Phosphatidylinositol 3-Kinase Inhibition Broadly Sensitizes Glioblastoma Cells to Death Receptor– and Drug-Induced Apoptosis

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Abstract

The aberrant activity of the phosphatidylinositol 3-kinase (PI3K) pathway has been reported to correlate with adverse clinical outcome in human glioblastoma *in vivo*. However, the question of how this survival network can be successfully targeted to restore the sensitivity of glioblastoma to apoptosis induction has not yet been answered. Here, we report that inhibition of PI3K by LY294002 broadly sensitizes wild-type and mutant *PTEN* glioblastoma cells to both death receptor– and chemotherapy-induced apoptosis, whereas mammalian target of rapamycin (mTOR) inhibition is not sufficient to restore apoptosis sensitivity. LY294002 significantly enhances apoptosis triggered by tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), agonistic anti-CD95 antibodies, or several anticancer drugs (i.e., doxorubicin, etoposide, and vincristine) in a highly synergistic manner. In addition, LY294002 cooperates with TRAIL or doxorubicin to suppress colony formation, thus also showing a strong effect on long-term survival. Similarly, genetic knockdown of PI3K subunits p110α and/or p110β by RNA interference (RNAi) primes glioblastoma cells for TRAIL- or doxorubicin-mediated apoptosis. In contrast to PI3K inhibition, pharmacologic or genetic blockade of mTOR by RAD001 (everolimus), rapamycin, or RNAi fails to enhance TRAIL- or doxorubicin-induced apoptosis. Analysis of apoptosis pathways reveals that PI3K inhibition acts in concert with TRAIL or doxorubicin to trigger mitochondrial membrane permeabilization, caspase activation, and caspase-dependent apoptosis, which are abolished by the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone. Most importantly, PI3K inhibition by LY294002 sensitizes primary cultured glioblastoma cells obtained from surgical specimens to TRAIL- or chemotherapy-induced cell death. By showing that PI3K inhibition broadly primes glioblastoma cells for apoptosis, our findings provide the rationale for using PI3K inhibitors in combination regimens to enhance TRAIL- or chemotherapy-induced apoptosis in glioblastoma. [Cancer Res 2008;68(15):6271–80]

Introduction

Despite aggressive treatment protocols, the patients' prognosis for glioblastoma, the most common primary brain tumor, is still extremely poor (1). Hence, there is a need to develop novel treatment strategies to improve outcome. Evasion of apoptosis, the intrinsic suicide program of the cell, is a hallmark of human cancers (2). Defects in apoptosis may confer resistance to therapy because most current treatment approaches (e.g., chemotherapy or γ-irradiation) primarily act by triggering apoptosis in cancer cells (3). Thus, current attempts to improve the survival of glioblastoma patients will have to include strategies that specifically target tumor cell resistance to apoptosis.

There are two major apoptosis signaling pathways, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway, which both lead to activation of effector caspases (4). In the death receptor (extrinsic) pathway, stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily, such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors, results in activation of caspase-8, which in turn directly cleaves effector caspase-3 and indirectly promotes caspase-3 activation by initiating the mitochondrial pathway via cleavage of Bid (5, 6). The mitochondrial (intrinsic) pathway can be engaged by treatment with anticancer drugs, which leads to the release of apoptogenic factors, such as cytochrome *c*, from the mitochondrial interspace into the cytosol, where cytochrome *c* initiates caspase-3 activation by the cytochrome *c*/caspase-9/apaf-1 containing apoptosome complex (7). In addition, caspase-independent apoptosis and nonapoptotic modes of cell death, such as autophagy, have also been described (8, 9). The concept of directly triggering apoptosis by engaging death receptors is attractive for cancer therapy because death receptors have a direct link to the cell intrinsic death program. TRAIL is a prime candidate for cancer therapy because it predominantly kills cancer cells while sparing normal cells (10). However, many tumors remain resistant to TRAIL, often as a result of aberrant activation of survival pathways (11).

Phosphatidylinositol 3-kinase (PI3K) sits at the apex of a complex, multi-armed signaling network that not only mediates survival signals but also regulates proliferation, glucose metabolism, angiogenesis, and apoptosis (12–14). Whereas PI3K is presumed to activate most of its downstream targets via the Akt protein, the network rapidly diversifies into many distinct arms thereafter, one of which leads to the activation of the mammalian target of rapamycin (mTOR) and its targets (15). Furthermore, intricate interactions between allegedly independent survival networks, such as the PI3K- and Raf/mitogen-activated protein kinase kinase/extracellular signal–regulated kinase (MEK/ERK)–activated pathways, have also been described (16, 17). Deregulation of the PI3K-mediated signaling network plays a central role in tumor formation and progression and is frequently observed in a variety of human cancers, including glioblastoma, as a result of, for example, genetic alterations or deregulated growth factor signaling

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(18, 19). Recently, a correlation between PI3K pathway activation and highest-grade tumors, decreased apoptosis in tumor samples, and adverse clinical outcome was shown in primary samples of human gliomas (20).

The PI3K network has attracted much attention because it provides promising targets for cancer drug discovery, and a series of inhibitors of the PI3K pathway are currently under development (21, 22). However, the question whether such inhibitors can be exploited to restore the sensitivity of glioblastoma to apoptosis induction has not yet been answered. To address this question, we investigated in the present study the role of PI3K-mediated signaling in the regulation of apoptosis in glioblastoma.

