Cilengitide induces autophagy-mediated cell death in glioma cells

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We studied the effect of the integrin inhibitor cilengitide in glioma cells. Cilengitide induced cell detachment and decreased cell viability, and induction of autophagy followed by cell apoptosis. In addition, cilengitide decreased the cell renewal of glioma stem-like cells (GSCs). Inhibition of autophagy decreased the cytotoxic effect of cilengitide. Pretreatment of glioma cells and GSCs with cilengitide prior to γ-irradiation resulted in a larger increase in autophagy and a more significant decrease in cell survival. We found that cilengitide induced autophagy collectively in glioma cells, xenografts, and GSCs, which contributed to its cytotoxic effects and sensitized these cells to γ-radiation.

Keywords: autophagy, cilengitide, glioma cells, radiation, beclin-1.

Malignant gliomas, including anaplastic astrocytomas and glioblastomas, are the most common primary brain tumors, occurring at a rate of approximately 6.08/100,000 individuals annually within the United States. Current treatment options include surgery, radiation therapy, and chemotherapy. Unfortunately, prognosis remains extremely poor, and the median survival of 12–14 months for patients with glioblastoma has not changed appreciably. Limitations to therapy include the distinctly infiltrative nature of these tumors and their prominent angiogenesis and vasculogenesis.

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Integrins control cell attachment to extracellular matrices (ECMs) and participate in cellular defense against genotoxic assaults. The integrin αvβ3 binds diverse ECM ligands with an exposed arginine-glycine-aspartic acid (“RGD”) sequence. In glioblastomas, αvβ3 and αvβ5 integrins and their ligands are overexpressed, and they regulate the cellular behavior of these tumors and support growth factor–mediated cell survival.

Pharmacological antagonists of the integrin αvβ3 have been used in glioma tumor models. Interference with integrin αvβ3 induces cytotoxic effects in glioma and endothelial cells and prolongs survival in orthotopic glioma models. Currently, αv integrin antagonists including cilengitide (EMD121974), which is a cyclic RGD-containing peptide, are in clinical trials. Cilengitide has also been shown to synergize with radiotherapy to increase efficacy in breast cancer, non–small cell lung carcinoma, and glioblastoma xenograft models. Although cilengitide has been shown to induce cytotoxic effects in glioma cells, the mechanisms underlying its effects and its ability to radiosensitize glioma cells are not completely understood.

Autophagy is a highly conserved process in which cellular organelles and long-lived proteins are sequestered into double-membrane vesicles, autophagosomes, and delivered to the lysosomes to be degraded or recycled. The triggering of autophagy is largely associated with the inhibition of mammalian target of rapamycin complex 1 (mTORC1), which leads to the activation of various autophagy-related proteins (Atgs) and different signaling pathways. Autophagosome formation requires the conjugation of ATG12 to ATG5 and that of phosphatidylethanolamine to light chain (LC)3/ATG8 via ubiquitin-like conjugation systems. Activation of the autophagy process is dependent on the cellular context and on the duration and strength...
of the inducing signals.\textsuperscript{17} Thus, in addition to maintaining cellular homeostasis, autophagy can either be cytoprotective or mediate a type II form of programmed cell death.\textsuperscript{17} Autophagy is induced in response to various anticancer therapies.\textsuperscript{18} Indeed, malignant gliomas undergo autophagy in response to various treatments, such as radiation,\textsuperscript{19,20} temozolomide (TMZ),\textsuperscript{21} arsenic trioxide,\textsuperscript{22} curcumin,\textsuperscript{23} cisplatin,\textsuperscript{24} and cannabinoids.\textsuperscript{25} In addition to autophagy induced by anticancer treatments, detachment of cells from the ECM, which is usually associated with anoikis and leads to apoptotic cell death, can also induce autophagy in some cells.\textsuperscript{26}

In this study, we found that cilengitide decreased cell viability via the induction of autophagy followed by cell apoptosis. Combined cilengitide and \( \gamma \)-radiation treatment induced a larger degree of autophagy and increased cell cytotoxicity.

**Materials and Methods**

**Materials**

Anti–beclin-1 antibodies were obtained from Santa Cruz Biotechnology. Anti-LC3 and active caspase-3 antibodies were obtained from Cell Signaling Technology. Cilengitide was provided by Merck KGaA. Vitronectin was obtained from Millipore and was used at a concentration of 5 \( \mu \text{g/mL} \).

