Synergistic Augmentation of Rapamycin-Induced Autophagy in Malignant Glioma Cells by Phosphatidylinositol 3-Kinase/Protein Kinase B Inhibitors

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Abstract
The mammalian target of rapamycin (mTOR) is a downstream effector of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway and a central modulator of cell proliferation in malignant gliomas. Therefore, the targeting of mTOR signaling is considered a promising therapy for malignant gliomas. However, the mechanisms underlying the cytotoxic effects of a selective mTOR inhibitor, rapamycin, on malignant glioma cells are poorly understood. The purpose of this study was thus to elucidate how rapamycin exerts its cytotoxic effects on malignant glioma cells. We showed that rapamycin induced autophagy but not apoptosis in rapamycin-sensitive malignant glioma U87-MG and T98G cells by inhibiting the function of mTOR. In contrast, in rapamycin-resistant U373-MG cells, the inhibitory effect of rapamycin was minor, although the phosphorylation of p70S6 kinase, a molecule downstream of mTOR, was remarkably inhibited. Interestingly, a PI3K inhibitor, LY294002, and an Akt inhibitor, UCN-01 (7-hydroxystaurosporine), both synergistically sensitized U87-MG and T98G cells as well as U373-MG cells to rapamycin by stimulating the induction of autophagy. Enforced expression of active Akt in tumor cells suppressed the combined effects of LY294002 or UCN-01, whereas dominant-negative Akt expression was sufficient to increase the sensitivity of tumor cells to rapamycin. These results indicate that rapamycin exerts its antitumor effect on malignant glioma cells by inducing autophagy and suggest that in malignant glioma cells a disruption of the PI3K/Akt signaling pathway could greatly enhance the effectiveness of mTOR inhibitors.

Introduction
Malignant gliomas are the most common primary brain tumor. Despite remarkable advances in surgical techniques and treatment options, including chemotherapy and radiotherapy, the prognosis of this disease is still very poor (1). In fact, the 5-year survival rate for the most malignant type, glioblastoma multiforme, is only 2% (1). To improve this poor prognosis, novel treatment strategies are needed.

The signaling pathway composed of phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and mammalian target of rapamycin (mTOR) plays a central role in the regulation of cell proliferation, growth, differentiation, and survival (2, 3). Dysregulation of this pathway is frequently observed in a variety of tumors, including malignant gliomas (2, 4). Therefore, inhibition of the PI3K/Akt/mTOR signaling is being investigated as a potential therapy for cancer. Interestingly, tumor cells in which the PI3K/Akt/mTOR pathway is dysregulated are more susceptible than normal cells to the inhibition of mTOR, which is a downstream effector of this signaling pathway (5, 6). These findings provide a strong basis for investigating mTOR inhibitors as potential tumor-selective therapeutic agents. Rapamycin and its derivatives, CCI-779 and RAD001, specifically inhibit the function of mTOR by blocking the phosphorylation of downstream molecules, such as p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), leading to G1-phase cell cycle arrest (7, 8). Accumulating evidence from preclinical and early clinical studies suggests that these mTOR inhibitors would be effective as growth inhibitors against a broad range of tumors (9, 10). However, the mechanisms by which mTOR inhibitors, such as rapamycin, exert their cytotoxic effect on malignant glioma cells are poorly defined.

In addition to its role in regulating cell proliferation, growth, differentiation, and survival, mTOR can control whether a cell undergoes programmed cell death type I (apoptosis) or type II (autophagy; ref. 11). Rapamycin induces apoptosis in a cell type–specific fashion (12–15), yet in some cells in which rapamycin inhibits mTOR it stimulates autophagy rather than apoptosis (16–19). It is still unclear whether cross-talk occurs between these two programmed cell death pathways and what determines why cells die from one type or the other.