Materials and Methods

Cell culture and chemicals. Glioblastoma cell lines were obtained from the American Type Culture Collection and cultured in DMEM or RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Biochrom), 1 mmol/L glutamine (Biochrom), 1% penicillin/streptavidin (Biochrom), and 25 mmol/L HEPES (Biochrom) as described previously (23). LN18 and LN229 cells express wild-type *PTEN*, whereas all the other cell lines used harbor mutant *PTEN* (24). For experiments, cells were seeded at $0.4 \times 10^5/\text{cm}^2$ to $0.8 \times 10^5/\text{cm}^2$, allowed to settle for 24 h, washed once with PBS, and serum starved for 24 h before treatment.

Primary cultured glioblastoma cells. Primary glioblastoma cells were isolated by mechanical disaggregation from surgical specimens obtained from three patients with grade 4 glioma (GB1–GB3) and cultured in DMEM supplemented with 10% FCS (Biochrom), 1 mmol/L glutamine (Biochrom), 1% penicillin/streptavidin (Biochrom), and 25 mmol/L HEPES (Biochrom). The study was approved by the Ethics Committee, Medical Faculty, University of Ulm. Clinical and histopathologic data on patients' tumors are summarized in Supplementary Table S1. Purity of cultured glioblastoma cells was >90% as assessed by expression of microtubule-associated protein 2 (examples are shown in Supplementary Fig. S5*A*; ref. 25). Expression of PTEN protein was not detectable in any of the cultured samples (Supplementary Fig. S5*B*).

Determination of apoptosis and necrosis. Glioblastoma cells were pretreated for 1 h with kinase inhibitors LY294002 (Calbiochem), wortmannin (Sigma), RAD001 everolimus; kindly provided by H. Lane (Novartis Institute for BioMedical Research, Oncology Basel, Novartis Pharma AG), or rapamycin (Sigma), and then treated with TRAIL (R&D Systems, Inc.), agonistic anti-CD95 (APO-1) antibody (23) and protein A (5 ng/mL; Sigma), doxorubicin, etoposide, or vincristine (all from Sigma), or *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk; Bachem) in the presence or absence of inhibitors in serum-free medium. Apoptosis was determined by fluorescence-activated cell sorting (FACS) analysis (FACScan, BD Biosciences) of DNA fragmentation of propidium iodide–stained nuclei as described previously (23). The percentage of specific apoptosis was calculated as follows: $100 \times$ [experimental apoptosis (%) − spontaneous apoptosis (%)] / [100% − spontaneous apoptosis (%)]. Necrosis was determined by assessment of cell membrane integrity. Briefly, cells were harvested, washed once with ice-cold PBS, incubated for 10 min with a 5% propidium iodide solution, and analyzed by flow cytometry. Untreated cells were used as a negative control, and cells treated for 12 h with 6% hydrogen peroxide (Mallinckrodt Baker) as a positive control.

Colony forming assay. Cells (10,000 or 20,000) were treated in suspension at 37°C with TRAIL for 12 h or with doxorubicin for 1 h, respectively, in the presence of either 20 μmol/L LY294002 or 0.2% DMSO (as control) and were then seeded in a 55-cm^2 tissue culture plate. Medium was exchanged 24 h after seeding and colonies were stained after 15 to 20 d with 0.75% crystal violet, 50% ethanol, 0.25% NaCl, and 1.57% formaldehyde. Colonies (>50 cells) were counted and the percentage of surviving colonies relative to solvent-treated controls was calculated.

Knockdown of PI3K and mTOR by RNA interference. Glioblastoma cells were seeded at $0.2 \times 10^5/\text{cm}^2$ in a 12-well tissue culture plate and allowed to settle overnight. Cells were transfected with 60 pmol of PIK3CA Stealth RNAi (PIK3CAHSS10800 4-6, Invitrogene), PIK3CB Stealth RNAi (PIK3CAHSS10800 7-9, Invitrogene), mTOR1 SMARTpool siRNA (M-003008-01, Dharmacon), or nontargeting control siRNA (D001206-13-20, Dharmacon) using TransMessenger transfection reagent (Qiagen) in a total volume of 378 μL/well. Transfection medium was replaced by complete medium after 3.5 h. Transfection was repeated the next day and cells were serum starved overnight before they were treated for 48 h with TRAIL or doxorubicin.

Western blot analysis. Western blot analysis was done as previously described (23) using rabbit anti–phospho-Akt (Ser^{473}) antibody (1:1,000), rabbit anti–phospho-S6 ribosomal protein ($\text{Ser}^{235/236}$) antibody (1:1,000), rabbit anti–S6 ribosomal protein antibody (1:1,000), mouse anti–phospho-ERK ($\text{Thr}^{202}/\text{Tyr}^{204}$) (E10) antibody (1:2,000), rabbit anti-mTOR antibody (1:1,000), rabbit anti–PI3K p110α antibody (1:1,000; Cell Signaling), mouse anti-Akt antibody (1:500; BD Bioscience), rabbit anti-ERK antibody (1:10,000; Sigma), and rabbit anti–PI3K p110β antibody (1:500, Abcam), followed by goat-anti-mouse IgG or goat-anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000; Santa Cruz Biotechnology). Enhanced chemiluminescence was used for detection (Amersham Bioscience). Mouse anti–β-actin antibody (1:5,000; Sigma) or mouse anti–α-tubulin antibody (1:3,000; Calbiochem) was used as a loading control. All Western blots shown are representative of at least two independent experiments. Densitometry analysis was done using ImageJ (provided online by the NIH[3]).