**Cell Transfection**

The glioma cell lines U251 and U87 were maintained as previously described.\textsuperscript{11} Cells were transfected with SureSilencing Beclin1 (Qiagen) or control small-hairpin (sh) RNA plasmids (SA Biosciences) by electroporation using the Nucleofector device program A027 as described\textsuperscript{20} (Amaxa Biosystems).

**Glioma Stem Cell–like Cells and Enrichment of CD133+ Cells**

The generation of the glioma stem-like cells (GSCs) and the enrichment of CD133+ cells and their characterization were recently described.\textsuperscript{20} All GSCs employed in this study exhibited self-renewal and multipotentiality and generated tumors in nude mice. Spheroids were maintained in neurosphere medium and examined for the expression of CD44, Bmi-1, CD133, Musashi-1, Sox2, and nestin.

**Detection of Acidic Vesicular Organelles Using Acridine Orange Staining**

For the detection of vacuoles, cells were stained with acridine orange at a final concentration of 1 \( \mu \text{g/mL} \) for 15 min, removed from the plate with trypsin-EDTA, and collected in phenol red–free growth medium, as previously described.\textsuperscript{20} Green (510–530 nm) and red (>630 nm) fluorescence emission from cells illuminated with a blue (488 nm) excitation light was measured with a FACS[fluorescence-activated cell sorting]Calibur from Becton Dickinson using CellQuest software.

**Cell Imaging**

An Olympus 1x50 fluorescent microscope attached to an Insight SPOT 4 camera was used to capture phase-contrast or fluorescent images at \( \times 60 \) using SPOT software. Fluorescence confocal microscopy was performed using a Nikon Confocal Microscope CI System and Nikon EZC1 2.30 software at \( \times 60 \) magnification. Composite images were prepared using Photoshop CS3 software.

**Water Soluble Tetrazolium-1 Assay**

A water soluble tetrazolium (WST)–1 assay (Roche Diagnostics) was performed according to the manufacturer’s instructions. Briefly, cells transfected with control shRNA or beclin-1 shRNA plasmids (SA Biosciences) were plated onto a 96-well plate at a concentration of 25 000 cells/mL. Cells were then irradiated (5 Gy) and incubated for 24 and 48 hours thereafter. To measure the effects of cilengitide on cell viability, equal numbers of cells (5 \( \times 10^4 \)) were plated onto vitronectin-coated plates and treated with cilengitide (25 \( \mu \text{g/mL} \)) for increasing times (from 0 to 48 h) or treated for 24 hours with increasing concentrations of cilengitide (0 to 25 \( \mu \text{g/mL} \)). The WST-1 assay was used to assess the number of viable cells, and the relative absorbance was measured at 595 nm.

**Preparation of Cell Homogenates and Immunoblot Analysis**

Cell lysates (30 \( \mu \text{g of protein} \)) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as previously described.\textsuperscript{15} Following incubation with the primary and secondary antibodies, the immunoreactive bands were visualized by using the ECL (enhanced luminal-based chemiluminescent) Western blotting detection kit (GE Healthcare/Amersham).

**Neurosphere Formation Assay**

The ability of CD133+ cells to form secondary neurospheres was determined as previously described.\textsuperscript{20} Briefly, disaggregated cells were subjected to the appropriate treatments, and cells were plated onto 24-well plates at a density of 100 cells/well through limiting dilution. The number of neurospheres per well was determined 14 days thereafter for 8 different wells. Spheres that contained more than 20 cells were scored. Results are presented as percentages of maximal neurospheres formed in control untreated cells.

**Colony Survival**

Cells were plated onto 100-mm dishes at different concentrations and were adjusted for plating efficiency to obtain 100 colonies. After 14 days of treatment
(25 μg/mL), the cultures were fixed (40% ethanol and 10% acetic acid) and stained with 0.05% Coomassie brilliant blue, and the number of colonies was determined. Colonies having at least 50 cells were counted.

Measurements of Cell Apoptosis

Cell apoptosis was measured using propidium iodide staining and analysis by flow cytometry as previously described. Briefly, cells were plated onto 6-well plates and treated as indicated for 24 or 48 hours. Detached cells and trypsinized adherent cells were pooled, fixed in 70% ethanol for 1 hour on ice, washed with phosphate buffered saline (PBS), and treated for 15 min with RNase (50 μM) at room temperature. Cells were then stained with propidium iodide (5 μg/mL) and analyzed on a Becton Dickinson cell sorter.

