


Autophagy inhibition synergizes with calcium mobilization to achieve efficient therapy of malignant gliomas

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Autophagy plays a critical role in tumorigenesis, but how autophagy contributes to cancer cells' responses to chemotherapeutics remains controversial. To investigate the roles of autophagy in malignant gliomas, we used CRISPR/CAS9 to knock out the *ATG5* gene, which is essential for autophagosome formation, in tumor cells derived from patients with glioblastoma. While *ATG5* disruption inhibited autophagy, it did not change the phenotypes of glioma cells and did not alter their sensitivity to temozolomide, an agent used for glioblastoma patient therapy. Screening of an anti-cancer drug library identified compounds that showed greater efficacy to *ATG5*-knockout glioma cells compared to control. While several selected compounds, including nigericin and salinomycin, remarkably induced autophagy, potent autophagy inducers by mTOR inhibition did not exhibit the *ATG5*-dependent cytoprotective effects. Nigericin in combination with *ATG5* deficiency synergistically suppressed spheroid formation by glioma cells in a manner mitigated by Ca^{2+} chelation or CaMKK inhibition, indicating that, in combination with autophagy inhibition, calcium-mobilizing compounds contribute to efficient anticancer therapeutics. *ATG5*-knockout cells treated with nigericin showed increased mitochondria-derived reactive oxygen species and apoptosis compared to controls, indicating that autophagy protects glioma cells from mitochondrial reactive oxygen species-mediated damage. Finally, using a patient-derived xenograft model, we demonstrated that chloroquine, a pharmacological autophagy inhibitor, dramatically enhanced the efficacy of compounds selected in this study. Our findings propose a novel therapeutic strategy in which calcium-mobilizing compounds are combined with autophagy inhibitors to treat patients with glioblastoma.

KEYWORDS

autophagy, calcium signaling, drug screening, glioblastoma, mitochondria

Abbreviations: AMPK, AMP-activated protein kinase; mTORC1, mechanistic target of rapamycin complex 1; CaMKK, calcium-calmodulin-dependent protein kinase kinase; GBM, glioblastoma; CQ, chloroquine; TMZ, temozolomide; ROS, reactive oxygen species.

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1 | INTRODUCTION

Autophagy is an intracellular catabolic mechanism that maintains cellular homeostasis by digesting damaged proteins and organelles which could interfere with normal cellular processes. This lysosome-mediated degradation of damaged cellular components also generates energy and macromolecule building blocks to support healthy cell growth. Autophagy is initiated by energy exhaustion, such as occurs when ATP or nutrients are limited, and so is an important energy source when nutrients are depleted. Under such conditions, upregulation of the AMP/ATP ratio activates AMPK, which, in turn, inhibits mTORC1, triggering autophagy. Autophagy is also controlled by changes in intracellular Ca^{2+} concentration. The Ca^{2+} -calmodulin complex activates calcium/calmodulin-dependent protein kinase kinase (CaMKK1/2), which activates AMPK and triggers autophagy.^{1,2} Lysosomal Ca^{2+} signaling can activate autophagy through calcineurin.³ Thus, autophagy is controlled by multiple signals generated by dynamic changes in nutrient levels and/or calcium mobilization.

Glioblastoma (GBM) is the most common high-grade malignant glioma in humans. GBM is categorized as a WHO grade IV astrocytoma, a very aggressive, invasive and destructive brain tumor.⁴ Alterations in several signaling pathways are associated with gliomagenesis, including the receptor tyrosine kinase (RTK)/RAS/phosphatidylinositol 3-phosphate (PI3K) pathway and the p53 and retinoblastoma tumor suppressor pathways, in combination with epigenetic modifiers.⁵ Because autophagy is reportedly required for gliomagenesis,⁶ chloroquine (CQ), an autophagy inhibitor used as an antimalarial agent, has been tested as a treatment for high-grade brain tumors. In *in vitro* and preclinical studies, CQ enhanced the cytotoxicity of temozolomide (TMZ), an alkylating agent clinically used to treat GBM, by inhibiting mitochondrial autophagy in glioma cells.⁷ This study also showed that knockdown of the autophagy-related gene *BECN1* or CQ treatment enhanced TMZ-induced cell death through a reactive oxygen species (ROS)-mediated mechanism.⁷ However, a different study reported that knockdown of *BECN1* or the autophagy-related gene *ATG7* prevented the death of GBM cell lines subjected to combined radiation/TMZ treatment.⁸ Although there have been several clinical trials of combined TMZ plus CQ therapy for patients with cancer, including patients with GBM, it is not clear whether this approach is effective. Thus, the effects of autophagy and its inhibitors or inducers on cancer treatment are complicated.

In this study, we successfully used CRISPR/CAS9 to disrupt the *ATG5* gene and so disable autophagy in glioma cell lines generated from patients with GBM. Unexpectedly, *ATG5* deficiency had no effect on the phenotypes of these glioma cells or on their sensitivity to TMZ *in vitro* or *in vivo*. We also conducted a chemical compound screening that revealed that *ATG5* deficiency can synergize with the activation of Ca^{2+} signaling to induce tumor cell death. Finally, we have demonstrated the clinical relevance of our findings by combining nigericin or salinomycin with the autophagy inhibitor CQ to suppress tumor growth *in vivo* by a patient-derived xenograft mouse

model. Our findings may lead to novel therapeutics for patients with GBM.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

Human glioma cell lines that were derived from 2 patients with GBM and termed TGS01 and TGS04 were established as described previously.⁹ An additional 2 human glioma cell lines (KGS01 and KGS03) that were derived from 2 patients with GBM were used in some experiments. Use of these human materials and protocols was approved by the Ethics Committees of Kanazawa University and the University of Tokyo. Cells were cultured as nonadherent spheroids in serum-free NSPC medium containing DMEM/F12 (Wako, Osaka, Japan), B27, GlutaMAX, penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), hEGF (10 ng/mL, Sigma-Aldrich, St. Louis, MO, USA), and hFGF (10 ng/mL, Wako).

For sphere formation assays, single-cell suspensions were prepared using Accutase (STEMCELL Technologies, Vancouver, BC, Canada). Suspensions were filtered through a 40- μm cell strainer (BD Biosciences, San Jose, CA, USA), and cells were cultured for 14 days in NSPC medium containing 1% methylcellulose (Wako), with or without drugs (see below). IC50 values were calculated using Prism 6 software.

2.2 | CRISPR/CAS9-mediated *ATG5* knockout

The target sequences of gRNA (*sgATG5_4*) were selected from a genome-wide single-guide RNA library.¹⁰ The forward and reverse oligonucleotides, including the 20-bp target sequence and a *BbsI* sticky end, were synthesized, annealed, and inserted into the pX330 plasmid digested with *BbsI* to create the pX330-*sgATG5* plasmid. TGS01 or TGS04 cells were transfected with 2 μg of the pX330-*sgATG5* plasmid using the Amaxa Mouse Neural Stem Nucleofector Kit (Lonza, Basel, Switzerland). Gene disruption in clones derived from single cells was confirmed by western blotting (see below).