Caspase activity. Caspase activity was determined using fluorimetric substrates for caspase-3 (DEVD-R110; Roche Diagnostics), caspase-8 (IETD-AFC; BioCat Inc.), caspase-9 (LEHD-AFC; Bachem), or caspase-2 (VDAVD-AFC; Bachem) according to the manufacturers' instructions. Caspase activity was measured with a 1420 Victor Multilabel Counter (Perkin-Elmer).

Determination of mitochondrial membrane potential and cytochrome *c* release. To determine mitochondrial transmembrane potential, cells were incubated with CMXRos (1 μmol/L; Molecular Probes) for 30 min at 37°C and immediately analyzed by flow cytometry. Cytochrome *c* release was determined in permeabilized cells using mouse anti–cytochrome *c* monoclonal antibody (1:40; BD Biosciences) as previously described (26).

Confocal microscopy. To visualize mitochondria, cells were cultured in the presence of 200 nmol/L MitoTracker Red CMXRos (Molecular Probes) for 40 min before paraformaldehyde fixation, permeabilization, and mounting, as previously described (27). Cells were stained with mouse anti–cytochrome *c* monoclonal antibody (1:100; BD Biosciences), followed by incubation with FITC-conjugated antimouse IgG (1:100; Chemicon International), and analyzed by confocal microscopy (Leica DM IRB).

Statistical analysis. Statistical significance was assessed by Student's *t* tests (two-tailed distribution, two-sample unequal variance).

Results

Sensitization of glioblastoma cells for death receptor– and chemotherapy-induced apoptosis by PI3K inhibition. To analyze the role of PI3K signaling in the regulation of apoptosis in human glioblastoma, we selected from the glioblastoma cell lines U87MG, U138MG, and A172, which express mutant *PTEN* and exhibit relatively high constitutive Akt phosphorylation, as well as LN18 and LN229 cells, which express wild-type *PTEN* and exhibit relatively low constitutive Akt phosphorylation (Fig. 1*A, top*). We defined constitutive Akt phosphorylation, which was taken as a surrogate for PI3K activity, as phosphorylation 24 hours after complete serum deprivation because growth factors in the serum are known to stimulate PI3K signaling (28). The low phosphorylation status of Akt under serum deprivation correlates with wild-type *PTEN* status in some cell lines (e.g., LN18 and LN229), but not in others (e.g., U373MG and T98G), indicating that additional factors besides PTEN are involved in the regulation of Akt

