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REVIEW ARTICLE

Autophagy inhibition is the next step in the treatment of glioblastoma patients following the Stupp era

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Abstract

It has now been nearly 15 years since the last major advance in the treatment of patients with glioma. "The addition of temozolomide to radiotherapy for newly diagnosed glioblastoma resulted in a clinically meaningful and statistically significant survival benefit with minimal additional toxicity". Autophagy is primarily a survival pathway, literally self-eating, that is utilized in response to stress (such as radiation and chemotherapy), enabling clearance of effete protein aggregates and multimolecular assemblies. Promising results have been observed in patients with glioma for over a decade now when autophagy inhibition with chloroquine derivatives coupled with conventional therapy. The application of autophagy inhibitors, the role of immune cell-induced autophagy, and the potential role of novel cellular and gene therapies, should now be considered for development as part of this well-established regimen.

You are what what you eat eats.

—— Michael Pollan, In Defense of Food: An Eater's Manifesto

Gliomas are a diverse and devastating group of primary brain tumors. The most common and the deadliest form of glioma is glioblastoma multiforme (GBM) World Health Organization grade IV [1], with a median survival of 15 months. GBM can be classified on the basis of molecular, genetic, and histological features, with the most salient finding being the presence or absence of isocitrate dehydrogenase (IDH1 and IDH2) mutations and O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation [2]. Further transcriptomal classification of GBM into proneural, neural, classical, and mesenchymal subtypes are characterized by *IDH1/PDGFRA* expression, neuron markers, *EGFR* amplification, and *NF1* expression, respectively, and may give insight into treatment response [3, 4].

Unfortunately, recent advances in our understanding of the genomic and clinical landscape of gliomas have yielded little improvement in patient improvement [5]. Cytoreduction through surgical resection plays a key role, but the diffuse nature of this disease makes a surgical cure impossible. The blood–brain barrier (BBB) prevents many small molecules from reaching tumor cells and associated neoangiogenic vasculature, and attempts to circumvent the BBB have been only minimally successful [6, 7]. It is therefore imperative to understand GBM-related mechanisms of drug and radiation resistance in order to prolong the efficacy of chemotherapy. Autophagy, a vital stress-induced cell survival response, is markedly upregulated in many malignancies [8–10]. Autophagic mechanisms of tumor cell survival represent a significant mechanism of resistance to traditional chemotherapy GBM; however, the molecular mechanisms that underlie autophagy in GBM are not fully understood. The role of this review is to describe the known mechanisms of autophagy during GBM pathogenesis and treatment responses.

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Principles of autophagy

Autophagy was initially described as visible focal cytoplasmic lesions in stressed cells by H.W. Altmann in 1955 [11]. In 1962, Hruban et al. [12] characterized focal cytoplasm degradation sites believed to limit cellular injury by sequestration of the damaged portions of cytoplasm. They found that the structure of these inclusions were influenced by the character of sequestered cytoplasmic area, the stage of degradation, the cell type, and the type and severity of noxious stimuli. They described this process as occurring in stages of sequestration, formation of complex dense bodies, and formation of lysosome-like bodies.

The term "autophagy" was first formally presented February 1963, during the Ciba Foundation Symposium on Lysosomes by Christian de Duve, a Nobel laureate and Professor in Biochemistry [13]. Among the pioneers of the field present at the symposium was Alex Novikoff, who described similar findings that he called, "cytolysomes", acid-phosphatase-positive structures containing cytoplasmic components and organelles, such as mitochondria, endoplasmic reticulum (ER) membranes, and ribosomes. These cytolysomes were found to be abundant in cells undergoing physiological or pathological autolysis. Hence, de Duve had suggested the name "autophagic" (eating self) vacuoles for these cytolysomes to distinguish them from heterophagic (eating others) vacuoles [13]. The process of autophagy was further characterized some time later by Russell Deter, who studied the hepatocyte response to glucagon as a postdoctoral fellow in de Duve's laboratory. Deter had confirmed previous findings by Thomas Ashford and Keith Porter, in which glucagon stimulation of hepatocytes caused striking morphologic alterations in cytoplasmic-dense bodies, suggesting autolysis [13]. However, in contrast to Ashford and Porter's belief that glucagon induced an increase in formation of lysosomes, Deter's biochemical approach suggested that lysosomes are recruited to facilitate degradation of already segregated cytoplasmic components, thereby forming autophagic vacuoles [13]. Subsequent studies of autophagy in yeast have led to the identification of 36 autophagy-related genes (ATGs), with 20 human orthologs [14, 15]. Study of these genes has enabled our current understanding of the molecular machinery and regulation of autophagy today.