In previous studies, we reported that malignant glioma cells treated with γ-irradiation or chemotherapeutic agents have a tendency to undergo autophagy rather than apoptosis (20–22). Based on these findings, we speculated that rapamycin also might induce autophagy but not apoptosis in malignant glioma cells. To test our hypothesis, in this study, we treated three malignant glioma cell lines, U87-MG, T98G, and U373-MG, with rapamycin. We found that rapamycin-sensitive U87-MG and T98G cells did indeed undergo autophagy instead of apoptosis. U373-MG cells, however, were resistant to rapamycin, although rapamycin suppressed phosphorylation of p70S6K. We also treated the cells with a PI3K inhibitor, LY294002 (23), and an Akt inhibitor, UCN-01 (7-hydroxystaurosporine; ref. 24). Both inhibitors synergistically augmented the effect of rapamycin in all of three tumor cell lines regardless of the sensitivity of the cells to rapamycin. Our findings suggest that treatment combining a mTOR inhibitor with the PI3K or Akt inhibitor can overcome the resistance of tumor cells to a mTOR inhibitor by stimulating autophagy and that this combination thus represents a promising new approach for treating malignant gliomas.
Materials and Methods

Reagents. We obtained rapamycin from Sigma Chemical Co. (St. Louis, MO) and dissolved it in ethanol. We purchased LY294002 from Sigma Chemical and dissolved it in DMSO. UCN-01, kindly provided by Kyowa Hakko Kogyo (Tokyo, Japan), was also dissolved in DMSO. The final concentrations of ethanol and DMSO in the culture medium did not exceed 0.2%.

Cell culture. Human malignant glioma cell lines U87-MG, T98G, and U373-MG were purchased from American Tissue Culture Collection (Rockville, MD). Tumor cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 µg/mL). Tumor cells were incubated at 37°C in humidified 5% CO₂.

Cell viability assay. To determine the effects of rapamycin and rapamycin plus LY294002 or UCN-01 on tumor cells, we determined cell viability after the treatments. We used a trypan blue dye exclusion assay as described previously (25). Tumor cells in exponential growth were harvested and seeded at 5 × 10⁶ cells per well (0.1 mL) in 96-well flat-bottomed plates (Corning, Inc., Corning, NY) and incubated overnight at 37°C. The cells were then treated with or without rapamycin with or without rapamycin plus LY294002 or UCN-01. After the cells were collected by trypan exclusion, they were stained with trypan blue, and the viable cells in each well were counted. The viability of the untreated cells (the control) was considered 100%. Survival fractions were calculated from the mean cell viability of the treated cells.

Cell cycle assay. For the cell cycle analysis, tumor cells treated as described above were trypsinized, fixed with 70% ethanol, and stained with propidium iodide using a flow cytometry reagent set (Roche Applied Science, Indianapolis, IN) as described previously (21, 22). Samples were analyzed for DNA content using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA).

Apoptosis detection assay. To determine whether apoptosis was induced in the tumor cells treated as described above, the cells were stained with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technique using an ApopTag apoptosis detection kit (Invitrogen) as described previously (21, 22). Cisplatin (5 µg/mL) was used as a positive control to induce apoptosis (26).

Electron microscopy. Tumor cells were grown on glass coverslips, treated as described above, and fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.3) for 1 hour. After fixation, the samples were postfixed in 1% OsO₄ in the same buffer for 1 hour and then subjected to the electron microscopic analysis as described previously (21, 22). Representative areas were chosen for ultrathin sectioning and viewed with a JEM 1010 transmission electron microscope (JEOL, Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained by AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA).

Detection and quantification of acidic vesicular organelles with acridine orange staining. Autophagy is the process of sequestrating cytoplasmic proteins into the lytic component and is characterized by the formation and promotion of acidic vesicular organelles (AVO). To detect the development of AVOs, we treated tumor cells as described above and then performed vital staining with acridine orange as described previously (21, 22). Briefly, the treated tumor cells were stained with acridine orange (1 µg/mL) for 15 minutes. Samples were examined under a fluorescence microscope. To quantify the development of AVOs, the tumor cells were stained with acridine orange (1 µg/mL) for 15 minutes, removed from the plate with trypsin-EDTA (Invitrogen), and analyzed using the FACScan flow cytometer and CellQuest software.

Results

Rapamycin inhibits the cell viability of malignant glioma cells. To examine the antitumor effect of rapamycin on malignant glioma cells, we treated three malignant glioma cell lines (U373-MG, U87-MG, and T98G) with rapamycin at concentrations ranging from 0.1 nmol/L to 25 µmol/L for 72 hours. As shown in Fig. 1A, rapamycin inhibited cell viability in all three cell lines in a dose-dependent manner, but their sensitivities varied. The IC₅₀ levels of T98G, U87-MG, and U373-MG cells were 2 nmol/L, 1 µmol/L, and >25 µmol/L, respectively.