[3] http://rsb.info.nih.gov/ij/

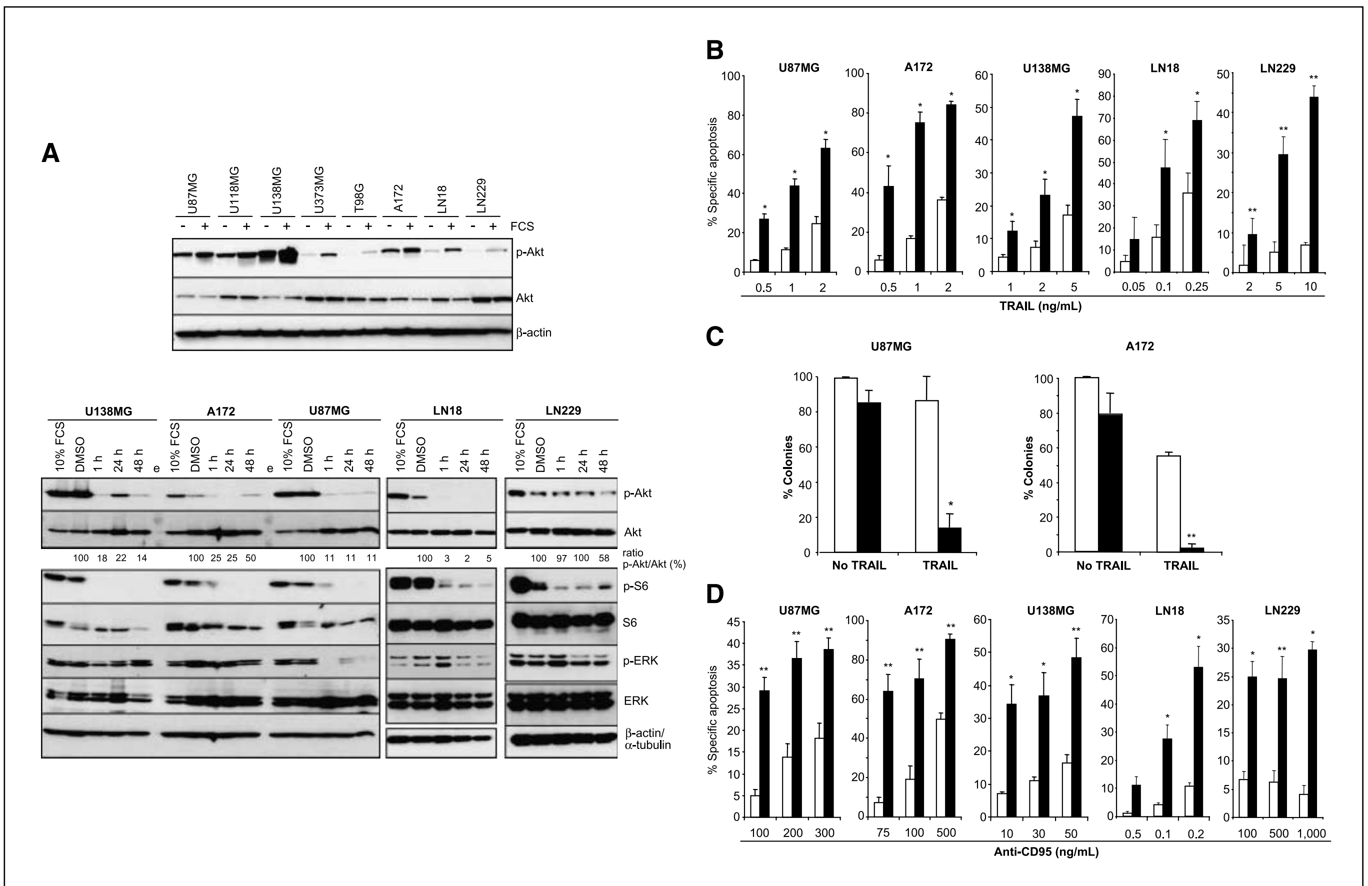

Figure 1. Sensitization of glioblastoma cells for death receptor–induced apoptosis by PI3K inhibition. *A,* activation of the PI3K pathway in glioblastoma cell lines. Protein expression levels and phosphorylation status of Akt, S6 ribosomal protein, ERK, β-actin, and α-tubulin were analyzed by Western blotting in glioblastoma cell lines cultured for 24 h in medium containing either 0% FCS (−) or 10% FCS (+; *top*) or treated with 20 μmol/L LY294002 for the indicated time points (*bottom*). The relative phosphorylation of Akt was densitometrically determined and is expressed in percent as phospho-Akt/Akt ratio, with the DMSO control set at 100%. *e,* empty lane. β-Actin and α-tubulin served as loading controls. *B,* inhibition of PI3K sensitizes glioblastoma cells to TRAIL-induced apoptosis. Glioblastoma cells were pretreated or not with 20 μmol/L LY294002 for 1 h and then treated for 48 h with TRAIL in the presence (■) or absence (□) of 20 μmol/L LY294002. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei; the percentage of specific apoptosis is shown. *Columns,* mean of three or two (for LN229) independent experiments done in triplicate; *bars,* SE. Apoptosis induced by LY294002 alone at 48 h: U87MG cells, 21%; A172 cells, 10%; U138MG cells, 20%; LN18 cells, 24%; LN229 cells, 14%. *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without LY294002 (Student's *t* test). *C,* inhibition of PI3K cooperates with TRAIL to suppress colony formation. Before seeding, cell suspensions were treated for 12 h with 20 μmol/L LY294002 (■) or DMSO (□) and/or TRAIL (1 ng/mL for U87MG; 2 ng/mL for A172). At day 20, colonies were stained with crystal violet and counted. Colonies are shown as percentage of solvent-treated controls. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without LY294002 (Student's *t* test). *D,* inhibition of PI3K sensitizes glioblastoma cells to anti-CD95-induced apoptosis. Glioblastoma cells were pretreated or not with 20 μmol/L LY294002 for 1 h and then treated for 24 h (48 h for LN229) with anti-CD95 agonistic antibodies in the presence (■) or absence (□) of 20 μmol/L LY294002. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei; the percentage of specific apoptosis is shown. *Columns,* mean of three or two (for LN18 and LN229) independent experiments done in triplicate; *bars,* SE. Apoptosis induced by LY294002 alone at 24 h: U87MG cells, 9%; A172 cells, 6%; U138MG cells, 16%; LN18 cells, 21%; LN229 cells, 14% at 48 h. *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without LY294002 (Student's *t* test).

phosphorylation (14). Initially, we used pharmacologic inhibitors of PI3K to interfere with constitutive Akt activity (Fig. 1*A, bottom*). Phosphorylation of S6 ribosomal protein, a surrogate readout for mTOR activity, was also inhibited by LY294002 for a prolonged time period of up to 48 hours, indicating that Akt and S6 ribosomal protein are phosphorylated in a PI3K-dependent manner (Fig. 1*A, bottom*). In U87MG cells, LY294002 also repressed phosphorylation of ERK (Fig. 1*A, bottom*), a target of MEK, which can also be activated as a consequence of interactions between the PI3K and Raf/MEK/ERK pathways (16, 17), pointing to a cell line–specific cross talk between the two signaling networks.