Autophagic pathways

Autophagy is a mechanism for cells to maintain homeostasis in a variety of situations. It recycles cytoplasmic contents to generate usable energy and macromolecular building blocks in times of metabolic stress, removing superfluous effete proteins, damaged organelles, and intracellular microbes, promoting antigen presentation [16]. There are two fundamental means to classify autophagy, by the content of its cargo and by the mechanism of cargo delivery. Autophagic cargo can be either selective or nonselective. Nonselective autophagy recycles bulk cytoplasmic contents under starvation conditions to provide energy and molecular building blocks. Selective autophagy specifically targets damaged or superfluous organelles, such as mitochondria (mitophagy), peroxisomes (pexophagy), ER (reticulophagy), and microorganisms (xenophagy) for targeted degradation. There are three methods to deliver autophagic cargo—via macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) [17].

Of these three types, macroautophagy is the most studied. The defining feature of macroautophagy is the formation of a double-membrane sequestering compartment called a phagophore [16]. The origin of the phagophore in mammalian cells is a debated topic with evidence of contribution from multiple membrane compartments, including the ER, mitochondria, the Golgi apparatus, and the plasma membrane [18–21]. In yeast, phagophore nucleation occurs at a perivacuolar region called the PAS (phagophore assembly site) and elongates by the delivery of membrane from various organelles [22, 23]. However, no defined PAS has been found in mammalian cells, although some believe that autocatalytic activated caspases could be one [24, 25]. Furthermore, the mammalian phagophore is elongated by an Ω (omega)-shaped membrane structures derived from phosphatidylinositol 3-phosphate-enriched ER subdomains called omegasomes [26]. The phagophore then further expands and closes off its cargo, fully engulfing the cytoplasmic contents to form a double-membrane vesicle called an autophagosome. The autophagosome is subsequently delivered to the lysosome/vacuole, where the outer membrane fuses with the vacuolar membrane, thereby releasing the inner membrane-bound cargo into the vacuolar lumen [16]. Here, the contents of the autophagosome are degraded and released into the cytosol for reuse.

Microautophagy involves the direct engulfment of cargo by lysosomes (in mammalian cells) or vacuoles (in plant and fungal cells) without the formation of an autophagosome [27]. Microautophagy is induced by starvation and by the mammalian target of rapamycin (mTOR), and has specific roles in maintaining organellar size, membrane composition, and survival under nitrogen restriction. Collectively, these events help prepare cells for a phase of logarithmic growth following starvation-induced growth arrest [28]. Currently, there is controversy over the role of microautophagy as a compensatory machinery for macroautophagy. In addition to selective macroautophagy [29], microautophagy is another self-eating mechanism that is able to degrade nonessential portions of the nucleus in a process known as piecemeal microautophagy of the nucleus [27]. Microautophagy is initiated by an invagination of the

lysosomal membrane mediated by dynamin-related GTPase Vps1p, which affects lateral movement of certain lipids and lipid-modifying proteins in the membrane [30]. Further invagination forms an autophagic tube mediated by two ATG7-dependent ubiquitin-like conjugation systems in an ATP-dependent process [31]. The autophagic tube has a characteristic constriction at the neck of the tube to distinguish it from an ordinary invagination. The lateral sorting mechanism of the autophagic tube causes lipid enrichment and integral protein depletion that causes phase separation to facilitate vesicle formation [32]. Various enzymatic activities further expand the vesicle until V-ATPase activity acidifies the lumen to establish an electrochemical gradient. This activates vacuolar transporter chaperone complex that initiates the scission process of the vesicle [33]. The released vesicle is free to move around inside the lysosomal lumen until various hydrolases degrade the vesicle to release the cargo contents [27].