Combination Effect of Rapamycin plus LY294002 or UCN-01

Analysis of combined effect. The combined effect of rapamycin plus LY294002 or UCN-01 was analyzed by the combination index (CI) isobologram using CalcuSyn software (Biosoft, Ferguson, MO) as described previously (23). Mutually exclusive equations (28) were used to determine the CI. A CI < 1 indicated synergism, a CI = 1 indicated additivity, and a CI > 1 indicated antagonism. Theoretically, an isobolographic analysis distinguishes the four most important types of interactions: pure additivity, supra-additivity (synergy), indifference, and infra-additivity (antagonism). The isobolograms were generated at IC₅₀ levels (concentrations resulting in cell viability in 50% of the control). Each plot represented values generated in at least three independent experiments for the simultaneous treatment of cells.

Transient transfection and immunofluorescent staining. Tumor cells were plated in chamber slides at a density of 3 × 10⁴ cells/mL and incubated at 37°C overnight. The cells were then transfected with 1 µg of a hemagglutinin (HA)–tagged, membrane-bound, wild-type, or dominant-negative Akt expression vector [Myr-HA-AKT1 or Myr-HA-AKT1 (AAA)] using FuGene 6 (Roche Applied Science) according to the manufacturer's instructions. After 24 hours of incubation, the tumor cells were subjected to treatment with rapamycin plus LY294002 or UCN-01 for an additional 72 hours. To detect the expression of Myr-HA-AKT1 or Myr-HA-AKT1 (AAA), the cells were fixed with 4% formaldehyde for 15 minutes, rinsed thrice with PBS, blocked by 3% rabbit serum for 30 minutes, and stained with an anti-HA-probe antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The cells were washed thrice with PBS and incubated with Texas red–conjugated avidin (Vector Laboratories, Burlingame, CA) diluted 1:1,000 for 1 hour at room temperature. The cells were then stained with 0.05 mmol/L monodansylcadaverine for 10 minutes and then observed under a fluorescence microscope. The autophagic index was determined as the percentage of the monodansylcadaverine-labeled cells out of 200 HA-positive cells from each treatment group.

Statistical analysis. Data were expressed as the mean ± SD. The statistical analysis was done using Student’s t test (two tailed). The criterion for statistical significance was P < 0.05.
Rapamycin-mediated inhibition of mammalian target of rapamycin signaling occurs independent of sensitivity of malignant glioma cells to rapamycin. To distinguish specific mTOR-mediated cell proliferation block from nonspecific toxicity of rapamycin, we measured the phosphorylation of one of the downstream targets of mTOR, p70S6K, using Western blotting for tumor cells treated with rapamycin at 1 to 100 nmol/L for 24 hours. As shown in Fig. 1B, the phosphorylation of p70S6K at Thr389 in three tumor cell lines was inhibited in a dose-dependent manner. In rapamycin most sensitive T98G cells, treatment with 100 nmol/L rapamycin (at IC_{50}) for 24 hours completely inhibited the mTOR-dependent phosphorylation of p70S6K. In rapamycin-sensitive U87-MG cells and rapamycin-resistant U373-MG cells, treatment with 100 nmol/L rapamycin (at IC_{50} and IC_{90}) remarkably suppressed the phosphorylation of p70S6K. These results suggest that the effect of rapamycin at least at 100 nmol/L was mTOR specific and that the sensitivity of malignant glioma cells to rapamycin was not related to the extent of the inhibition of rapamycin of mTOR signaling. In other words, targeting a single molecule in signaling pathway may not be sufficient to effectively suppress the growth of tumor cells at least in malignant glioma cells. Taken together, we decided to treat tumor cells with rapamycin at concentrations of up to 100 nmol/L for subsequent experiments.