To explore the role of PI3K in the regulation of apoptosis, we selected two prototypic classes of stimuli that are known to trigger apoptosis via distinct pathways: death-inducing ligands that stimulate the death receptor (extrinsic) pathway of apoptosis and anticancer agents that activate the mitochondrial (intrinsic) pathway. Inhibition of PI3K by LY294002 significantly enhanced TRAIL-induced apoptosis in a dose- and time-dependent manner (Fig. 1*B*; Supplementary Fig. S1*A*). Calculation of combination index revealed that the interaction between LY294002 and TRAIL to trigger apoptosis is synergistic (Supplementary Table S2). To further verify sensitization provided by PI3K inhibition, we used wortmannin as an additional PI3K inhibitor, which also sensitized glioblastoma cells to TRAIL-induced apoptosis (Supplementary Fig. S1*B*). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay, which was used as another method to monitor cytotoxicity, also showed that PI3K inhibition and TRAIL acted in concert to reduce viability of glioblastoma cells (Supplementary Fig. S1*C*). Moreover, we conducted clonogenic assays to explore whether PI3K inhibition has an effect on long-term survival as well. Importantly, LY294002 cooperated with TRAIL to suppress colony formation of glioblastoma cells

compared with either treatment alone (Fig. 1*C*). Besides TRAIL, we extended our studies to CD95 as another death receptor system. LY294002 also significantly increased CD95-induced apoptosis in a synergistic fashion (Fig. 1*D*; Supplementary Table S2). This shows that PI3K inhibition sensitizes glioblastoma cells to death receptor–triggered apoptosis.

We then tested the effect of PI3K inhibition in combination with anticancer drugs, prototypic stimuli that trigger apoptosis via the mitochondrial pathway. LY294002 synergized with several chemotherapeutic drugs (i.e., doxorubicin, etoposide, and vincristine) to induce apoptosis in a dose-dependent fashion (Fig. 2*A* and *B*; Supplementary Fig. S2*A*). Interactions between LY294002 and cytotoxic drugs were synergistic, as indicated by combination index (Supplementary Table S2). LY294002 and doxorubicin also cooperated to reduce cell viability when cytotoxicity was assessed by an MTT viability assay (Supplementary Fig. S2*B*). Furthermore, LY294002 and doxorubicin acted in concert to suppress colony formation of glioblastoma cells, showing that the combination treatment was superior to either treatment alone in inhibiting long-term survival (Fig. 2*C*). Together, this set of experiments shows that PI3K inhibition primes glioblastoma cells for both death receptor– and chemotherapy-induced apoptosis. In subsequent experiments, we used TRAIL as a prototypic trigger of the extrinsic apoptosis pathway and doxorubicin as a prototypic stimulus that activates the intrinsic pathway.

Effect of mTOR inhibition on TRAIL- or doxorubicin-induced apoptosis. Next, we blocked a downstream arm of the PI3K network at the level of mTOR using pharmacologic inhibitors. Control experiments confirmed that the mTOR inhibitor everolimus inhibited phosphorylation of S6 ribosomal protein (Fig. 3*A, top*). Everolimus also decreased the expression levels of the S6 ribosomal protein (Fig. 3*A, top*), possibly due to inhibition of protein translation. Furthermore, everolimus caused a transient increase in Akt phosphorylation at 1 and 4 hours in U87MG cells