CMA, the third autophagic mechanism, is a type of self-eating that targets client proteins, which contain the specific KFERQ protein motif within its structure [34]. The KFERQ motif is present in ~30% of cytosolic proteins [35], and includes proteins such as glyceraldehyde 3-phosphate dehydrogenase, E3 ubiquitin ligase ITCH, calcineurin inhibitor RCAN1, neuronal α-synuclein, and tau proteins among others [36–38]. The KFERQ motif is recognized by the cytosolic chaperone protein HSPA8/HSC70 and is delivered to the lysosomal receptor LAMP2A (lysosome-associated membrane protein type 2A) along with its associated co-chaperone protein complex [34]. The binding of HSPA8-substrate complex to LAMP2A causes multimerization and subsequent unfolding of the substrate before it reaches the lysosomal lumen through LAMP2A-enriched translocation complex [36]. Within the lumen, substrate proteins are degraded and recycled. Like macro- and microautophagy, CMA is activated under conditions of metabolic stress to provide alternate sources of energy. In addition, recent studies have delineated CMA's important role in major histocompatibility complex II-mediated antigen processing and presentation, as well as in preventing autoimmune pathologies [34]. We have recently demonstrated that immune cells, including T cells and natural killer (NK) cells, can induce autophagy [39] in a manner known as immune cell-mediated autophagy (iCMA). Our study showed that iCMA is a phenomenon that can be promoted by human peripheral blood lymphocytes, primarily by NK cells. In a series of experiments, we were able to demonstrate that autophagy was increased in human cancer cell lines when co-cultured with primary human lymphocytes [39]. This effect of autophagy was further amplified in the presence of interleukin-2 (IL-2) with lymphocytes. In our study, we were able to demonstrate lymphocyte-induced autophagy in multiple cancer cell lines, including colorectal, pancreatic, kidney, and bladder [39]. Furthermore, we observed similar cell-mediated upregulation of autophagy when using NK cells, macrophages, and T cells, all of which promoted autophagy in an ATG5-dependent manner. While cytokines such as IL-2 and interferon-γ help promote iCMA, we found that the full effects of iCMA occurred when there was cell-to-cell contact of lymphocytes to cancer cells. The effects of iCMA ultimately promote cancer cell survival by allowing these cells to gain resistance to cancer treatment modalities such as radiation [39]. Given the broad cancer survival effects of iCMA, some consideration should be given when applying modern immunotherapies or gene therapies to patients with glioma to limit this survival pathway [40, 41]. Importantly, guidelines have been updated and published for the measure of autophagy [42].

Upstream signaling pathways regulating autophagy are influenced by a multitude of intracellular conditions, which include the nutrient status of the cell, availability of growth factors, and detection of intracellular metabolic stress [43]. TOR (target of rapamycin) acts as an inhibitor of autophagy in both yeast and mammalian cells depending on the nutrient status of the cell [44]. In a nutrient adequate state, extracellular amino acids enter mammalian cells, and via different modalities, activate mTOR complex 1 (mTORC1), which subsequently inhibits autophagy [45, 46]. In yeast, the inhibition of TOR during a nutrient-deprived state, or with rapamycin treatment, leads to the activation of Atg1 kinase activity and increased affinity of Atg1 to bind Atg13 and Atg17, promoting the recruitment of multiple Atg proteins to the PAS and markedly inducing autophagosome formation [47]. In mammals, Unc-51-like kinase 1 and 2 (ULK1 and ULK2), homologs of the yeast Atg1, serve to phosphorylate mammalian Atg13 and FIP200, forming a stable trimeric complex [48, 49]. The formation of this complex occurs regardless of the cell nutritional status; however, under nutrient adequate conditions, mTOR phosphorylates and inactivates ULKs and Atg13, thus inhibiting autophagy [48]. Inhibition of mTOR in the starvation state allows the formation of the ULK–Atg13–FIP200 complex, which then localizes to the phagophore-inducing autophagy [48, 50].

Autophagy mediated by the depletion of growth factors, or due to other forms of intracellular metabolic stress, have been shown to have upstream signaling pathways that also converge on mTORC1 [43]. Insulin and insulin-like growth factors (IGFs) are shown to regulate mTORC through the class I PtdIns3K (class I phosphatidylinositol 3-kinase), generating PIP_3 and activating protein kinase B (PKB) and Akt. PKB/Akt leads to phosphorylation of tuberous sclerosis complex 2 protein (TSC2), preventing the formation of the TSC1/2 complex, which allows activation of mTORC1 through Rheb (a Ras family GTPase) [51–53]. In

the absence of the insulin or IGFs, mTOR is inactivated, which releases the inhibitory effect on autophagy. Similarly, in situations with increased intracellular metabolic stress, reduction in ATP levels activate AMPK (AMP-activated protein kinase), leading to phosphorylation of and activation of the TSC1/2 complex inhibiting mTOR through Rheb [43, 54]. Cellular stress in mammalian cells can also lead to the ER-releasing Ca^{2+} to the cytosol and activating calmodulin-dependent kinase kinase-β, which can further activate AMPK and induce autophagy via the inhibition of mTOR [43].