Apoptosis is undetectable in malignant glioma cells treated with rapamycin. Because it has been shown that rapamycin induces apoptosis in some cell lines (12–15), we investigated whether rapamycin could also induce apoptosis in malignant glioma cells. As shown in Fig. 2A, the TUNEL analysis detected few apoptotic cells in U87-MG cells untreated (a) or treated with rapamycin for 72 hours at concentrations of 10 nmol/L (c) or 100 nmol/L (d). For a positive control for apoptosis, we treated tumor cells with cisplatin (5 μg/mL) for 72 hours (Fig. 2A and B). Although cisplatin significantly induced apoptosis in U78-MG cells compared with no treatment (P < 0.01), no significant apoptosis was detected in rapamycin-treated tumor cells (Fig. 2B). Because it has also been established that rapamycin causes G_{1}-phase cell cycle arrest (7, 8), we did a cell cycle analysis using untreated and treated U87-MG cells. Treatment with rapamycin (10 or 100 nmol/L) for 72 hours increased the cell population in the G_{1} phase and decreased it in the S and G_{2}-M phases (Fig. 2C), indicating that rapamycin induces G_{1} arrest. When tumor cells were treated with cisplatin (5 μg/mL) for 72 hours, a sub-G_{1} population characteristic of apoptosis was prominent (19.6%; Fig. 2C). In contrast, no significant sub-G_{1} population was detected in tumor cells treated with rapamycin (10 or 100 nmol/L), whereas the percentage of sub-G_{1} population caused by cisplatin was significant (P < 0.01; Fig. 2D). A similar tendency was observed in T98G cells treated with rapamycin (data not shown). These results indicate that in malignant glioma cells rapamycin induced G_{1} arrest but not apoptosis.

Rapamycin induces autophagy in malignant glioma cells. Increasing evidence indicates that inhibition of mTOR is associated with the induction of autophagy (16–19). We therefore investigated whether treating malignant glioma cells with rapamycin would result in autophagy. After exposing U87-MG cells to rapamycin (100 nmol/L) for 3 days, the ultrastructure was analyzed by electron microscopy. As shown in Fig. 3A, b, numerous autophagic vacuoles and empty vacuoles were observed in U87-MG cells treated with rapamycin. However, no chromatin condensation or fragmentation that was characteristic of apoptosis was observed. On the other hand, untreated tumor cells showed few autophagic features (Fig. 3A, a).

To detect the development of AVOs, untreated or treated U87-MG cells were stained with acridine orange. The untreated tumor cells exhibited no cytoplasmic staining (Fig. 3B, a), whereas the rapamycin-treated tumor cells showed abundant cytoplasmic AVO formation, which is characteristic of autophagy (Fig. 3B, b). To quantify the rapamycin-induced increase in the fractional volume and acidity of the AVOs, we did a flow cytometric analysis. As shown in Fig. 3C, a and b, rapamycin (100 nmol/L) increased the strength of the bright red fluorescence (Y axis) in U87-MG cells from 5.98% to 20.04%, indicating the development of AVOs. 3-Methyladenine (1 mmol/L), which inhibits autophagosome sequestration (22, 29), suppressed the development of AVOs in untreated and treated U87-MG cells (Fig. 3C, c and d). Rapamycin significantly developed AVOs in U87-MG cells compared with no treatment (P < 0.01), whereas the inhibitory effect of 3-methyladenine on development of AVOs was also significant (P < 0.05; Fig. 3D).

**Figure 1.** Effect of rapamycin on malignant glioma cells. A, cytotoxic effect of rapamycin on U373-MG, U87-MG, and T98G tumor cells. Tumor cells were seeded at 5 × 10^{5} cells per well (0.1 mL) in 96-well flat-bottomed plates and incubated overnight at 37°C. After exposure to rapamycin for 72 hours, the cells were trypsinized and the viable cells were counted. The viability of the untreated cells was regarded as 100%. Points, mean of three independent experiments; bars, SD. B, effect of rapamycin on mTOR activity in U373-MG, U87-MG, and T98G cells. Tumor cells were treated with rapamycin at concentrations ranging from 0 to 100 nmol/L for 24 hours. Cell lysates were subjected to Western blot analysis using an antibody against phospho-Thr^{389}-specific p70S6K. An anti-total p70S6K antibody was used to confirm equal loading of proteins.
Because monodansylcadaverine accumulates in mature autophagic vacuoles, such as autophagolysosomes, but not in the early endosome compartment (27), monodansylcadaverine staining can be used to detect autophagic vacuoles. As shown in Fig. 3F, in U87-MG cells treated with rapamycin (100 nmol/L) for 3 days, the accumulation of monodansylcadaverine was noticeable, whereas in untreated tumor cells monodansylcadaverine-labeled cells were undetectable. To quantify the number of monodansylcadaverine-labeled cells after they were treated with rapamycin, we determined the percentage of autophagic cells out of 200 that had monodansylcadaverine-labeled vacuoles. As shown in Fig. 3F, treatment with rapamycin dose-dependently induced autophagy in U87-MG cells (34.8% at 10 nmol/L and 61.5% at 100 nmol/L) and T98G cells (36.3% at 10 nmol/L and 65.0% at 100 nmol/L). Rapamycin treatment significantly increased the incidence of autophagy in U87-MG and T98G cells compared with the control (P < 0.005). Conversely, in rapamycin-resistant U373-MG cells, the incidence of autophagic cells was <15% even after treatment with rapamycin (100 nmol/L). No difference was seen between control (no treatment) and rapamycin-treated U373-MG cells (Fig. 3F). These results indicate that rapamycin-induced autophagy in rapamycin-sensitive U87-MG and T98G cells but not in rapamycin-resistant U373-MG cells suggest that in tumor cells the sensitivity to rapamycin is related to the extent of autophagy induction.