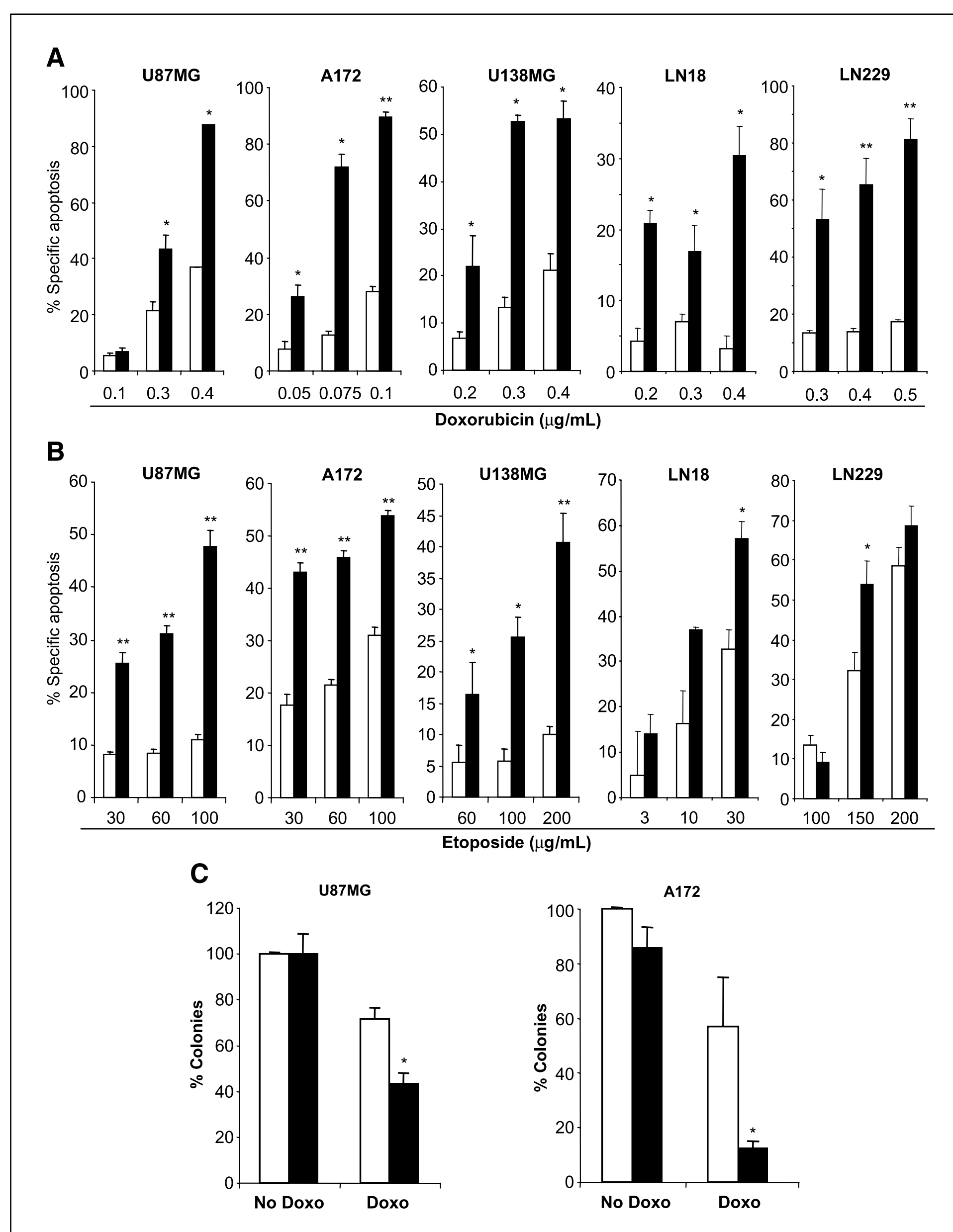

Figure 2. Sensitization of glioblastoma cells for chemotherapy-induced apoptosis by PI3K inhibition. *A* and *B,* inhibition of PI3K sensitizes glioblastoma cells to doxorubicin- and etoposide-induced apoptosis. Glioblastoma cells were pretreated or not with 20 μmol/L LY294002 for 1 h and then treated for 48 h with doxorubicin (*A*) or for 48 h (72 h for U138MG) with etoposide (*B*) in the presence (■) or absence (□) of 20 μmol/L LY294002. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei; the percentage of specific apoptosis is shown. *Columns,* mean of three or two (for *LN18* and *LN229*) independent experiments done in triplicate; *bars,* SE. Apoptosis induced by LY294002 alone: U87MG cells, 21%; A172 cells, 10%; U138MG cells, 20%; LN18 cells, 24%; LN229, 14%. For statistical analysis, Student's t test was done, comparing treatment with and without LY294002 (*, $P < 0.05$; **, $P < 0.001$). *C,* inhibition of PI3K cooperates with doxorubicin to suppress colony formation. Before seeding, cell suspensions were treated for 1 h with 20 μmol/L LY294002 (■) or DMSO (□) and/or doxorubicin (*Doxo*; 0.1 μg/mL for U87MG, 0.03 μg/mL for A172). At day 20 (U87MG) or day 16 (A172), colonies were stained with crystal violet and counted. Colonies are shown as percentage of solvent-treated controls. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without LY294002 (Student's t test).