2.3 | Tumor xenografts

ATG5-WT or *ATG5*-KO cells were mixed with NSPC medium and Matrigel matrix (Corning, Cambridge, MA, USA) (1:1 ratio) at a concentration of 1×10^4 cells/ μL . Cells (1×10^6 cells/100 μL) were subcutaneously transplanted into each of the 2 flanks of anesthetized female Balb/c nu/nu mice. Transplanted mice were randomly distributed into groups (4 mice/group) for drug treatments: control (DMSO, every day); TMZ (Sigma-Aldrich) (0.1 mg/kg/d, every day); nigericin (Sigma-Aldrich) (1 mg/kg/d, every day); or salinomycin (Cayman Chemical, Michigan, USA) (5 mg/kg/d, every 2 days). Drugs dissolved in DMSO were mixed with corn oil and intraperitoneally (ip) injected 1 day after transplantation. CQ dissolved in 20% DMSO/PBS(-) were injected (ip 25 mg/kg/d, every day). All drugs were

injected into mice for 2 weeks starting on day 1 after transplantation. The widths (W) and lengths (L) of tumors developing in recipients were measured using calipers. Tumor volumes were calculated using the formula: volume (V) = $(W^2 \times L)/2$. For intracranial glioma cell injections, cells (1×10^5 /mouse) were transplanted into anesthetized 4-week-old female Balb/c nu/nu mice as described previously.¹¹ All animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University and performed following the university's guidelines for the care and use of laboratory animals.

2.4 | Western blotting

PBS(-)-washed cells were lysed in a RIPA buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 2 mmol/L EDTA) containing 0.1% PMSF protease inhibitor. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were loaded onto 10% or 15% SDS-PAGE gels, separated by SDS-PAGE, and transferred to 0.45-mm PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skim milk/TBST, incubated with primary antibodies in Can Get Signal Solution (TOYOBO, Osaka, Japan) overnight at 4°C, and then incubated with HRP-conjugated secondary antibodies before detection with ECL Prime (GE Healthcare, Piscataway, NJ, USA). Primary antibodies recognizing the following proteins were used: pAMPK α (Thr-172) (catalog no. 2535), AMPK α (catalog no. 2532) (all from Cell Signaling Technologies, Danvers, MA, USA; 1:1000), LC3 (NanoTools, Teningen, Germany; clone 5F10, 0231; 1:200), SQSTM1 (p62) (Abnova, Taipei, Taiwan, H00008878-M01), β -actin (A5441; 1:2000), α -tubulin (T6199; 1:2000) (Sigma-Aldrich), ATG5 (Novusbio, Littleton, CO, USA, NB110-53818; 1:500), NESTIN (Santa Cruz Biotechnology, CA, USA, sc-377380; 1:1000) and OLIG2 (IBL, Gunma, Japan, 18953; 1:1000).

2.5 | Autophagy flux

The pMRX-IP-GFP-LC3-RFP-LC3 Δ G probe, which was kindly provided by Dr Noboru Mizushima (University of Tokyo),¹² was retrovirally transduced into TGS04 WT or TGS04 ATG5-KO cells. For preparation of retrovirus, 293gp cells were transfected with pMRX-IP-GFP-LC3-RFP-LC3 Δ G and pVSV-G. Retrovirus-containing supernatants were concentrated by centrifugation at 6000 g for 16 hours. Transduced cells were treated with drugs as appropriate and dissociated with Accutase as above before flow cytometric analysis to detect GFP.

2.6 | Cell viability

Cell viability was assessed using the WST-8 Cell Counting Kit (Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Cells were dissociated using Accutase and seeded into 96-well plates (10 000 cells/well) or 384-well plates (2000 cells/well). After

48-hour culture, cells were incubated with WST-8 Reagent for 3 hours followed by measurement of absorbance at 450 nm using an Infinite Pro 200 Reader (Tecan).

2.7 | Drug screening

Libraries used for drug screening were the SCADS Inhibitor Kit-1, 2, 3 and 4 libraries (Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Innovative Areas, Scientific Support Programs for Cancer Research, from The Ministry of Education, Culture, Sports, Science and Technology, Japan). TGS04 WT and ATG5-KO cells were seeded into 384-well plates (2000 cells/well) and cultured with or without library compounds diluted to 1/100, 1/400 and 1/2000. After 48 hours, cell viability was measured as above. The "Index of drug sensitivity of ATG5-KO glioma cells" was calculated as the ratio of value 2/value 1 at each dose of a compound, where value 1 was the drug efficacy in ATG5-KO cells (eg 0.2 means 80% reduction) and value 2 was the drug efficacy in WT cells (eg 0.8 means 20% reduction), compared with DMSO treatment. An index >1.0 meant that ATG5-KO cells were more sensitive than WT cells to the drug. An index <1.0 meant that ATG5 deficiency induced drug resistance.

2.8 | Intracellular Ca²⁺

Cells were incubated with 2.5 mmol/L Fluo-4 Direct calcium reagent (Fluo-4 Direct Calcium Assay Kit, Thermo Fisher Scientific) for 30 minutes and washed twice with 5% FBS/PBS(-) buffer. Washed cells were treated with or without drugs for 15 minutes in assay buffer, followed by flow cytometric analysis.

2.9 | Mitochondrial reactive oxygen species

Cells were cultured with or without drugs for 6 hours, washed with PBS(-), and incubated for 15 minutes with 2.5 μ mol/L MitoSox (Thermo Fisher Scientific) in 5% FBS/PBS(-) buffer. Cells were washed twice with PBS(-) before flow cytometric analysis.

2.10 | Quantitative RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was reverse-transcribed to cDNA using SuperScript Reverse Transcriptase (Thermo Fisher Scientific). Real-time quantitative PCR was performed with Mx3000P (Stratagene, CA, USA). The following cycle parameters were used: denaturation at 95°C for 10 seconds, annealing for 30 seconds at 60°C, and elongation for 30 seconds at 72°C. Sequences of sense and antisense primers used were as follows^{13,14}: HMOX1 sense, 5'-CCAGCAACAAAGTGCAAGATTC-3'; HMOX1 antisense, 5'-TCACATGGCATAAAGCCCTACAG-3'; NQO1 sense, 5'-GAAGAGCACTGATC GACTG GC-3'; NQO1 antisense, 5'-GGATACTGAAAGTTCGCAGGG-3'; actin sense, 5'-AGAGCTACGAGCTGCCTGAC-3'; actin antisense, 5'-AGCACTGTGTTGGCGTACAG-3'.

2.11 | Cell cycle

Cells were cultured with or without drugs for 6 hours, incubated with 10 $\mu\text{mol/L}$ BrdU for 30 minutes, washed with PBS(-), vortexed in 70% cold ethanol, and incubated at -20°C overnight. The next day, cells were stained with BrdU-FITC (BD Biosciences, San Jose, CA, USA) for 1 hour and 7AAD for 20 minutes before flow cytometric analysis.

2.12 | Apoptosis

Cells treated with drugs for 6 hours were dissociated with Accutase, washed with 3% FBS/PBS(-), and incubated for 15 minutes with FITC-Annexin V and 7AAD in Annexin V Binding Buffer (BD Biosciences, San Jose, CA, USA). Apoptotic cells were measured by flow cytometry.