Antitumor properties of autophagy

Autophagy has been identified to have a dual role in cancer, featuring both in the early anti-oncogenic and late pro-oncogenic periods, and supporting both pro-tumorigenic and anti-tumorigenic properties. Mutations in core autophagy genes do not independently drive tumorigenesis; epigenetic regulation of autophagic genes is abundant in cancer and promote tumor progression [55–57]. Moreover, defective autophagy mechanisms cause oxidative stress, DNA damage, and genome instability, all of which contribute to cancer initiation and progression [58]. For instance, mutations in *Beclin-1/ATG6* gene, an upstream regulator of autophagy and a tumor-suppressor gene [59], have been identified in multiple cancer types, including 50% of breast cancers, in up to 75% of ovarian, 40% of prostate cancers, and many cervical cancers [60, 61]. Decreased autophagic protein expression is observed in many malignant brain tumors (high-grade astrocytic, ependymal neoplasms, and atypical meningiomas), but not in benign meningiomas or in medulloblastoma. For instance, the expression level of Beclin-1 messenger RNA is significantly lower in all glial tumors when compared to all meningiomas ($p < 0.0001$) [62].

The consequence of such a defect in autophagy is the accumulation of protein aggregates, damaged mitochondria, and other organelles, which generate reactive oxygen species (ROS) that induce DNA damage [63]. Of these protein aggregates, accumulation of p62/SQSTM1 is of particular importance as it has been hypothesized to act as a molecular link between autophagy and tumorigenesis. Indeed, the absence of p62/SQSTM1 in p62/SQSTM1$^{-/-}$ mice is protective against developing Ras-induced lung carcinomas compared to wild-type mice [64]. Conversely, the accumulation of p62/SQSTM1 in autophagy-defective cells is associated with increased ROS generation, DNA damage, and genomic instability [63]. In gastric cancer cells, autophagy inhibition by ATG5 and ATG7 knockdown has been shown to increase PD-L1 expression through a p62/SQSTMI-dependent pathway [65]. The knockdown of p62/SQSTM1 in autophagy-defective cells attenuates ROS and the DNA damage response [66], indicating autophagic degradation of p62/SQSTM1 protein aggregates may promote tumorigenesis by permitting autophagic cell death. Furthermore, we have shown that autophagy limits the release of proinflammatory HMGB1 protein to limit necrosis and chronic inflammation associated with tumorigenesis [66].

All Atg5- or Atg7-null mice develop premalignant pancreatic lesions without progression to malignant tumors. In the setting of autophagy inhibition, progression from premalignant tumor to invasive cancer was limited by p53 activity [67]. While Atg5 or Atg7 knockout mice were protected from developing pancreatic ductal adenocarcinoma (PDAC) compared to their wild-type counterparts, Atg7 deletion in an already p53-deficient mice accelerated its transformation to PDAC rather than delaying it, emphasizing the unusual aspects of p53 loss which normally occurs late, not early, in tumor progression[68]. Recent studies have shown that there exists an intricate link between Beclin-1 mutation, p53 activity and spontaneous tumor generation. In Beclin-1 haploinsufficient cells, there is a downregulation of Beclin-1-associated de-ubiquinating enzymes leading to loss of p53 activity and tumor generation.

Pro-tumorigenic properties of autophagy

Paradoxically, autophagy also acts as a tumor survival mechanism by dampening the effects of high metabolic demands in tumor cells, inhibiting apoptotic signals and modulating ROS cytotoxicity [69]. As such, increased basal autophagy activity is found in human pancreatic cancer cell lines. Moreover, exposure to metabolic stress in autophagy-deficient cells impairs survival of tumor cells [66] and leads to tumor regression and extended survival in pancreatic cancer xenografts in murine models [58]. Enhanced autophagic activity is observed in states of nutrient deficiency and hypoxia, including malignancies, and can broadly promote tumor cell survival [70]. Autophagy appears to play an important role in the persistence of tumor cells following chemotherapy and/or radiation therapy and thus contributes to tumor recurrence and progression. Furthermore, human cancer cell lines with activating mutations of H-ras or K-ras display higher than basal levels of autophagy, even in the abundance of nutrients [71]. Inhibition of autophagy in these cells also inhibits cancer cell growth and progression. In summary, the tumor-suppressive or -promoting properties of autophagy are likely dependent on a multitude of factors, including cellular nutrient states, the presence and types of genetic alterations, cell of origin, and as yet undiscovered mechanisms.

Gene therapy and autophagy

Gene therapy as a therapeutic target has frequently focused on microRNAs (miRNAs). Multiple miRNAs are repressed in cancer, due to downregulation of miRNA-processing enzymes, such as Drosha and Dicer [72]. There have been two approaches to miRNA-related gene therapy studied thus far: miRNA replacement and miRNA inhibition through administration of miR target sequences [72]. miR-502, which inhibits the RAB1B autophagy regulatory gene, is downregulated in colorectal cancers. Ectopic expression of miR-502 levels have been associated with decreased proliferation of colon cancer in vitro and in vivo, and mir-502 is a potential therapeutic target for miRNA inhibition via gene therapy [73].