Inhibition of phosphatidylinositol 3-kinase or Akt synergistically sensitizes malignant glioma cells to rapamycin. Because mTOR is one of the downstream effectors of the PI3K/Akt signaling pathway and the PI3K/Akt pathway is constitutively activated in many gliomas, we speculated that inhibitors of PI3K/Akt signaling might augment the effect of rapamycin on malignant glioma cells. To test our hypothesis, we combined rapamycin with LY294002 (a PI3K inhibitor) or UCN-01 (an Akt or protein kinase C inhibitor).

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To assess whether these combined effects were synergistic or additive, we calculated the CI value and plotted the isobologram at IC50. As shown in Table 1, the CI values of all the combinations tested were <1.0, indicating that the combination treatments had synergistic interactions. As shown in Fig. 5b, in the IC50 isobolograms, the plots were in the "synergistic" portion in all three cell lines for any drug combination. These results indicate that LY294002 and UCN-01 synergistically sensitized both rapamycin-sensitive U87-MG and T98G cells and rapamycin-resistant U373-MG cells to rapamycin.

**Combination treatment does not induce apoptosis.** Our findings that the combined effects were synergistic prompted us to investigate whether the antitumor effects were due to the induction of apoptosis or to the promotion of autophagy. As shown in Fig. 6a, few, if any, apoptotic cells were detected in U87-MG cells treated with LY294002 (5 μmol/L), UCN-01 (50 nmol/L), or each combined with rapamycin (10 nmol/L). There was no significant apoptosis in treated U87-MG cells (Fig. 6b). Furthermore, the cell cycle assay showed that treatment for 3 days with LY294002 (5 μmol/L), UCN-01
(50 nmol/L), or each combined with rapamycin (10 nmol/L) increased the G₁-phase cell population and decreased the S- and G₂-M-phase populations in U87-MG cells as did treatment with rapamycin alone (Fig. 6C). As expected from the results of the TUNEL study, treated tumor cells did not have a significant sub-G₁ population (Fig. 6C and D). Similar tendencies were observed in U373-MG and T98G cells treated as described above (data not shown). These results indicate that the effects of the combined treatments were not due to the induction of apoptosis.

**Combined effects are through promotion of autophagy.** To confirm that the combinatorial interactions could be attributed to the stimulation of autophagy, we did monodansylcadaverine staining. After treating the cells for 3 days with LY294002, UCN-01, or each combined with rapamycin, we stained them with monodansylcadaverine. In all three cell lines, treatment with LY294002 induced autophagy, whereas treatment with UCN-01 did not (Fig. 7). In rapamycin-sensitive U87-MG and T98G cells, the combination treatments significantly increased the incidence of autophagy compared with the single-agent treatments (P < 0.005-0.05). Interestingly, >50% of the cells treated with the combinations were autophagic, even rapamycin-resistant U373-MG cells. The development of AVOs also increased in all the tumor cells treated with the combinations (data not shown). These results indicate that the combined effects depend on the stimulation of autophagy.

**Combined effects are through inhibition of the Akt pathway.** It was shown recently that LY294002 and UCN-01 both inhibit Akt pathway (23, 24). Therefore, we speculated that the combined effects we observed were due to suppression of the Akt pathway. To test our hypothesis, we analyzed the effects of the combination treatments on Akt and its downstream mTOR signaling. As shown in Fig. 8A, the phosphorylation of p70S6K at Thr389 but not of Akt at Ser473 was suppressed in U373-MG cells by rapamycin (100 nmol/L). Treatment with LY294002 (5 μmol/L) alone or UCN-01 (50 nmol/L) alone suppressed the phosphorylation of Akt at Ser473 and p70S6K at Thr389 to some extent, although the inhibitory effect of LY294002 on phospho-p70S6K was greater than that of UCN-01. As predicted, combination of rapamycin plus UCN-01 and rapamycin plus LY294002 suppressed the levels of phospho-Akt and phospho-p70S6K, but the extent was similar to that by each agent alone. These results suggest that the combined effects are due at least in part to inhibition of the Akt/mTOR pathway, although the target molecules of LY294002 and UCN-01 are not the same.