Brain Xenografts

Using a protocol approved by the Institutional Animal Care and Use Committee, we implanted U251 cells expressing light chain (LC)3–green fluorescent protein (GFP) into the brains of nude rats (National Cancer Institute) as previously described. Briefly, cells were plated onto 6-well plates and treated as indicated for 24 or 48 hours. Detached cells and trypsinized adherent cells were pooled, fixed in 70% ethanol for 1 hour on ice, washed with phosphate buffered saline (PBS), and treated for 15 min with RNase (50 μM) at room temperature. Cells were then stained with propidium iodide (5 μg/mL) and analyzed on a Becton Dickinson cell sorter.

Statistical Analysis

The results are presented as means ± standard errors. Data were analyzed by analysis of variance and Student’s t-test using Prism 4 software (GraphPad Software).

Results

Cilengitide Induces Cell Detachment and a Decrease in Viability of Glioma Cells

Cilengitide has been shown to induce cytotoxicity in glioma cells; however, the mechanisms underlying this effect are currently not understood. We therefore used the glioma cell lines U251 and U87 to examine the antitumor effect of cilengitide on glioma cells. Treatment of the cells with cilengitide (25 μg/mL) induced a time-dependent detachment of the cells from the plates (Fig. 1A). Cilengitide effects were observed as soon as 1 hour after treatment; the majority of the cells exhibited a rounded morphology and detached from the plate after 4 hours. To further characterize the effect of cilengitide, we examined its effect on the viability of the cells. We found that the detachment of the cells by cilengitide preceded its cytotoxic effect. Using trypan blue staining (data not shown) and the WST assay (Fig. 1B), we found that cilengitide did not induce cell death up to 24 hours after cilengitide treatment. At that time we observed a decrease in U87 and U251 cell viability that further increased after 48 hours (Fig. 1B) in a dose-dependent manner (Fig. 1C).

To determine whether the decrease in cell viability by cilengitide was due to cell apoptosis, we employed 2 approaches. In the first, we used propidium iodide and determined the sub-G1 cell population using FACS analysis. As shown in Fig. 1D, cilengitide did not induce apoptosis of the U251 cells up to 24 hours, even at high concentrations. In contrast, stimulation of these cells with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resulted in cell apoptosis (data not shown). We also examined the effect of cilengitide on the levels of active caspase-3. For these experiments, U251 cells were treated with cilengitide (25 μg/mL) for 24 hours, and the expression of active caspase-3 was measured using Western blot analysis. Similar to the results obtained with propidium iodide staining and FACS analysis, cilengitide did not induce activation of caspase-3 in the U251 cells (Fig. 1E) or the U87 cells (data not shown) after 24 hours. In contrast, we found that the cytotoxic effects of cilengitide after 48 and 72 hours (data not shown) were associated with the induction of cell apoptosis in both U251 and U87 cells (Fig. 1D) and with activation of caspase-3 (Fig. 1E). Collectively, these results indicate that the initial cytotoxic effects of cilengitide were not mediated by the induction of cell apoptosis, whereas some level of apoptosis was induced at only later time points.

Cilengitide Induces Autophagy in Glioma Cells

Autophagy has been recently reported to be induced in glioma cells by different anticancer therapies. To examine the induction of autophagy by cilengitide, we first characterized the expression of the autophagosome-specific protein LC3 using LC3-GFP in glioma cells. Induction of autophagy is characterized by the localization of LC3 in the autophagosome membranes, which appear as LC3-GFP dots. For these experiments, we employed U251 and U87 cells transfected with LC3-GFP. The cells were treated with cilengitide for 4–24 hours, and the percentage of cells with dotted GFP staining was calculated. As shown in Figure 2A, treatment of the U251 cells with cilengitide induced an increased punctal staining already at 8 hours. After 12 hours, about 42% of the U251 cells and 37% of the U87 cells that were treated with cilengitide...
(25 μg/mL) exhibited punctal staining compared with about 5%–7% of the control cells (Fig. 2B). As previously reported, we found that TMZ induced autophagy in the 2 cell lines examined (Fig. 2B).