The use of miRNAs for cancer treatment is a rapidly growing field that holds great potential for success, even in the treatment of glioblastomas. Notably, multiple miRNAs have been associated with inducing resistance to temozolomide (TMZ) chemotherapy in GBM, and a number of these miRNA-dependent chemotherapy resistance mechanisms involve a regulation of autophagic pathways. For instance, miR-138 upregulation is seen in glioma-initiating stem cells [74] and promotes resistance to TMZ in a manner that is at least partially dependent on its ability to target Beclin-1 and inhibit autophagic cell death [75]. In yet another example, elevated miR-21 levels in GBM is proportionally correlated with radiation resistance in glioblastoma cell lines. miR-21 positively regulates the PI3K/AKT pathway to inhibit autophagy and subsequently confer cell survival following radiation. Finally, delivery of anti-miR-21 successfully reverses radiation resistance and promotes apoptosis in glioma cells [76]. Although a full review of miRNA regulation of autophagy in GBM is outside the scope of this review, a thorough evaluation of this topic has been performed by Palumbo et al. [77].

microRNA gene therapy holds a promising future as a therapeutic pathway for autophagy. Delivery of miRNAs and antagomirs (inhibitory miRNA sequences) can occur through a variety of systems, including viral vectors, liposomal formulations, and electroporation [78]. Interestingly, miRNA recognitions sequences can be used to enhance the cellular specificity of viral vectors in GBM. For instance, our group recently developed an EGFR-retargeted HSV vector encoding the recognition sequence for miR-124 to ensure activation of the virus only in GBM cells, and dormancy in neurons that express high levels of mir-124 [79]. Restrictive properties of BBB make it a significant challenge in the development of miR-based therapies. A number of strategies have been proposed with various efficacy, safety, and limitations. Most commonly studied methods include convection-enhanced delivery, chemical disruption (i.e., intra-arteral mannitol), and ligand-mediated transcytosis [80]. A study in 2017 by Kim et al. [81] investigated novel delivery methods in a xenograft glioblastoma model, comparing therapeutic efficacy between intratumoral, intrathecal, and intraventricular routes. Using the anti-miR, anti-Let-7, they demonstrate a significant decrease in the expression of anti-Let-7 target genes only when using the intratumoral and intraventricular delivery routes. The study also demonstrated that although the intratumoral delivery method was efficient, there was limited delivery to the entire brain when compared to the intraventricular route. When considered in the context of current glioma therapy of maximal surgical resection followed by radiotherapy and TMZ treatment, the authors make the case that the intraventricular delivery method appears to be a more practical clinical option as surgical resection makes the intratumoral route unfavorable for repetitive administration of miR therapy [81]. While microRNA gene therapy has shown promise as a new frontier in GBM therapy, further research must be done to address possible off-target effects. Transfection of miRNA lowers expression of many genes, and some genes are unexpectedly upregulated as well, which can result in dramatic and sometimes unexpected phenotypic changes [82, 83]. Khan et al. [82] hypothesize that these effects may be due to a loss of function of endogenous miRNAs, as the transfected miRNA must compete with endogenous miRNAs for the RNA-induced silencing complex, relieving repression of target genes of these endogenous miRNAs [84]. In summary, although microRNA gene therapy encoding ATGs can be engineered with high specificity for cancer cells, and shows much promise as a therapeutic modality in GBM, delivery of miRNA therapy and limitation of miRNA off-target effects remain a significant hurdle in the realization of miRNA gene therapy.

Role of autophagy in GBM

GBM is the most common primary human brain cancer. Median survival does not exceed 15 months in most series, despite aggressive surgical resection combined with TMZ administration and radiation therapy [85]. In order for an aggressive cancer to survive and progress, it must develop strategies to survive prolonged periods of stress induced by chemotherapy, radiation therapy, nutrient deprivation, and oxidative stress [86]. Autophagy has emerged as an intriguing mechanism for promoting tumor cell survival and has become an area of active research in GBM over the past decade. Topics of particular interest include the use of autophagy inhibitors in conjunction with TMZ and the role of autophagy in GBM tumor stem cell survival.

Biomarkers of autophagy in GBM

As discussed above, autophagy plays a dual role in cancer: while in most cases it confers resistance to treatment, it may also promote cell death. Initial studies of autophagy in GBM surveyed the expression levels of autophagy-associated genes [87]. A primary candidate is Beclin-1 (ATG6), which is important in the initiation of autophagy [73] and has a decreased expression in a variety of cancers [60, 88, 89], including GBM [62]. In a series of 76 patients with newly diagnosed GBM, high levels of cytosolic Beclin-1 correlated with an improved survival from 10 to 15 months among patients with a Karnofsky performance score >80 [90], suggesting that autophagic mechanisms are intact in patients whose tumors are susceptible to adjuvant therapy in GBM.