To further ascertain the involvement of Akt in the combination treatments, we transiently transfected tumor cells with an active or a dominant-negative Akt expression vector [Myr-HA-AKT1 or Myr-HA-AKT1 (AAA)] 1 day before the tumor cells were treated for 3 days with rapamycin (100 nmol/L) plus LY294002 (5 μmol/L) or UCN-01 (50 nmol/L). As shown in Fig. 8B, expression of the active or dominant-negative Akt in itself did not induce autophagy in U373-MG cells (a-d). Active Akt when expressed in U373-MG cells suppressed the incidence of autophagy by treatment with rapamycin plus LY294002 (Fig. 8B, e and f) or UCN-01 (Fig. 8B, i and j), whereas dominant-negative Akt promoted the autophagy induced by treatment with rapamycin plus LY294002 (Fig. 8B, g and h) or UCN-01 (Fig. 8B, k and l).

To quantify the effect of active or dominant-negative Akt on the incidence of autophagy after the combination treatments, we counted 200 HA-positive cells from each group and determined the ratio of autophagic cells to these HA-positive cells. As shown in
Figure 5. Combination effect of rapamycin plus LY294002 or UCN-01 on malignant glioma cells. A, cell viability after the combination of rapamycin and LY294002 or UCN-01. Tumor cells were treated with a combination of rapamycin (1-100 nmol/L) and LY294002 (1-10 μmol/L) or UCN-01 (5-100 nmol/L) for 72 hours and the viable cells were counted as described above. Columns, mean of three independent experiments; bars, SD. B, IC50 isobologram of the combination treatments. In the isobologram, a plot on the diagonal line indicates that the combination is simply additive. A plot to the left under the line indicates that the combination is synergistic, whereas a plot to the right above the line indicates that it is antagonistic. IC50 isobolograms were plotted as described in Materials and Methods.
Fig. 8C, expression of active Akt inhibited the incidence of autophagy induced by rapamycin plus LY294002 from 58% to 38% (P < 0.05). In contrast, expression of dominant-negative Akt increased the incidence of autophagy induced by rapamycin plus LY294002 from 58% to 70% (P < 0.05). A similar effect was detected in the cells treated with rapamycin plus UCN-01 (Fig. 8C). Expression of active Akt decreased the incidence of autophagy from 52% to 26% (P < 0.05), whereas expression of dominant-negative Akt increased the incidence of autophagy from 52% to 62% (P < 0.05). These results indicate that the inhibition of Akt signaling is involved in the combined effect of rapamycin plus LY294002 or UCN-01.

### Discussion

In this study, we showed that rapamycin induces autophagy but not apoptosis in rapamycin-sensitive malignant glioma cells. We also showed that both LY294002 and UCN-01 synergistically sensitize rapamycin-sensitive and rapamycin-resistant malignant glioma cells to rapamycin by promoting autophagy. The effect of rapamycin plus LY294002 or UCN-01 is reversed by activation of Akt, which is a molecule upstream of mTOR in the PI3K/Akt/mTOR signaling pathway. These findings suggest that inhibition of the PI3K/Akt/mTOR signaling pathway at different points is a potential therapeutic strategy for managing malignant gliomas.