2.13 | Immunohistochemistry

Tumors derived from xenografted patient-derived GBM cells were fixed with 4% paraformaldehyde at 4°C overnight and embedded in paraffin. Sections were stained with H&E (Wako). For immunostaining, sections were treated with target retrieval solution (Dako) and stained with antibodies recognizing NESTIN (1:200) or OLIG2 (1:250), followed by visualization using HRP-conjugated secondary antibodies (GE Healthcare) and the DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). Immunostained sections were counterstained with hematoxylin and viewed using an Axio ImagerA1 microscope (Carl Zeiss).

2.14 | Statistical analyses

Student's *t* test was used to compare 2 groups. One-way analysis of variance followed by Bonferroni's post-hoc test was used to compare more than 2 groups. Differences in survival rate were analyzed using the log-rank test. Significance calculations were performed using Prism 6 software: **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

3 | RESULTS

3.1 | Autophagy inhibition due to ATG5 gene disruption does not affect the proliferation, survival or differentiation of glioma cells in vitro or in vivo

To investigate the roles of autophagy in the survival, proliferation and differentiation of glioma cells, we used CRISPR/CAS9 to disrupt the ATG5 gene, which encodes a molecule essential for autophagosome formation, in glioma cell lines (TGS01 and TGS04) derived from 2 patients with GBM.⁹ Using spheroid cultures, we successfully obtained several single-cell-derived ATG5-KO clones from each patient cell line.

Western blotting of all ATG5-KO clones confirmed that ATG5 protein had disappeared and that the LC3-I/LC3-II ratio had dramatically increased, as expected (Figure 1A and Supplementary

Figure S1a). Control WT glioma cells treated with the V-ATPase inhibitor bafilomycin A1 accumulated LC3-II due to inhibition of autophagy flux, whereas LC3-II accumulation was absent in ATG5-KO cells. The p62 protein, an autophagic target, was markedly upregulated in the deficient cells. To reinforce these results, we next utilized a novel fluorescent probe, GFP-LC3-RFP-LC3 ΔG , which is the most accurate means developed to date of precisely detecting autophagic flux.¹² WT and ATG5-KO cells were incubated with the potent autophagy activator Torin1, which inhibits mTOR kinase activity. Use of an autophagic flux probe (GFP-LC3-RFP-LC3 ΔG) showed that GFP-LC3 digestion was enhanced in Torin1-treated WT cells but not in deficient cells (Figure 1B). Furthermore, ATG5-KO cells showed normal proliferation (Figure 1C and Supplementary Figure S1b) and differentiation, judging by their expression of CD133 (Figure 1D), NESTIN and OLIG2 (Figure 1E). These data established that our ATG5-KO clones were truly deficient in ATG5-dependent autophagy but that this process was dispensable for glioma cell proliferation and differentiation.

Nutrient levels in cell cultures can influence autophagy. To investigate ATG5 deficiency in vivo, we inoculated WT or ATG5-KO glioma cells into the basal ganglia of brains of immunosuppressed mice. Consistent with our in vitro observations, ATG5 deficiency did not prolong the survival of recipient mice (Figure 2A). Histological analysis of recipient brains confirmed no differences in glioma cell growth or differentiation status (Figure 2B), and the volumes of tumors derived from ATG5-KO cells were comparable to controls (Figures 2C and Supplementary Figure S1c). Thus, autophagy is dispensable for the growth and differentiation of glioma cells in vivo.

3.2 | Autophagy does not affect the therapeutic efficacy of temozolomide

Although it was reported that TMZ induced autophagy in glioma cells, and pharmacological inhibition of autophagy enhanced the sensitivity of glioma cells to TMZ, the effectiveness of this approach in clinical trials is controversial.¹⁵⁻¹⁹ We subjected WT and ATG5-KO cells to sphere formation assays in the presence of TMZ but unexpectedly found no differences (Figure 3A). When we inhibited autophagy in TMZ-treated control cells by adding CQ, only 10 $\mu\text{mol/L}$ CQ suppressed spheroid formation, and lower CQ concentrations did not show any additive or synergistic effects with TMZ (Figure 3B). In mice xenografted with WT or ATG5-KO cells, TMZ suppressed tumor growth to the same extent in both sets of recipients (Figure 3C,D). Thus, a lack of autophagy does not affect TMZ efficacy in vivo.

3.3 | Identification of compounds synergizing with autophagy inhibition

To identify other agents that might synergize with autophagy inhibition to reduce GBM cell growth, we subjected WT and ATG5-KO cells to in vitro drug screening using the SCADS Inhibitor Kit Library of 357 small molecule anticancer compounds. We first evaluated each

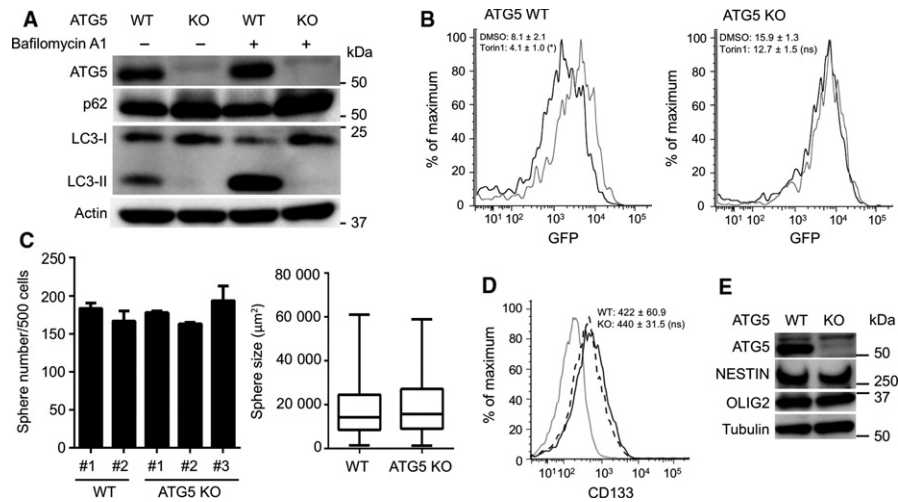


FIGURE 1 ATG5 deficiency does not affect the proliferation or differentiation of patient-derived glioma cells in vitro. **A**, Western blotting to detect the indicated proteins in TGS04 WT and ATG5-KO cells treated with or without 0.1 $\mu\text{mol/L}$ bafilomycin A1 for 3 h. Actin, loading control. Data are representative of 3 independent experiments. **B**, Autophagic flux in TGS04 WT and ATG5-KO cells that were retrovirally transduced with GFP-LC3-RFP-LC3 ΔG probe and treated with 0.1 $\mu\text{mol/L}$ Torin1 (black solid line) or DMSO (gray line) for 16 h. Fluorescence levels of GFP and RFP were measured by flow cytometry. Data are the mean \pm SD of the GFP/RFP peak intensity ratio and are representative of 3 independent experiments. **C**, Quantitation of the number (left) and size (right) of spheres formed by individual TGS04 WT and ATG5-KO clones. Data are the mean \pm SD ($n = 3$). **D**, Flow cytometric analysis of CD133 expression in TGS04 WT (black solid line) and ATG5-KO (black dashed line) cells. Gray line, unstained control. Data are representative of 3 independent experiments. **E**, Western blotting to detect the indicated stem cell markers in TGS04 WT and ATG5-KO cells. Tubulin, loading control. Data are representative of 3 independent experiments

compound's ability to reduce the viability of the WT and ATG5-KO cells and then calculated the ratio of the inhibitory effect on ATG5-KO cells compared to its effect on control cells (Figure 4A,B). Although rapamycin, an mTORC1 inhibitor, is a potent autophagy inducer, it did not affect the proliferation/survival of ATG5-KO cells. Genotoxic agents, such as doxorubicin and cisplatin, were similarly ineffective. These screening data were bolstered by spheroid formation assays, confirming that mTOR inhibitors and genotoxic agents do not synergize with autophagy inhibition to kill GBM cells in this setting (Figure 4C).