TMZ-induced autophagy

TMZ, in conjunction with involved field radiation, is the only chemotherapeutic agent that has shown statistically significant benefit in on overall survival for GBM patients following resection. TMZ is a well-tolerated small molecule with the ability to cross the BBB. Initial research in hematopoietic malignancies showed that TMZ induced DNA crosslinking through the formation of O^6-methylguanine (O^6MeG) and the subsequent induction of apoptosis following irreparable damage [91]. Interestingly, similar experiments in GBM revealed little to no caspase-3-dependent apoptosis in GBM cells [92], suggesting that other cell death mechanisms may contribute to the therapeutic efficacy of TMZ in GBM.

Initial in vitro studies into the mechanism of action of TMZ showed significant cell cycle arrest following treatment [93], but it was not until 2004 that autophagy was implicated [94]. Several lines of evidence emerged to link late autophagic inhibition with TMZ-mediated glioma cell death: (i) Treatment of glioma cells with clinically relevant doses of TMZ significantly increased the number and intensity of LC3 puncta as well as acidic vesicular organelles in the absence of apoptosis; (ii) apoptosis was induced in TMZ-treated cells following inhibition of late autophagy with bafilomycin A1; and (iii) treatment with an early autophagy inhibitor, 3-methyladenine, promoted cell survival instead of cell death. Together, these results demonstrate that late inhibitors, such as chloroquine (CQ), could limit tumor cell survival and promote cell death following the onset of autophagy induction, thus opening the door for combination therapy. Although these findings have since been confirmed in other reports, controversy remains as to the exact nature of the role of autophagy in TMZ-mediated cell death [95]. Notably, induction of autophagy has also been observed in fresh surgical specimens following TMZ treatment. While more autophagy was noted in the samples previously treated with TMZ, it is difficult to conclude if the effect is related to TMZ alone or other subsequent intervention [96].

More recent investigations have attempted to elucidate the mechanisms of TMZ-induced autophagy in glioblastoma cell lines. The initial investigations evaluated the role of TMZ-induced mitochondrial damage in U87 cells [97]. In this work, TMZ was noted to open mitochondrial depolarization transition pores, leading to a decrease in mitochondrial mass and an increase in autophagy secondary to stress. Treatment with electron transport chain inhibitors prevented this effect and led to increased apoptosis. While this work is intriguing, cells were treated with 400 μM of TMZ, levels significantly higher than found clinically.

Research into the mechanism of DNA damage induced by TMZ and the repair mechanisms involved implicate autophagy as an important pathway. In an elegant series of experiments, Knizhnik et al. [98] showed that TMZ-induced autophagy is dependent on the formation of O^6MeG adducts [98]. In addition, the presence of MGMT prevented the induction of autophagy and directed TMZ-treated cells towards apoptosis. This provides strong evidence for a critical role of the mismatch repair (MMR) system in TMZ-induced autophagy. They postulated that autophagy may regulate the cell fate by diverting cells to either senescence or apoptosis. In addition, a role for MMR genes (particularly ATM) during TMZ-induced autophagy has been shown in subsequent studies [87].

Chloroquine

These observations have led to interest in autophagy inhibitors in combination with TMZ to improve clinical efficacy. Currently, the most rigorously studied agents are the quinolone derivatives: hydroxychloroquine (HCQ) and CQ. These compounds were initially used in the treatment of malaria and inhibit autophagy through preventing lysosomal fusion and acidification, but the exact mechanism is controversial [99]. CQ acts in a synergistic fashion with TMZ in U251 and LN229 glioma lines in a GRP78- as well as a PI3K-BECN1-dependent fashion [100]. Others have shown an increase in apoptosis, particularly in p53 wild-type cell lines, when treated with combinations of CQ and TMZ [101, 102]. Mitophagy has also been supported as a mechanism induced by TMZ. Using a coral-derived fluorescent molecule, mito-Keima, directed to the mitochondria has allowed direct assessment of mitophagy [103, 104]. At high TMZ doses, CQ enhances cytotoxicity in both the rat C6 glioma and patient-derived glioma stem cell (GSC) lines [105]. At this time, most evidence indicates that CQ exerts an effect through autophagy-related pathways; however, a

recent study in KRAS-driven tumors suggests that the effect may be the direct result of lysosomal interference [106].