mTOR, also known as FRAP (FKBP-12/rapamycin-associated protein), RAFT1 (rapamycin and FKBP-12 target-1), and RAPT1 (rapamycin target-1), is a 289-kDa serine/threonine kinase (30–32). It is a member of the PI3K-related kinase family and acts as a master switch for cellular catabolism and anabolism (11). Because dysregulation of mTOR signaling occurs in a variety of cancers, mTOR is considered a promising target for cancer therapy (7, 8). Three potent mTOR inhibitors were identified recently: rapamycin and its derivatives, CCI-779 and RAD001 (7, 8). The two derivatives of which are characteristic of autophagy. Although it was suggested recently that autophagy plays different roles (defensive or destructive) in cancer therapy (39–41), its actual role is unknown. Three possible mechanisms have been proposed to explain how these inhibitors work. First, binding of the FKBP-12/rapamycin complex to mTOR may activate certain protein phosphatases, resulting in dephosphorylation of downstream effectors, such as p70S6K and 4E-BP1 (31). Second, the FKBP-12/rapamycin complex may compete with phosphatidic acid to bind the FRB domain of mTOR, leading to the inhibition of mTOR signaling (33). Third, the FKBP-12/rapamycin complex that binds mTOR may lead to the dephosphorylation of p70S6K and 4E-BP1 by destabilizing the mTOR/p70S6K/4E-BP1 complex (34). Because p70S6K and 4E-BP1 are translation regulators, their dephosphorylation by mTOR inhibitors suppresses overall cellular protein synthesis (up to ~5%) as well as G1 cell cycle progression (7). The detailed mechanisms by which mTOR inhibitors cause their antitumor effects are poorly defined, however.

mTOR has profound effects on the control of apoptosis and autophagy (11). Rapamycin induces apoptosis in dendritic cells (14) and in certain tumor cells (12, 13, 15), and combined with cisplatin, it sensitizes some tumor cells to apoptosis induction (35). In the present study, however, malignant glioma cells treated with rapamycin alone or rapamycin plus LY294002 or UCN-01 did not undergo apoptosis. We reported recently that γ-irradiation and some chemotherapeutic agents cause autophagy rather than apoptosis in malignant glioma cells (20–22). Because malignant glioma cells are resistant to apoptosis (36), they may have a tendency to undergo nonapoptotic autophagy after anticancer therapy. Rapamycin has been found to induce autophagy rather than apoptosis in yeast and cultured mammalian cells, such as hepatocytes (16–18, 37). Autophagy, which is characterized by the accumulation of autophagic vacuoles, is defined as the degradation of normal proteins in response to nutrient deprivation or other types of stresses (29, 38). In the present study, treatment with rapamycin alone or rapamycin plus LY294002 or UCN-01 induced AVO development and monodansylcadaverine accumulation, both of which are characteristic of autophagy. Although it was suggested recently that autophagy plays different roles (defensive or destructive) in cancer therapy (39–41), its actual role is unknown. Further investigation is thus necessary to determine the function of autophagy in rapamycin-treated tumor cells.

| Table 1. CI values at IC_{50} for LY294002 or UCN-01 combined with rapamycin in the treatment of malignant glioma cells |
|-----------------|-------------|-------------|-------------|-------------|
| Rapamycin (nmol/L) | U373-MG | U87-MG | T98G |
| LY294002 (μmol/L) |          |          |          |          |
| 1              | 0.24      | 0.14      | 0.1      | 0.12      |
| 5              | 0.43      | 0.34      | 0.25     | 0.24      |
| 10             | 0.64      | 0.46      | 0.34     | 0.24      |
| UCN-01 (nmol/L) |          |          |          |          |
| 5              | 0.42      | 0.25      | 0.13     | 0.09      |
| 10             | 0.38      | 0.1       | 0.08     | 0.11      |
| 20             | 0.51      | 0.41      | 0.04     | 0.06      |

NOTE: The CI values of each of the combination treatments were calculated as described in Materials and Methods. CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.
In our study, the failure of rapamycin-resistant U373-MG cells to respond to low-dose rapamycin could not be explained by a failure of inhibition of mTOR kinase activity, because rapamycin (100 nmol/L) was equally effective at inhibiting p70S6K phosphorylation in rapamycin-resistant U373-MG cells as well as in rapamycin-sensitive U87-MG and T98G cells. Recent investigations have shown that PTEN-deficient tumor cells are more sensitive to rapamycin than are PTEN wild-type tumor cells (42). In our study, however, all the malignant glioma cells we used have a mutant PTEN (43, 44). This discrepancy in PTEN-related rapamycin