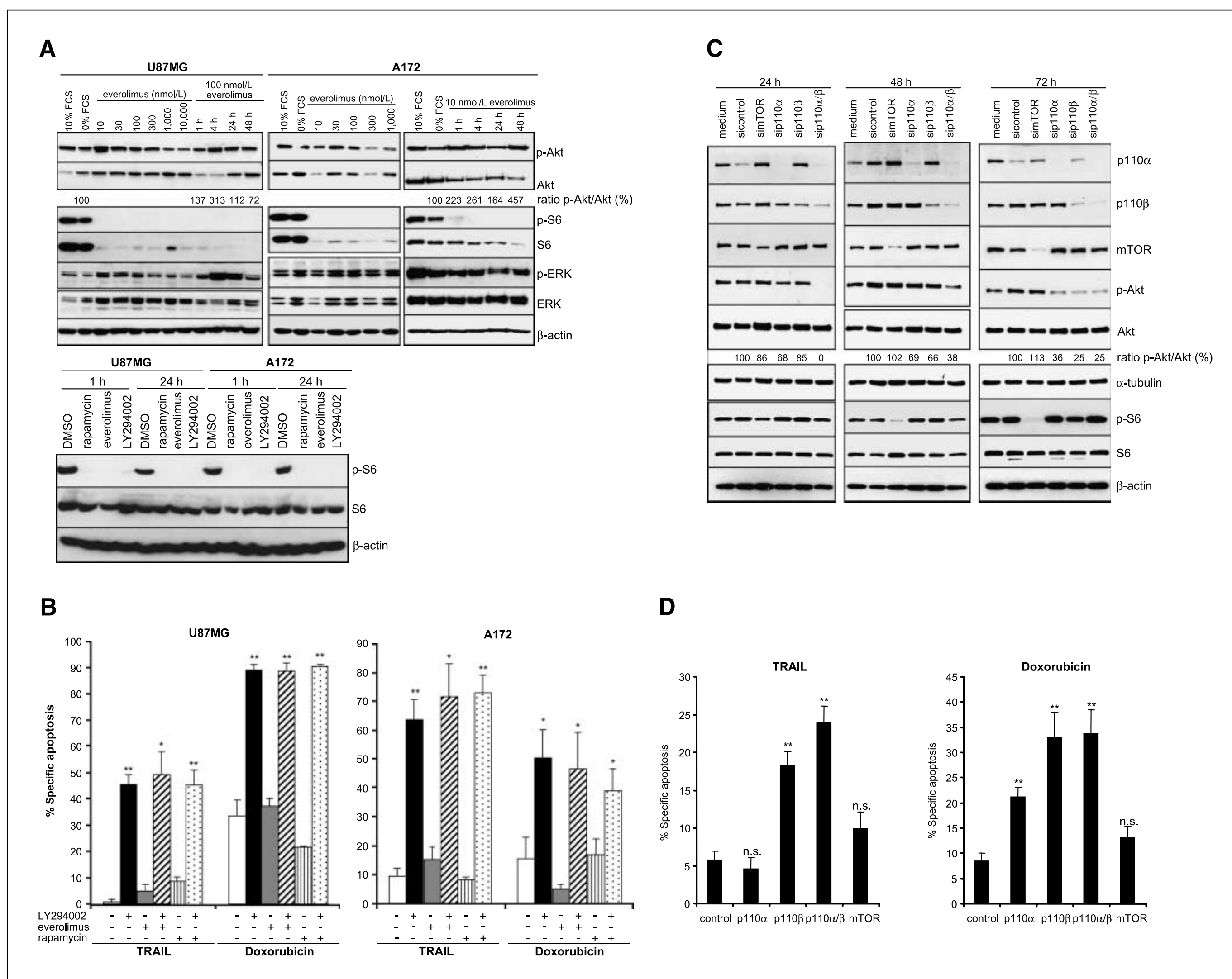

Figure 3. Inhibition of PI3K, rather than of mTOR, sensitizes glioblastoma cells to doxorubicin- and TRAIL-induced apoptosis. *A,* inhibition of mTOR by everolimus and rapamycin. *Top,* glioblastoma cells were incubated in medium containing 10% or 0% FCS for 24 h, with everolimus for 24 h at the indicated concentrations, or with 100 nmol/L (U87MG) or 10 nmol/L everolimus (A172) at the indicated time points. *Bottom,* glioblastoma cells were treated with 100 nmol/L rapamycin, 10 nmol/L (U87MG) or 100 nmol/L (A172) everolimus, 20 μmol/L LY294002 or 0.2% solvent (DMSO) for 1 and 24 h. Protein expression levels and phosphorylation status of Akt, S6 ribosomal protein, ERK, and β-actin were analyzed by Western blotting. The relative phosphorylation of Akt was densitometrically determined and is expressed in percent as phospho-Akt/Akt ratio, with the DMSO control set at 100%. *B,* inhibition of mTOR fails to enhance TRAIL- or doxorubicin-induced apoptosis. Glioblastoma cells were pretreated for 1 h with kinase inhibitors [i.e., 20 μmol/L LY294002, 10 nmol/L (A172) or 100 nmol/L (U87MG) everolimus, or 100 nmol/L rapamycin, or with 20 μmol/L LY294002 plus 10 nmol/L (A172), 100 nmol/L (U87MG) everolimus, or 100 nmol/L rapamycin] and then incubated with 1 ng/mL TRAIL for 24 h or with 0.3 μg/mL doxorubicin (U87MG) or 0.075 μg/mL doxorubicin (A172) for 48 h, with (■) or without (□) 20 μmol/L LY294002, 100 nmol/L (U87MG), or 10 nmol/L (A172) everolimus (■); 100 nmol/L (U87MG) or 10 nmol/L (A172) everolimus plus 20 μmol/L LY294002 (▨); 100 nmol/L rapamycin (▥); or 100 nmol/L rapamycin plus 20 μmol/L LY294002 (⬚). Apoptosis was measured by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei; the percentage of specific apoptosis is shown. *Columns,* mean of two independent experiments done in triplicate; *bars,* SE. Apoptosis induced by LY294002 alone: U87MG cells, 9% (24 h) and 21% (48 h); A172 cells, 6% (24 h) and 10% (48 h). Apoptosis induced by everolimus alone: U87MG cells, 7% (24 h) and 21% (48 h); A172 cells, 16% (24 h) and 18% (48 h). Apoptosis induced by rapamycin alone: U87MG cells, 7% (24 h) and 9% (48 h); A172 cells, 22% (24 h) and 30% (48 h). *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without kinase inhibitors (Student's *t* test). *C* and *D,* knockdown of PI3K sensitizes glioblastoma cells to TRAIL- or doxorubicin-induced apoptosis. U87MG cells were transfected with siRNA duplex oligoribonucleotides against p110α (*sip110α*) and/or p110β (*sip110β*), mTOR complex 1 (*simTOR*) or nontargeting siRNA (*sicontrol*). *C*, knockdown of p110α, p110β, and mTOR expression and protein expression levels and phosphorylation status of Akt and S6 ribosomal protein were analyzed by Western blotting at the indicated time points. α-Tubulin and β-actin were used as loading controls. The relative phosphorylation of Akt was densitometrically determined and is expressed in percent as phospho-Akt/Akt ratio, with the nontargeting siRNA set at 100%. *D*, U87MG cells were treated 24 h after transfection with 2 ng/mL TRAIL (*left*) or with 0.2 μg/mL doxorubicin (*right*) for 48 h. Apoptosis was determined by analysis of DNA fragmentation of propidium iodide–stained nuclei; the percentage of specific apoptosis is shown. *Columns,* mean of three independent experiments done in triplicate; *bars,* SE. Apoptosis induced by siRNA duplex oligoribonucleotides alone: control, 14%; p110α, 3%; p110β, 17%; p100α/β, 15%; mTOR, 19%. *, $P < 0.05$; **, $P < 0.001$, comparing nontargeting siRNA with target-specific siRNA on TRAIL or doxorubicin treatment (Student's *t* test); *n.s.,* not significant.