The effect of CQ/HCQ + TMZ has shown mixed results in clinical series. An initial randomized double blind placebo-controlled trial treated a total of 30 patients with 150 mg/day of oral CQ or placebo starting on postoperative day 5 and continued for 1 year [107]. The CQ-treated group experienced a median survival of 24 months compared to 11 months in the placebo group. Unfortunately, this difference was not statistically significant likely due to the small sample size. More recently, a phase I/II study of HCQ with TMZ and concurrent radiation in 75 patients found 600 mg/day of HCQ to be the maximal-tolerated dose (MTD) [108]. Patients receiving higher doses experienced severe thrombocytopenia and neutropenia. The median survival was observed to be 15.6 months, with 25% living beyond 24 months, similar to outcomes in the EORTC (European Organisation for Research and Treatment of Cancer) phase III trial of concurrent TMZ and radiation [1]. The authors note the doses of HCQ tolerated in this study, with hematologic toxicity primarily, are significantly lower than MTDs in other disease processes, and alternative dosing strategies may be necessary to reach therapeutic levels in glioma patients. The studies regarding TMZ-induced autophagy and the effect of autophagy inhibition are summarized in Table 1 [109–111].

Both studies provide useful insight into the future of autophagy inhibition, but may be affected by suboptimal dosing of HCQ. These studies also do account for the status of DNA repair mechanism, such as *MGMT* status, which may play an important role in initiating autophagy as outlined above. Increased dosing, if tolerated, may provide a more profound treatment effect. Treatment with second- and third-generation autophagy inhibitors against ULK1, ATG7, and VPS34 are also novel avenues for exploration and may result in meaningful clinical gains particularly in recurrent disease [112–115]. Targeting noncanonical autophagy pathways, vesicular exocytosis, and the intersection of autophagy and immunology [116] offer relatively unexplored opportunities for progress.

TMZ-induced autophagy plays a critical role in chemoresistance in glioblastoma, particularly in tumors with intact DNA repair mechanisms. Future study will be essential to further define these pathways and devise strategies to improve responsiveness to traditional therapy. More clinical studies are planned to explore therapeutic options, but it is critical for these studies to incorporate molecular statuses of tumors, particularly IDH1, p53, and MGMT methylation status, as these pathways have a critical role in the preclinical studies. CQ/HCQ in combination with known agents such as bevacizumab, irinotecan, carmustine, or cisplatin may ultimately prove beneficial at recurrence, but have not been explored.

Radiation

Radiation therapy is an integral component of the treatment of patients with glioblastoma in combination with TMZ. Radiation induces cell death through apoptosis, and possibly necroptosis [117], via radiation-induced DNA damage in a variety of cancer types [118, 119]. Radiation induces autophagy and thus autophagy inhibitors may have a role in radiosensitization in GSCs [120] as well as established cell lines [121, 122]. Other studies have suggested that radiation induces autophagy as a gateway to apoptosis with little senescence [123]. It has been hypothesized that a dose of radiation insufficient to induce apoptosis may lead to delayed, autophagic cell death [124]. A subsequent investigation in glioma cell lines showed that the induction of autophagy and apoptosis increased with increasing radiation dose. In the same study, knockdown of Atg5 led to a dramatic decrease in both autophagy and apoptosis following radiation, consistent with the known dual role of Atg5 in both processes. Autophagy inhibitors indeed promote cell death in most studies following radiation therapy [125]. Table 2 summarizes the literature surrounding the effects of radiation-induced autophagy [126, 127].

Conclusions

Autophagy is an evolutionarily ancient complex cellular process that subserves multiple biological pathways, including catabolism/metabolism, apoptosis, ferroptosis, cell cycle progression, and cellular senescence. While it has been recognized since the 1960s, its role in cancer has only recently been investigated. In GBM it appears to play a role in chemoresistance and possibly radiation resistance in a subset of patients. Therefore, autophagy inhibitors suggest a promising role in the treatment of patients bearing these tumors, particularly in those with intact DNA repair mechanisms. Tumors with disruptions in MMR, such as MGMT-methylated tumors, IDH mutations, and p53 mutations, may enjoy less benefit from autophagy inhibition strategies.

While our understanding of autophagy has significantly progressed, a number of important questions remain. A large, prospective study that controls for molecular variables is needed to clarify the clinical role of autophagy inhibition in GBM. It is also critical for future investigations to identify factors and pathways that divert cancer cells from enhanced autophagy and promote apoptosis. There is a need to include innate immune effectors such as natural killer cells and myeloid cells in studying GBMs, as they can also induce autophagy and may lead to sustained T cell responses. Understanding and targeting novel molecular regulators of autophagy that limits anoikis, such as MDA-9,

Table 1 Summary of studies evaluating the effects of temozolamide on autophagy and apoptosis in glioblastoma cell lines.