Our screening revealed several compounds that were highly effective in inhibiting the growth of ATG5-KO cells compared to controls, including nigericin and valinomycin (Figure 4A,D). To examine the effects of these compounds on tumor initiation capacity, we performed sphere formation assays. The strong inhibitory effect of nigericin on sphere formation of ATG5-KO cells was found at much lower concentration (0.05 $\mu\text{mol/L}$), compared to that on cell viability, presumably because sphere formation requires an extended period of cell culture (14 days vs 48 hours). Our previous work²⁰ had determined that these compounds cause mitochondrial damage, resulting in an energy imbalance and reduction in sphere formation. Thus, impairing the mitochondrial metabolism of cells lacking autophagy may seal their fate. In the same study, we identified 5 compounds, including nigericin and valinomycin, able to preferentially inhibit mitochondrial activity, dramatically reduce ATP levels, and block glioma sphere formation. When we treated WT and ATG5-KO cells with other previously identified compounds (rottlerin, A23187 or auranofin), we found that these drugs showed greater efficacy in

suppressing the growth of ATG5-KO cells compared to WT cells (Figure 4D). In addition, a previous chemical screening performed by Gupta *et al*²¹ revealed that salinomycin selectively killed breast cancer stem-like cells, as well as nigericin. Moreover, as other previous studies showed that salinomycin increased mitochondrial membrane potential ($\Delta\Psi$), associated with a decrease in cellular ATP levels,^{22,23} we assumed that salinomycin has a similar effect to nigericin. As expected, we found that salinomycin could, indeed, effectively reduce sphere formation by ATG5-KO cells (Figure 4D). While several publications have reported that salinomycin suppresses autophagy,^{24,25} others have claimed that it induces autophagy.^{22,25,26} This controversy may be due to technical differences in the methods used to detect autophagy. To resolve this question, we used the GFP-LC3-RFP-LC3 ΔG probe to analyze the effects of compounds on autophagy flux. Our data definitively show that 5 compounds (nigericin, valinomycin, rottlerin, A23187 and salinomycin) induce autophagy (Supplementary Figure S2a,b) and greater efficacy against glioma cells when combined with autophagy inhibition.

3.4 | Autophagy protects glioma cells against calcium mobilization

Because A23187 is a potent calcium ionophore, and nigericin elevates cytoplasmic Ca^{2+} ,²⁷ we hypothesized that Ca^{2+} mobilization was involved in the autophagy-dependent survival/proliferation of glioma cells. Nigericin treatment of WT glioma cells increased intracellular Ca^{2+} levels (Figure 5A), as did treatment with valinomycin, rottlerin, A23187, auranofin or salinomycin (Supplementary

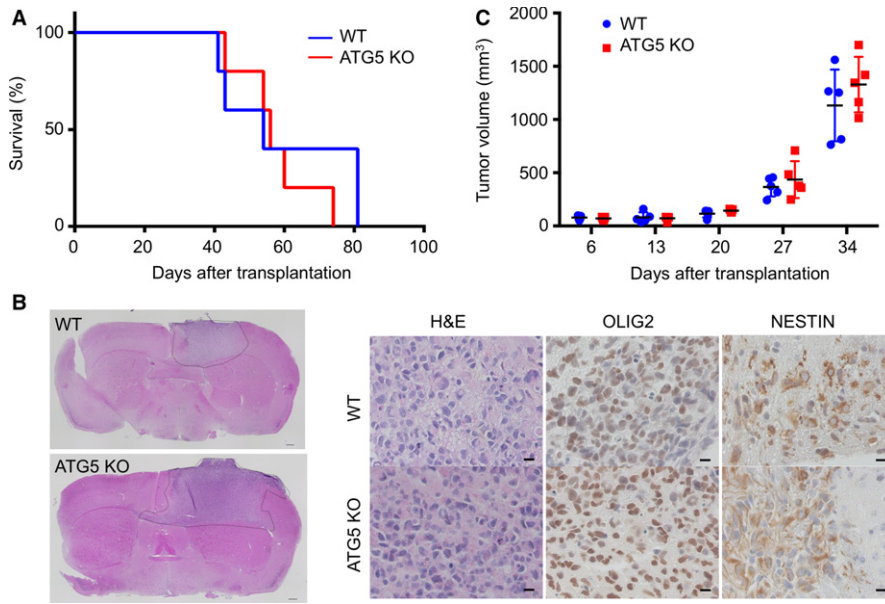


FIGURE 2 ATG5 deficiency does not affect the proliferation or differentiation of patient-derived glioma cells in vivo. A, Kaplan-Meier analysis of survival of recipient mice inoculated in the brain with 1×10^5 TGS04 WT or ATG5-KO cells ($n = 5$ mice/group). B, Left: Gross view of brains isolated from the mice transplanted with TGS04 WT or ATG5-KO cells. Tumor regions are indicated by dotted lines. Right: Sections of the brains in the left panel subjected to H&E staining or immunostaining to detect OLIG2 or NESTIN. Scale bars, 10 μm . C, Quantitation of volume of tumors in mice subcutaneously inoculated with TGS04 WT or ATG5-KO cells (1×10^6). Data are values for individual mice. The mean \pm SD are indicated ($n = 5$)