We also examined the induction of autophagy in cilengitide-treated cells by the expression of endogenous polyethylene–lipid conjugated LC3 (LC3II), which exhibits faster migration compared with LC3I in Western blot analysis. We found that cilengitide induced an increase in the expression of LC3II that was observed after 12 hours in the U251 cells and after 24 hours in the U87 cells (Fig. 2C). The increased expression persisted up to 72 hours after treatment.

To further determine the effect of cilengitide on the induction of autophagy, we quantified acidic vesicular organelles (AVOs), which include autophagic vacuoles and lysosomes, by acridine orange staining. For a positive control in this experiment, we used 100 μM TMZ. Treatment of the U251 cells with cilengitide or TMZ increased the red fluorescence from 3.3% in control cells to 38.9% and 60.7%, respectively (Fig. 2D). Similar results were obtained with the U87 cells (data not shown). Collectively, these results indicate that cilengitide induces autophagy in glioma cells.

**Inhibition of Autophagy Decreases the Cytotoxic Effect of Cilengitide**

To examine the role of autophagy in the cytotoxic effect of cilengitide in glioma cells, we first used 2 autophagy inhibitors, 3-methyladenine ([3-MA] (a phosphatidylinositol3-phosphate kinase inhibitor) and bafilomycin A1 (a specific vacuolar type H⁺-ATPase inhibitor), which inhibits autophagy at a late stage by inhibiting fusion between the autophagosome and lysosome. Treatment of both U251 and U87 cells with cilengitide (25 μg/mL) decreased the survival of the cells by approximately 40%, whereas the 2 autophagy inhibitors did not exert a significant effect. Combined treatment of cilengitide with 3-MA or bafilomycin A1 significantly abolished the cytotoxic effect of cilengitide (Fig. 3A).

In parallel, we found that both 3-MA and bafilomycin A1 inhibited the autophagy induced by cilengitide, as indicated by a decrease in AVO formation (Fig. 3B).

To further determine the role of autophagy in the effect of cilengitide, we silenced the expression of beclin-1 in the U251 and U87 cells. Transfection of the cells with a beclin-1 shRNA plasmid decreased the expression of this protein following 72 hours of...
Fig. 3. Inhibition of autophagy decreases the cytotoxic effect of cilengitide in glioma cells. U251 and U87 cells were treated with 3-methyladenine (3-MA) (1 mM) or bafilomycin (10 nM) for 30 min followed by cilengitide (25 μg/mL). The cells were then incubated for 24 h and examined for cell viability using the WST-1 assay (A) or for the presence of AVOs using acridine orange staining and FACS analysis (B). U251 and U87 cells were transfected with control or beclin-1 shRNA plasmids using electroporation. The expression of beclin-1 was examined in the cells after 72 h (C). Cells in which the expression of beclin-1 was silenced were treated with cilengitide (25 μg/mL), and the presence of AVOs (D) or the viability of the cells using the WST-1 assay (E) was examined after 24 h. The results are the mean ± SE of 3 different experiments (A, B, D and E) or are representative of 3 different experiments (C). *P < .001.

Fig. 2. Cilengitide induces autophagy in glioma cells. U251 cells were transfected with LC3-GFP, and after 24 h the cells were treated with cilengitide for 8 h (25 μg/mL). The U251 cells were visualized using a confocal microscope (C1, Nikon), and cells with punctated LC3-GFP appearance were observed (A). U87 and U251 cells were transfected with LC3-GFP and treated with either different concentrations of cilengitide for 12 h or with temozolomide (TMZ, 100 μM) for 48 h. The percentage of cells with puncta LC3-GFP of the total cells was determined for control and treated cells (B). The expression of LC3I and LC3II was measured in U251 and U87 cells treated with cilengitide (25 μg/mL) for different time points (C). U251 cells were treated with cilengitide (25 mg/mL) for 24 h and with TMZ (100 ng/mL) for 48 h. The development of AVOs in control and treated cells was determined using acridine orange staining and FACS analysis (D). The results are representative of 3 different experiments (A, C, and D) or are the mean ± SE of 3 different experiments (B). *P < .01, **P < .001.
transfection (Fig. 3C). Silencing of beclin-1 reduced the induction of autophagy (Fig. 3D) and abolished the cytoprotective effect of cilengitide (Fig. 3E), similar to the results obtained with the autophagy inhibitors. In contrast, cilengitide decreased the survival of the U251 or U87 cells transfected with a control shRNA plasmid, similar to its effect in the parental cells (Fig. 3E).