Figure 6. Apoptosis detection in tumor cells treated with the combination of rapamycin and LY294002 or UCN-01. A, TUNEL staining. After treatment with LY294002 (5 μmol/L) or UCN-01 (50 nmol/L) in the presence or absence of rapamycin (10 nmol/L) for 72 hours, U87-MG cells were fixed, labeled with Br-dUTP, and stained with an anti-Br-dUTP antibody for TUNEL analysis. Bar, 10 μm. B, quantification of cells undergoing apoptosis. U87-MG cells were treated as described above and the percentage of apoptosis was calculated as the percentage of TUNEL-positive cells out of 200 cells from each group. Columns, mean of three independent experiments; bars, SD. C, effect of rapamycin and LY294002 or UCN-01 on the cell cycle. U87-MG cells treated with LY294002 (5 μmol/L) or UCN-01 (50 nmol/L) in the presence or absence of rapamycin (10 nmol/L) for 72 hours were stained with propidium iodide and analyzed using a FACScan. D, quantification of the sub-G1 population. U87-MG cells were treated as described above and the percentage of the sub-G1 population was calculated based on the results of cell cycle analysis. Columns, mean of three independent experiments; bars, SD.

Figure 7. Autophagy detection in the combination treatments. To quantify the incidence of autophagy, U373-MG, U87-MG, or T98G cells treated with or without rapamycin (1-100 nmol/L) for 72 hours in the presence or absence of LY294002 (1 or 5 μmol/L) or UCN-01 (5-50 nmol/L) were stained with monodansylcadaverine and observed under a fluorescence microscope. The autophagic index was calculated as described above. Columns, mean of three independent experiments; bars, SD. *, P < 0.01; **, P < 0.005.
sensitivity could be because the FRB domain of mTOR lies outside its catalytic domain (45). Thus, the binding of FKBP-12/rapamycin to the FRB domain does not sufficiently inhibit the catalytic activity of mTOR. The findings of our study support the notion that mTOR is a biologically relevant target of rapamycin in rapamycin-sensitive tumors but not in rapamycin-resistant tumors. Accordingly, treatment with rapamycin combined with inhibitors of other signaling pathways may overcome the resistance of certain tumors to rapamycin.

LY294002 is known as an inhibitor of autophagy as well as of PI3K signaling (46, 47). It suppresses the activity of the downstream Akt. Interestingly, in this study, LY294002 induced rather than inhibited autophagy in malignant glioma cells by inhibiting Akt activity. One recent investigation showed that LY294002 induced autophagy during the mitotic stage of serum-starved normal rat kidney cells (48). Whether autophagy is induced or inhibited by LY294002 may depend on the cell type or treatment conditions, such as concentration used and exposure time. Another recent investigation reported that treatment with rapamycin plus LY294002 increased growth inhibition in prostate cancer cells (49), a finding consistent with our results.

In contrast to LY294002, UCN-01 is an Akt inhibitor that can modulate the PI3K/Akt pathway (24). This staurosporine analogue was originally isolated as a selective protein kinase C inhibitor from the culture broth of Streptomyces sp. (50). Because UCN-01 exhibits potent antitumor activity in vitro and in vivo in tumor models, it has been investigated as a possible anticancer agent (51). In a previous study, high concentrations of UCN-01 induced apoptosis in malignant glioma cells (52); at the concentrations used in our study, however, we did not detect a significant number of apoptotic cells. We found that treatment combining rapamycin with LY294002 or UCN-01, however, had a synergistic effect on development of autophagy and that the simultaneous inhibition of mTOR and PI3K/Akt activity has a cytotoxic effect on both rapamycin-sensitive and rapamycin-resistant tumor cells. The effects on autophagy indicate that the targeting of two such points within a complex signaling cascade could well have a synergistic effect. Our results also suggest that treatments combining these and other biologically targeted agents will make possible the use of toxic agents at concentrations below those reported to have dose-limiting side effects. Neshat et al. reported that tumor cells expressing high levels of Akt activity are more sensitive to rapamycin than are those expressing low levels (42). Although their results seem to disagree with ours, the discrepancy could be because of differences in the expression levels of cyclin D1 and c-myc regulated by Akt activity in tumor cells, as Gera et al. showed regarding the sensitivity to rapamycin (53).
In conclusion, we found that the sensitivity of malignant glioma cells to rapamycin is closely tied to the extent of autophagy induction and that the combination of rapamycin plus LY294002 or UCN-01 synergistically increases the sensitivity of malignant glioma cells to rapamycin. More importantly, in our study, this combination regimen sensitizes even rapamycin-resistant tumor cells. Taken together, our findings provide a strong rationale for using therapies that combine rapamycin and LY294002 or UCN-01 to treat patients who have malignant gliomas.

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