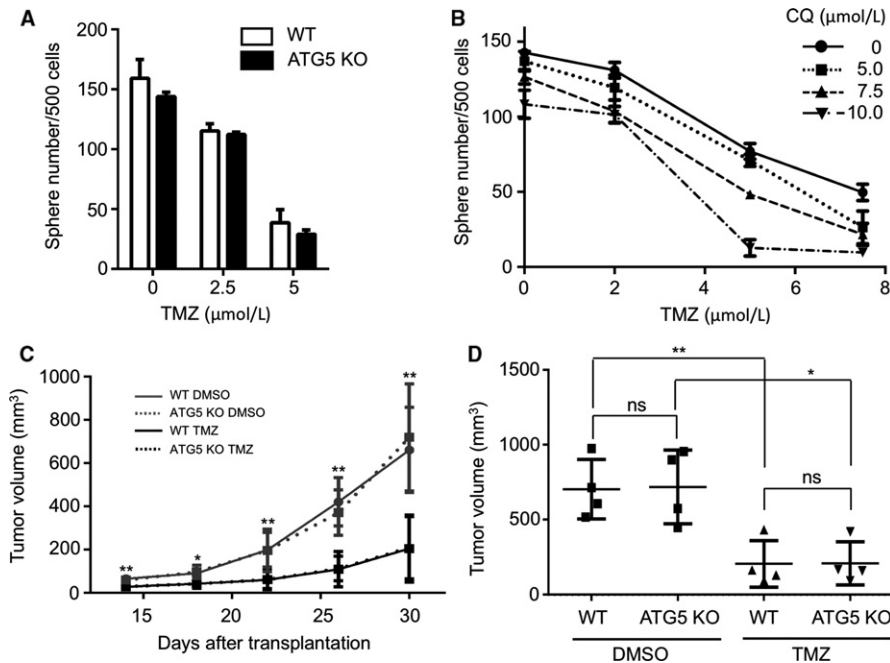


FIGURE 3 Autophagy is not involved in therapeutic effect of TMZ. A, Quantitation of sphere formation by TGS04 WT and ATG5-KO cells treated with the indicated TMZ concentrations. Data are the mean \pm SD ($n = 3$). B, Quantitation of sphere formation by TGS04 WT cells treated with the indicated combinations of TMZ and CQ. Data are the mean \pm SD ($n = 3$). C, Quantitation of volume of tumors in mice subcutaneously inoculated with TGS04 WT or ATG5-KO cells (1×10^6) and treated with DMSO or TMZ (ip daily injection of 0.1 mg/kg) starting 1 d after tumor cell inoculation. Data are the mean \pm SD ($n = 4$). D, Quantitation of tumor volumes in the mice in (C) at 30 d post-tumor cell inoculation. ns, not significant

Figure S3a). In this experiment, a relatively high concentration of nigericin (15 $\mu\text{mol/L}$) was required to achieve Ca^{2+} mobilization, perhaps reflecting the limited sensitivity of Fluo-4 assay to detect changes to Ca^{2+} levels in this experimental setting. To better address this question, we examined whether inhibitors of Ca^{2+} signaling affected nigericin efficacy. The autophagy induced by nigericin (0.5 $\mu\text{mol/L}$) in WT glioma cells could be markedly reduced by BAPTA-AM, a Ca^{2+} chelator (Figure 5B [left] and Supplementary Figure S3b [left]). Because increased intracellular Ca^{2+} triggers signaling by CaMKK and calcineurin, we treated WT glioma cells with nigericin (0.5 $\mu\text{mol/L}$) plus STO-609 (CaMKK inhibitor) or cyclosporine A (calcineurin inhibitor) to block Ca^{2+} signaling. STO-609 clearly

suppressed autophagy induced by nigericin (Figure 5B [right] and Supplementary Figure S3b [right]) but cyclosporine A did not (Supplementary Figure S3c). We then compared WT and ATG5-KO cells cotreated with nigericin (0.025–0.075 $\mu\text{mol/L}$) plus BAPTA or STO-609 and found that neither drug affected the efficacy of nigericin in suppressing the proliferation/survival of WT cells (Figure 5C). However, both drugs partly restored sphere formation by nigericin-treated ATG5-KO cells (Figure 5C). These data indicate that, although intracellular Ca^{2+} levels are modestly increased by nigericin, this level of Ca^{2+} signaling is sufficient and essential to create the synergism between nigericin and ATG5 deficiency that suppresses glioma cell survival/proliferation.

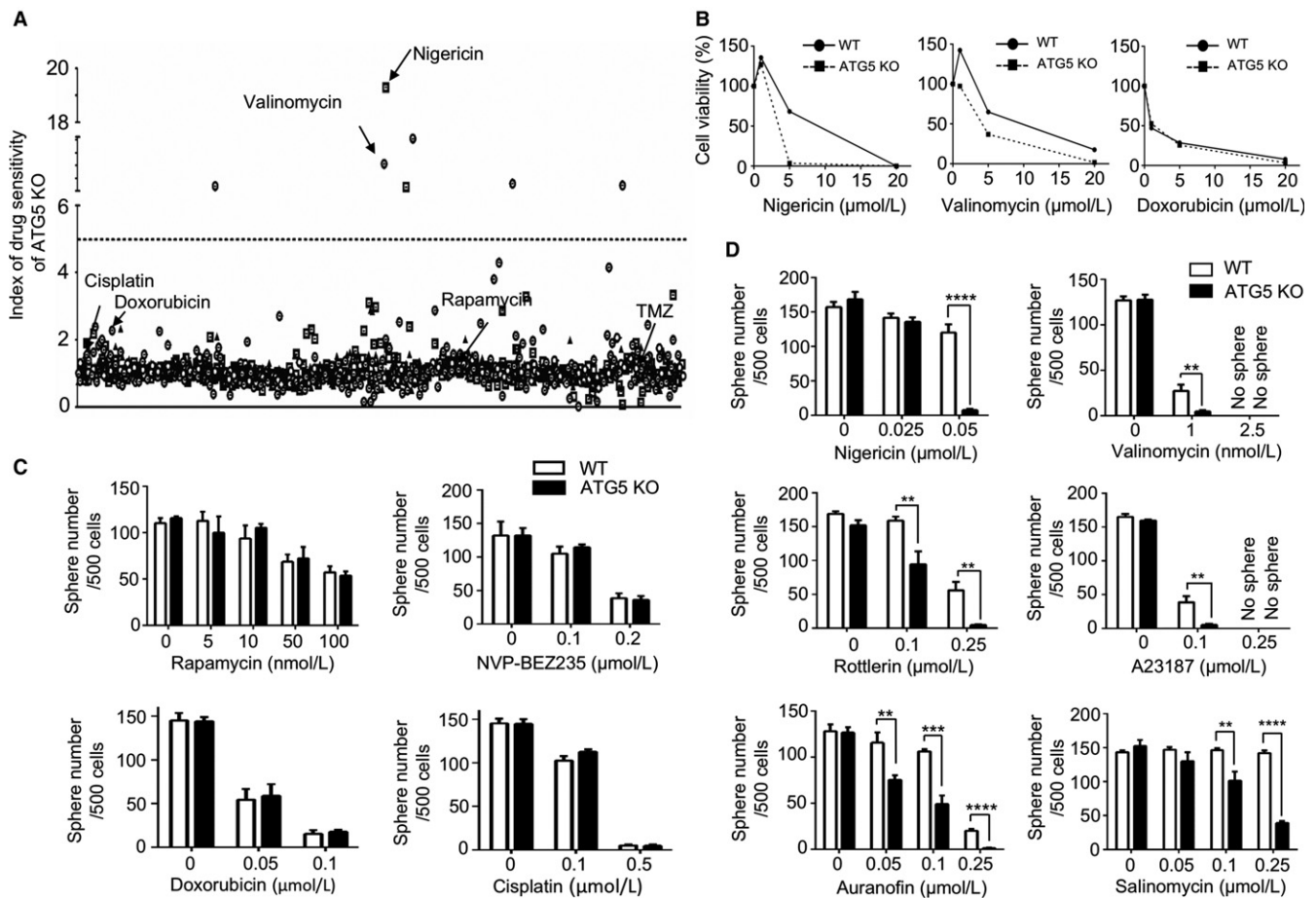


FIGURE 4 Identification of compounds whose efficacies are enhanced by autophagy inhibition. A, Index of sensitivity of TGS04 ATG5-KO cells to 357 library compounds (1:100 (▲), 1:400 (□), or 1:2000 (○) dilution). B, Examples of percentage viability from the screening data of TGS04 WT and ATG5-KO cells treated with indicated compounds. C, D, Quantitation of sphere formation by TGS04 WT and ATG5-KO cells treated with the indicated concentrations of the indicated compounds. Data are the mean \pm SD ($n = 3$)