**Treatment with Cilengitide and γ-Radiation Induces Enhanced Cytotoxicity in Glioma Cells**

Recent studies suggest that cilengitide sensitizes gliomas to γ-radiation. Since both γ-radiation and cilengitide induce autophagy in glioma cells, we examined their combined effect on the induction of autophagy and on cell survival. As shown in Figure 4A, both cilengitide and γ-radiation (1 and 2 Gy) induced autophagy in the U251 and U87 cells, as indicated by the increased expression of LC3II. Pretreatment of the cells with cilengitide followed by γ-radiation induced a larger increase in the expression of LC3II in both cell types (Fig. 4A). Similarly, we found that cilengitide decreased cell survival, whereas only a small effect was exerted by γ-radiation. However, treatment with cilengitide followed by γ-radiation (2 Gy) induced a larger decrease in cell survival than that obtained by treatment with either γ-radiation or cilengitide alone (Fig. 4B). Similar results were obtained using a clonogenic assay: the surviving fraction of cells treated with cilengitide and γ-radiation was significantly decreased compared with results for each treatment alone (Fig. 4C).

**Cilengitide Inhibits Self-renewal of Glioma Stem Cell–Like Cells**

Recently, a small subpopulation of CD133+ GSCs has been identified in specimens of glioblastomas, which has been implicated in enhanced chemo- and radioresistance and in tumor recurrence following these treatments. To examine whether cilengitide also exerts cytotoxic effects in this cell subpopulation, we first examined the effect of this treatment on the secondary neurosphere formation of these cells. Cilengitide at both 10 and 25 μg/mL decreased the self-renewal of both the HF2414 (Fig. 5A) and the HF2354 (Fig. 5B) GSCs that were examined, and the ability of the treated cells to form secondary neurospheres was significantly decreased. The addition of cilengitide to the GSC neurospheres resulted in the dissemination of the large spheroids to smaller clusters of 5–10 cells following 24–48 hours of treatment (Fig. 5C). Similar to the effect of cilengitide in glioma cells, it also induced autophagy in the GSCs after 24 hours (Fig. 5D).

We examined the effect of cilengitide on the response of the GSCs to γ-radiation and found that both cilengitide and γ-radiation induced autophagy in the GSCs, as indicated by the increased expression of LC3II. Pretreatment of the cells with cilengitide followed by γ-radiation induced a larger increase in the expression of LC3II in both GSC cells (Fig. 5E). In addition, we found that radiation alone had a marginal effect on the cell renewal of the GSCs (Fig. 5E). However, pretreatment of the cells with cilengitide (25 μg/mL) increased the response of GSCs to radiation, similar to the results obtained in the glioma cell lines, albeit to a smaller degree (Fig. 5E).

**Cilengitide Induces Autophagy in U251-Derived Xenografts**

We further examined the effect of cilengitide on the induction of autophagy in U251-derived xenografts. U251 cells stably expressing LC3-GFP were used to generate glioma xenografts in nude rats as previously described. At 21 days after implantation, the rats received 1 dose of cilengitide (4 mg/kg) and then were
killed 48 hours later. The induction of autophagy in frozen sections of the U251 xenografts was examined under a fluorescent microscope in frozen sections of the U251 xenografts. The control sections exhibited scattered fluorescence, whereas more punctate fluorescence was observed in the cilengitide-treated xenografts (Fig.6A). Similar results were obtained using confocal microscopy in sections that were stained with propidium iodide (Fig.6B).

Discussion

In this study, we examined the mechanisms of cilengitide-induced cytotoxicity in glioma cells. Cilengitide is an angiogenesis inhibitor that targets the integrins \( \alpha v \beta 3 \) and \( \alpha v \beta 5 \), which bind ECM proteins such as vitronectin and fibronectin. 8,9 In addition, recent studies reported that cilengitide exerts direct cytotoxic effects on glioma cells via an as yet unknown mechanism 11–13 and exhibits antitumor effects against gliomas in preclinical and clinical studies. 32,33 Our results indicate that cilengitide treatment induced cell detachment in glioma cells plated on vitronectin in a dose-dependent manner and decreased cell viability with increasing doses. These early cytotoxic effects of cilengitide were not associated with the induction of cell apoptosis, as indicated by lack of sub-G1 cell population and active caspase-3 expression. In contrast, we found that treatment of glioma cells with cilengitide induced autophagy in these cells, as demonstrated by puncta LC3-GFP appearance, the increased expression of LC3II, and increased acidic vacuole formation. Some level of cell apoptosis was observed in the cilengitide-treated cells only after 48 hours of treatment, following the induction of autophagy.