Author/year	Cell lines used	TMZ concentration	TMZ exposure time	Autophagy	Apoptosis	Cell cycle arrest	Study drug(s)	Drug effect
Hirose 2001	U87, LN-Z308	300 μM	3 h	NA	NA	Yes	NA	
Kanzawa 2003	T98G U373- MG	100 μM	72 h	Yes—U373 cells No—T98G cells	No—T98G cells	No—T98G cells	O^6-benzylguanine (BG)	BG induced in autophagy in T98G cells
Kanzawa 2004	U373, U251,U87, GB-1, T98G, A172	100 μM	72 h	U373 cells only	No—U373 cells only	Yes	3-MA, bafilomycin A1	Bafilomycin A1-induced apoptosis
Aoki 2008	U87, U373, T98G	200 μM	72 h	Yes	No—U373 cells	NA	NA	
Natsumeda 2011	14 Patient-derived lines	Clinical dosing	Clinical dosing	Yes	No	NA	NA	
Lin 2012	U87	400 μM	72 h	Yes	Yes	NA	ETC inhibitors	Decreased autophagy Increased apoptosis
Knizhnik 2013	U87, LN229	100 μM	96 h, 120 h	Yes	Following autophagy	NA	3-MA, bafilomycin A1	3-MA increased apoptosis; bafilomycin A1 increased cell cycle arrest
Gratas 2014	U251	50 μM	48 and 72 h	No	Yes	NA	NA	
Shen 2014	U251	100 μM	72 h	Yes	No	Yes	NA	
Zou 2014	U87, U251	100 μM	72 h	Yes	NA	NA	Compound C KU-55933	Compound C decreased autophagy and increased apoptosis
Sesen 2015	U87, LN-18, U251, SF767	100 μM	48 h	Yes	Yes	Yes	Metformin	Metformin increased autophagy
Zhang 2015	SKMG-4, U251, U373, T98G	31.25–2000 μM	120 h	Yes	No	NA	Dihydroartemisinin (DHA)	DHA increased autophagy when combined with TMZ
Bak 2016	C6	10 μM	24 h	Yes	No	NA	Vitamin D	Vitamin D increased autophagy, but no difference in apoptosis compared to TMZ alone

Table 2 Summary of studies evaluating the effects of radiation on autophagy, apoptosis, and cell cycle arrest in glioblastoma cell lines.

Author/year	Cell lines	Radiation dose (Gy)	Autophagy post radiation	Apoptosis post radiation	Cell cycle arrest post radiation	Autophagy inducer	Effect of inducer treatment	Autophagy inhibitor	Effect of inhibitor treatment
Zhuang 2003	Patient-derived lines	2, 4, 6	No	Mild increase	NA	Rapamycin	Decreased viability, increase apoptosis	NA	NA
Yao 2003	A171, T98G	5, 30	Yes	No	A171 only	NA	NA	NA	NA
Lomonaco 2009	Patient-derived lines	5	Yes	NA	NA	NA	NA	Bafilomycin A1 Beclin-1 shRNA ATG5 shRNA	Decreased cell viability and neurosphere formation
Jinno-Oue 2010	NP-2	6	Yes	Yes	Yes	NA	NA	NA	NA
Palumbo 2012	T98G, U373	0.35, 1.2, 2, 3, 5, 7	Yes	No	NA	Rapamycin	Decreased cell viability and clonogenic capbilities	Bec-1/ ATG7 siRNA	Increased cell viability and clonogenic capbilities
Palumbo 2014	T98G, U373	2	NA	NA	NA	Rapamycin	Inhibits cells clonogenic migration capability	ATG7 siRNA	Enhances cell clonogenic migration capability
Choi 2014	U251, T98G	2, 4, 6, 8	NA	NA	NA	TMZ + rapamycin TMZ + PPI103, 17DMAG, LBH589	No change in clonogenic survival fraction Decreased clonogenic survival fraction	NA	NA
Jo 2015	U87, U373, LN229	10	Yes	Yes	Yes	NA	NA	ATG5 knockdown	Decreased apoptosis
Yuan 2015	U251	8	Yes	Yes	NA	NA	NA	3-MA, siATG-5	Increased apoptosis
Talarico 2016	A172, ADF, LI	5, 8, 10	NA	NA	Yes	SI113	Decreased cell viability	Chloroquine	Increased cell viability

appear promising. Development of novel, molecularly targeted treatments focusing on second- and third-generation autophagy inhibitors alone [128] or in combination with radiation therapy [129] and/or chemotherapy may enable a long-lasting impact on patients with this devastating disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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