To investigate how autophagy inhibition enhances nigericin efficacy, we evaluated mitochondrial functions in nigericin-treated WT and ATG5-KO cells. Nigericin (2.5 $\mu\text{mol/L}$) increased mitochondrial ROS in glioma cells as measured by MitoSox. To investigate whether lower concentration of nigericin (0.5 $\mu\text{mol/L}$) affected ROS, we evaluated the mRNA expression levels of HMOX1 and NQO1, which are target genes of the key transcriptional factor NRF2 that is activated by elevated ROS. As a result, lower concentration of nigericin (0.5 $\mu\text{mol/L}$) was sufficient to induce expression of these genes (Figure 6B), suggesting that ROS are physiologically upregulated by nigericin. Consistent with this observation, nigericin treatment induced glioma cell apoptosis in a manner enhanced by ATG5 deficiency (Figure 6C), although the cell cycle profiles of WT and ATG5-KO cells were comparable (Figure 6D). Thus, autophagy is critical for protecting glioma cells from mitochondrial damage.

3.5 | Enhanced efficacy of selected anticancer compounds by pharmacological autophagy inhibition

We next determined whether pharmacological autophagy inhibition by CQ could enhance the anticancer effects of Ca^{2+} -mobilizing compounds. CQ cotreatment dramatically increased the ability of our

candidate compounds to suppress sphere formation by WT glioma cells, even at the minimum concentration needed to inhibit autophagy (less than 5 $\mu\text{mol/L}$) (Figure 7A and Supplementary Figure S2c). Although there were variations among patient samples, the enhancement of nigericin's or salinomycin's efficacy by CQ cotreatment was observed in 3 other patient-derived glioma cells (Supplementary Figure S4). In contrast, no mTOR inhibitor or genotoxic agent showed such synergism with CQ (Figure 7A). Strikingly, recipient mice that were transplanted with WT glioma cells and treated with nigericin or salinomycin in combination with CQ suppressed tumors growth in vivo (Figures 7B,C and Supplementary Figure S5a). No obvious adverse effects due to administration of CQ, such as loss of mouse body weight, were observed during the monitoring period (Supplementary Figure S5b,c). These data clearly indicate that autophagy inhibition can efficiently sensitize glioma cells to anticancer therapies associated with Ca^{2+} mobilization.

4 | DISCUSSION

The precise roles of autophagy in GBM have been unclear. In a KRAS-driven GBM mouse model, inhibition of autophagy genes reduced tumorigenesis.⁶ In another study, NVP-BE2235 synergized

with CQ to induce apoptosis and regression of established human gliomas in mouse flank xenografts.²⁸ Huang et al (2017) report that MST4, a member of the mammalian sterile20-like serine/threonine kinase (STK) family, is highly expressed in high-grade and mesenchymal GBM and upregulates autophagy by phosphorylating ATG4B, and that autophagy inhibition decreases the self-renewal, proliferation and tumorigenicity of GBM cells.²⁹ Thus, the function of autophagy in tumorigenesis depends on the cancer's developmental stage and cellular context.

Unexpectedly, our data showed that ATG5-dependent autophagy is totally dispensable for the proliferation, survival and differentiation of human GBM cells *in vivo*. Nevertheless, it remains possible that autophagy dependency may vary among GBM subtypes. Another reason for the discrepancy may be the existence of ATG5-independent autophagy, in which autophagosomes are formed in a Rab9-dependent manner by the fusion of isolation membranes with vesicles derived from the trans-Golgi and late endosomes.^{30,31} Therefore, ATG5-independent autophagic activity could have compensated for the lack of ATG5 function in our mutant glioma cells. However, as our CQ treatment data are consistent with ATG5 deficiency, we

believe that ATG5 plays a dominant role in the autophagy of the glioma cells in our experiments.

To further bolster our conclusions, we carefully investigated whether TMZ induces autophagy in the patient-derived glioma cells used in this study. TGS01 and TGS04 cells treated with various TMZ concentrations showed no obvious changes in their LC3I/II ratios (data not shown). In addition, we investigated autophagic flux using the highly accurate GFP-LC3-RFP-LC3ΔG probe. Consistent with our LC3I/II ratio data, TMZ did not induce any significant change in autophagic flux in TGS04 cells, whereas nigericin or salinomycin did (Supplementary Figure S2). We concluded that, although several previous publications have demonstrated that TMZ can induce autophagy in some cases,^{7,32,33} this agent did not induce autophagy in the cells examined in our study. Indeed, the effect of TMZ on autophagy remains controversial.³⁴ A contributing factor may be that patient-derived glioma cells are highly heterogeneous due to their variable genetic aberrations. That being said, it is interesting that the compounds selected in this study, notably nigericin, consistently induced the autophagy of several different patient-derived glioma cells. Therefore, we believe that our findings may be very valuable in