Recent studies have shown that various cells are dependent on integrin-mediated adhesion to specific ECMs for their growth and survival and that detachment induces an apoptotic cell death recognized as anoikis. 34 In addition to apoptosis, detachment has also been associated with the induction of autophagy, which has been implicated in the protection from anoikis in different cellular systems. 35,36 Indeed, a recent report by Fung et al. reported increased survival of epithelial cells after anoikis of epithelial acini through autophagy. 35 We
found that inhibition of both early and late stages of autophagy increased glioma cell survival after cilengitide treatment, suggesting that autophagy contributed to the cytotoxic effect of cilengitide. Autophagy has dual functionality, with a role in cell death as well as a cytoprotective role during stress conditions, such as nutrient starvation and growth factor depletion.37,38 Our results indicate that cilengitide-induced ECM detachment activates autophagy, which leads to cell death, as opposed to the protective effect that autophagy serves in the detachment of epithelial cells. These differences may be due to the different cellular systems that were employed—nontumorigenic epithelial cells versus tumor cells—and suggest that the role of detachment-induced autophagy may be dependent on cell type and context.

Cilengitide has been shown to increase the efficacy of radiation therapy in various cancer cells.5,11 γ-Radiation has been reported to induce autophagy in glioma cells and in other cancer cell types.19,20,39 Interestingly, in most of these cells, the induction of autophagy by γ-radiation contributes to the radioresistance of the cancer cells, and inhibition of this process radiosensitizes cancer cells.19,20,39 Thus, autophagy plays an opposite role in the cytotoxicity induced by cilengitide and γ-radiation in glioma cells. Because γ-radiation also induces autophagy in glioma cells, we examined the effect of combined treatment of cilengitide and γ-radiation on the autophagy and viability of glioma cells. We found that pretreatment of the cells with cilengitide increased the level of autophagy induced by γ-irradiation and significantly decreased the viability of the cells. Our results differ from those of Maurer et al.,12 and this difference may be attributed to the glioma cell lines employed and the timing of cilengitide and γ-radiation treatments.

It has been suggested that the duration and degree of stimulation that induces autophagy may determine whether this process is involved in the promotion of cell survival or cell death.40,41 Our results suggest that the excessive level of autophagy induced by the combined treatment of cilengitide and γ-radiation further contributes to the enhanced cytotoxic effect in glioma cells.

Similar to its effect on glioma cells, cilengitide also induced autophagy and decreased the self-renewal of GSCs. The GSCs are a small subpopulation of stem-like cells in specimens of glioblastoma. These cells exhibit various characteristics of neural stem cells,30,31 including the ability to generate clonal neurospheres, the expression of stem cell markers such as nestin and CD133, and the ability to differentiate to the different neural lineages. GSCs can generate tumors with characteristics similar to those of the tumors of origin when injected into immunodeficient mice.30,42 GSCs have been reported to exhibit resistance to radiotherapy and chemotherapy and are responsible for tumor regrowth.30,31 Our results indicate that cilengitide can affect the self-renewal of these cells and also sensitize them to the cytotoxic effect of γ-radiation. Thus, our data suggest that cilengitide induces autophagy in both glioma cells and GSCs and can exert cytotoxic effects in both cell types.

We found that in addition to its effect in cultured cells, cilengitide also induced autophagy in U251-derived xenografts, suggesting that induction of autophagy also occurs in vivo and may play an important role in the therapeutic potential of cilengitide.

In summary, we showed that the cytotoxic effect of cilengitide on glioma cells and GSCs is associated with the induction of autophagy and that cilengitide sensitizes both cell types to γ-radiation, probably by inducing a stronger induction of autophagy, which may lead to overactivation of this pathway and consequently to increased cell death.

Conflict of interest statement. None declared.

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References