diagnosis, surgical technology and adjuvant treatment will significantly improve the clinical outcome of these devastating lesions.

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