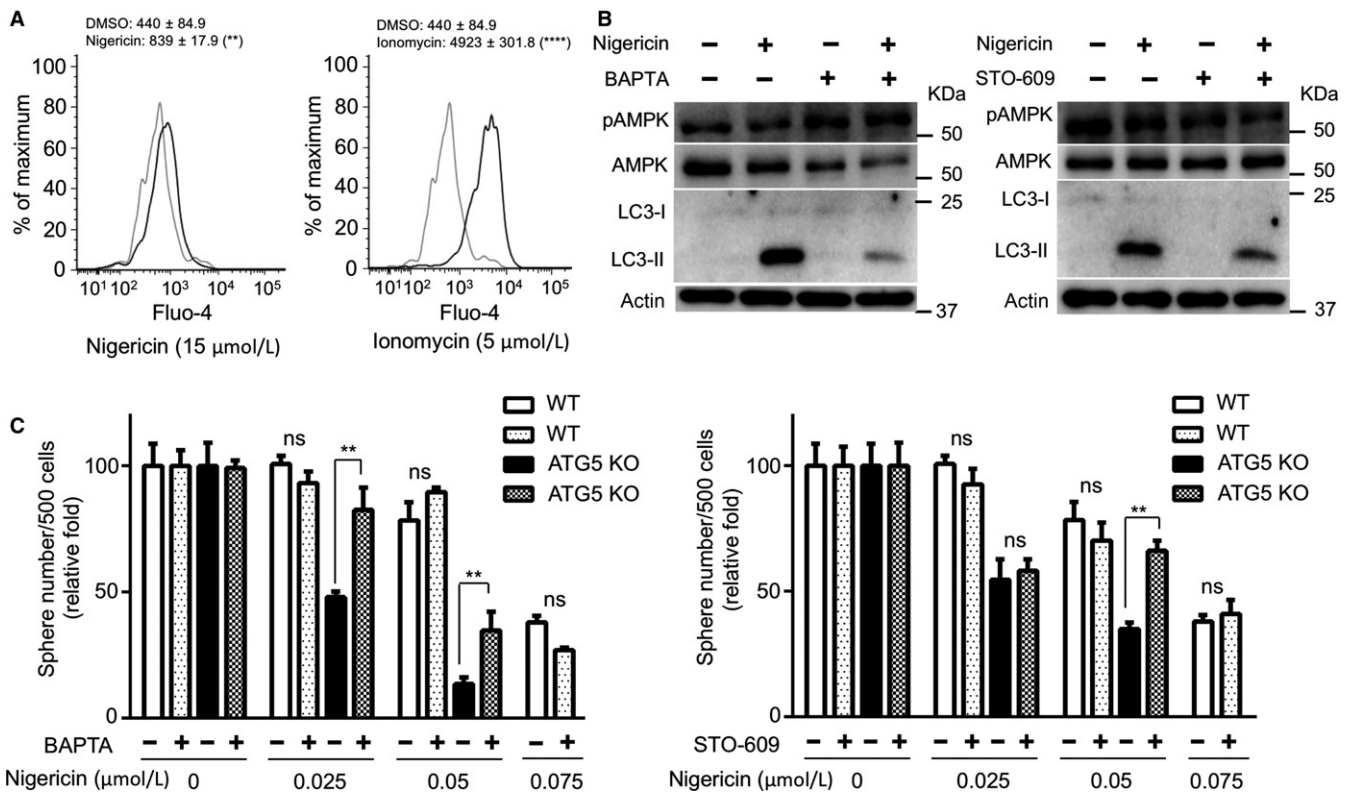


FIGURE 5 Autophagy protects glioma cells against Ca^{2+} mobilization. **A**, Fluo-4 flow cytometric analysis of intracellular Ca^{2+} in TGS04 WT cells treated for 15 min with 15 μmol/L nigericin (left) or 5 μmol/L ionomycin (right) as a positive control (black lines). Gray line, DMSO control. Data are the mean ± SD of Fluo-4 peak intensity and representative of 3 independent experiments. **B**, Western blotting to detect the indicated proteins in TGS04 WT cells that were pretreated with/without 10 μmol/L BAPTA for 5 h before addition of 0.5 μmol/L nigericin for 1 h (left) or pretreated with/without 25 μg/mL STO-609 for 5 h before addition of 0.5 μmol/L nigericin for 1 h (right). Data are representative of 3 independent experiments. **C**, Quantitation of sphere formation by TGS04 WT and ATG5-KO cells treated with the combinations of nigericin and 0.1 μmol/L BAPTA (left) or 2 μg/mL STO-609 (right). Results are expressed relative to sphere numbers in individual cultures without nigericin taken as 100%. Only WT cells were treated with 0.075 μmol/L nigericin to confirm the effects of BAPTA or STO-609 with a higher concentration of nigericin. Data are the mean ± SD (n = 3)

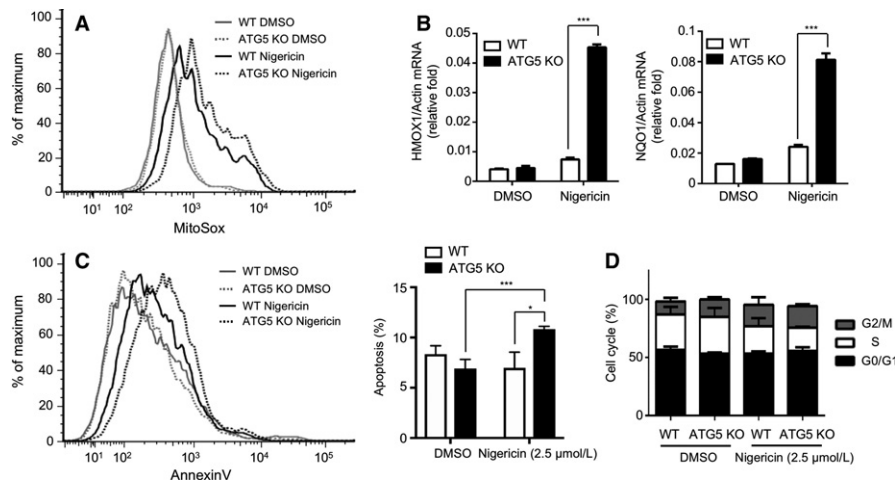


FIGURE 6 Autophagy deficiency induces mitochondrial damage and apoptosis. A, MitoSOX flow cytometric analysis of mitochondrial reactive oxygen species (ROS) in TGS04 WT and ATG5-KO cells treated with DMSO or 2.5 μmol/L nigericin for 6 h. Data are representative of 3 independent experiments. B, qPCR determination of mRNA levels of HMOX1 and NQO1 in TGS04 WT and ATG5-KO cells treated with DMSO or 0.5 μmol/L nigericin for 24 h. Data are representative of 3 independent experiments. C, Left: Flow cytometric analysis of apoptosis using Annexin V-FITC in the cells in (A). Right: Quantitation of percentages of Annexin V-positive cells in the left panel. Data are the mean ± SD (n = 3). D, Cell cycle analysis of TGS04 WT and ATG5-KO cells treated with 2.5 μmol/L nigericin for 6 h. Data are the mean ± SD (n = 3) for the indicated cell cycle stages

terms of the discovery of unique compounds able to induce autophagy in the GBM context.

Our results have demonstrated that, although mTOR inhibitors promote autophagy, only calcium mobilization signals synergize with autophagy inhibition to induce glioma cell death. While the exact mechanism is unclear, we hypothesize that mitochondria are an important target and that proper Ca²⁺ levels are needed for normal mitochondrial function. Ca²⁺ overload achieved by knock-down of the mitochondrial protein MICU1 triggers excessive ROS generation and sensitivity to apoptotic stress.³⁵ Conversely, inhibition of mitochondrial Ca²⁺ uptake by knockout of the endoplasmic reticulum-localized inositol trisphosphate receptor (InsP3R) Ca²⁺ release channel impairs oxidative phosphorylation and reduces ATP production.³⁶ This disruption of mitochondrial bioenergetics induces the necrotic death of cancer cells but not normal cells. Moreover, this cancer cell death can be prevented by the addition of mitochondrial metabolites.³⁷ In contrast, autophagy is involved in the maintenance of healthy mitochondria. Unnecessary or damaged mitochondria are eliminated by selective autophagy, termed mitophagy, which proceeds mainly via the alternative autophagy pathway.³⁸ PINK1/Parkin, which are frequently mutated in Parkinson's disease (PD), are stably localized to depolarized mitochondria and initiate mitophagy, suggesting that clearance of damaged mitochondria by mitophagy prevents PD pathogenesis.^{39,40} Conventional autophagy is also involved in mitophagy in a Parkin-independent and ATG5-dependent manner.⁴¹ Accordingly, T lymphocytes from *Atg7*-KO mice show increased mitochondrial content, ROS production and apoptosis.⁴² Liver tumors in *Atg5*-KO or *Atg7*-KO mice contain swollen mitochondria and 8-OHdG DNA damage, which is induced by ROS.⁴³ Mitochondria isolated from

Atg7-KO mouse skeletal muscle exhibit defective mitochondrial respiration, and *Atg7*-KO mouse embryonic fibroblasts show decreased resting mitochondrial oxygen consumption and increased ROS.⁴⁴ Taken together, these observations suggest that autophagy controls the number and quality of mitochondria in a cell and, thus, ROS production. Therefore, combining activation of calcium signaling with inactivation of autophagy may efficiently induce tumor cell death.

Although previous studies have reported the existence of a synergistic effect between salinomycin and autophagy inhibition,^{22,26} the exact mechanism by which this interaction triggered cell death has not been elucidated. Our data fill this gap by clearly showing that inhibition of Ca²⁺ signaling reduces the synergistic effects of our selected compounds plus ATG5 deficiency. Therefore, we believe that our results have made a significant contribution to elucidating the molecular mechanism by which autophagy supports the survival of glioma cells; that is, autophagy protects glioma cells against the stress caused by Ca²⁺ mobilization. In addition, our study has provided important data implicating several drugs that may have novel uses in the treatment of GBM. As there are multiple autophagy inhibitors that are available for use in a clinical setting, including CQ, our findings should accelerate the development of novel therapeutic approaches for the treatment of this disease.

In conclusion, our data support the concept that compounds promoting Ca²⁺ mobilization may be combined with autophagy inhibition as a novel therapy for patients with GBM. Further intensive screening of compounds will identify promising candidates as clinically available anticancer drugs in future, and investigation at the molecular level of how autophagy controls mitochondrial functions should reveal new therapeutic targets for this deadly malignancy.

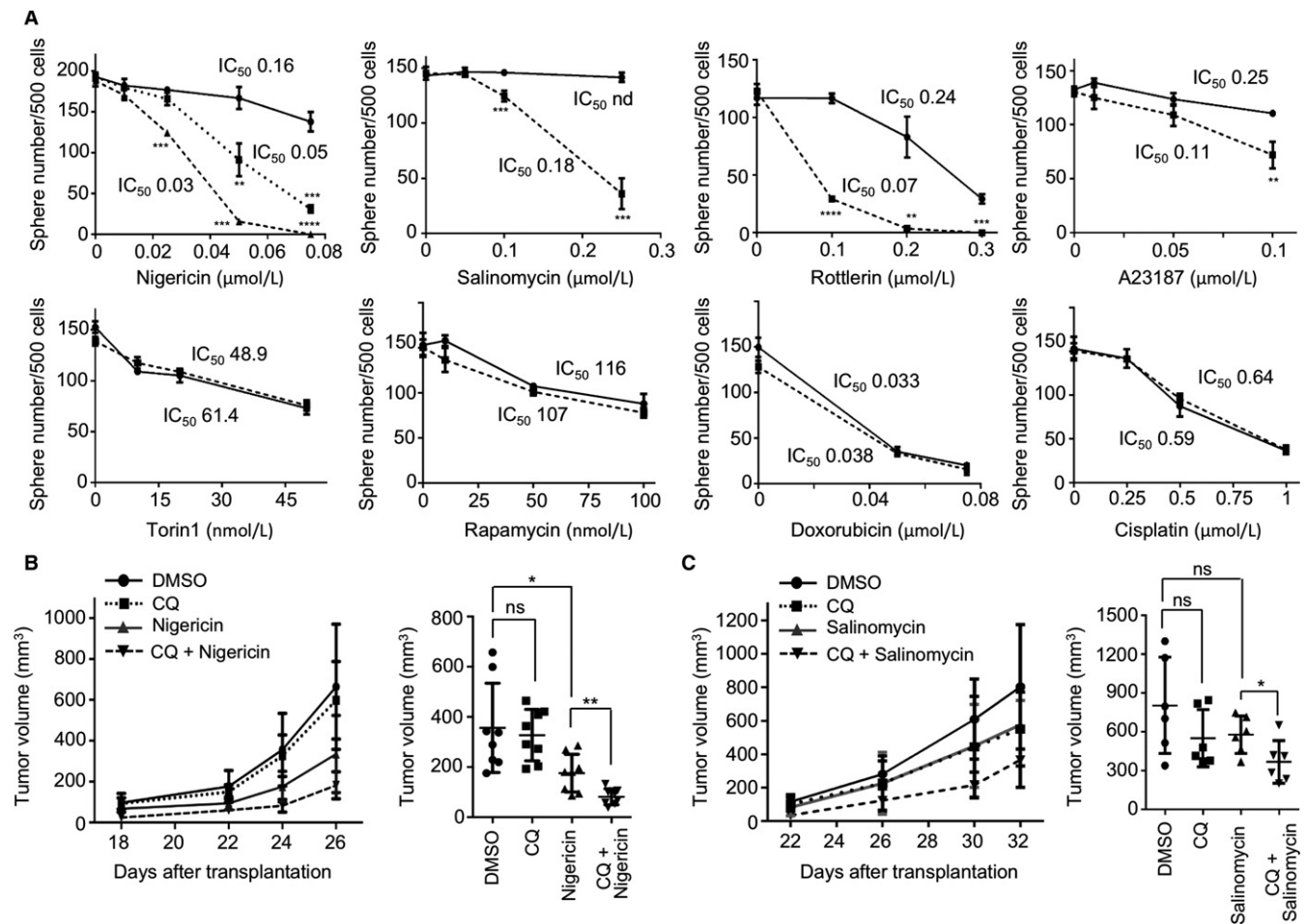


FIGURE 7 Pharmacological autophagy inhibition enhances the efficacy of selected anticancer compounds. **A**, Quantitation of sphere formation by TGS04 WT cells treated with the indicated compounds without CQ (solid line) or in combination with 1 $\mu\text{mol/L}$ (dotted line) or 5 $\mu\text{mol/L}$ (dashed line) CQ. Data are the mean \pm SD ($n = 3$). The IC_{50} of each compound with/without CQ is indicated. Statistical analyses were performed to detect differences between CQ-treated and untreated groups at each drug concentration after conversion to relative values to the drug-untreated control in each group. **B**, Left: Quantitation of volume of tumors in mice subcutaneously inoculated with TGS04 WT cells (1×10^6) and treated with DMSO, CQ only (ip daily injection of 25 mg/kg), nigericin only (ip daily injection of 1 mg/kg) or nigericin plus CQ. Data are the mean \pm SD ($n = 8/\text{group}$). Right: Quantitation of tumor volumes in the mice at 24 d post-tumor cell inoculation. **(c)** Left: Quantitation of volume of tumors in mice subcutaneously inoculated with TGS04 WT cells (1×10^6) and treated with DMSO, CQ only (ip daily injection of 25 mg/kg), salinomycin only (ip every 2 d injection of 5 mg/kg) or salinomycin plus CQ. Data are the mean \pm SD ($n = 6/\text{group}$). Right: Quantitation of tumor volumes in the mice at 32 d post-tumor cell inoculation

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CONFLICT OF INTEREST

The authors declare that no conflict of interests exists.

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