and a sustained increase in Akt phosphorylation up to 48 hours in A172 cells (Fig. 3*A*), a phenomenon previously described for mTOR inhibitors (29–31). In addition, we used a second mTOR inhibitor, rapamycin, which also blocked phosphorylation of S6 ribosomal protein (Fig. 3*A, bottom*). Despite inhibition of mTOR activity, both everolimus and rapamycin failed to sensitize glioblastoma cells to TRAIL- or doxorubicin-induced apoptosis (Fig. 3*B*), whereas everolimus sensitized neuroblastoma cells to apoptosis (Supplementary Fig. S3). In addition, combining everolimus or rapamycin with LY294002 did not further increase

TRAIL- or doxorubicin-mediated apoptosis compared with LY294002 alone (Fig. 3*B*). These findings indicate that mTOR inhibition is not sufficient to restore the apoptosis sensitivity of glioblastoma cells to TRAIL or doxorubicin.

Knockdown of PI3K, rather than of mTOR, sensitizes glioblastoma cells to TRAIL- or doxorubicin-induced apoptosis. In a genetic approach to interfere with aberrant PI3K signaling, we knocked down individual components of the network by RNA interference (RNAi). Transfection of glioblastoma cells with siRNA against PI3K subunits p110α and p110β (individually or combined) or mTOR1 led to selective down-regulation of the respective siRNA targets and to a concomitant decrease in phosphorylation of Akt (in case of p110α and/or p110β) or of S6 ribosomal protein (in case of mTOR; Fig. 3*C*). Interestingly, knockdown of p110β, alone or in combination with p110α, significantly increased TRAIL-induced apoptosis compared with cells transfected with control siRNA, whereas silencing of mTOR did not alter sensitivity to TRAIL-induced apoptosis (Fig. 3*D, left*). Furthermore, knockdown of p110α and p110β, individually or combined, but not knockdown of mTOR, significantly enhanced doxorubicin-induced apoptosis (Fig. 3*D, right*). These findings indicate that both p110α and p110β are involved in regulating doxorubicin-induced apoptosis, whereas TRAIL-induced apoptosis is predominantly regulated by p110β. This is in line with recent findings suggesting that p110α and p110β have distinct, yet overlapping, functions. Whereas p110α was reported to be the major isoform driving proliferation in glioblastoma (32), p110β was found to be a key regulator of apoptosis and proliferation in endometrial carcinomas (33). Together, this set of experiments shows that genetic silencing of PI3K, rather than of mTOR, sensitizes glioblastoma cells to TRAIL- or doxorubicin-mediated apoptosis.

Effect of mTOR inhibition on necrotic cell death. To explore the possibility that mTOR inhibition sensitizes glioblastoma cells to necrotic cell death, we assessed cell permeability by flow cytometry. No significant changes in cell permeability were observed on treatment with mTOR inhibitors alone or in combination with TRAIL or doxorubicin (Fig. 4). Hydrogen peroxide was used as a positive control for induction of necrosis (Fig. 4). These findings indicate that mTOR inhibitors do not enhance necrotic cell death on treatment with TRAIL or doxorubicin in glioblastoma cells.

Inhibition of PI3K sensitizes glioblastoma cells to TRAIL- or doxorubicin-induced caspase activation and mitochondrial perturbations. To gain insight into the molecular mechanisms mediating apoptosis sensitization by PI3K inhibition, we used the broad range caspase inhibitor zVAD.fmk to test the requirement of caspases. Apoptosis on treatment with LY294002 and TRAIL or doxorubicin was almost completely blocked in the presence of zVAD.fmk (Fig. 5*A*), showing that apoptosis was mediated by caspases. Next, we monitored activation of the caspase cascade by enzymatic caspase assays using fluorogenic caspase substrates. Notably, LY294002 significantly increased TRAIL- or doxorubicin-induced activation of caspase-2, caspase-3, caspase-8, and caspase-9 (Fig. 5*B*).

To investigate whether apoptosis sensitization by LY294002 involved the mitochondrial pathway, we analyzed parameters of mitochondrial membrane permeabilization. LY294002 significantly enhanced TRAIL-induced loss of mitochondrial membrane potential and also cooperated with TRAIL or doxorubicin to induce cytochrome *c* release from mitochondria (Fig. 5*C*). Confocal microscopy studies confirmed that LY294002 acted in concert with TRAIL or doxorubicin to trigger mitochondrial cytochrome *c* release (Fig. 5*D*; Supplementary Fig. S4). This set of experiments shows that PI3K inhibition sensitizes glioblastoma cells to TRAIL- or doxorubicin-induced caspase activation and mitochondrial perturbations.

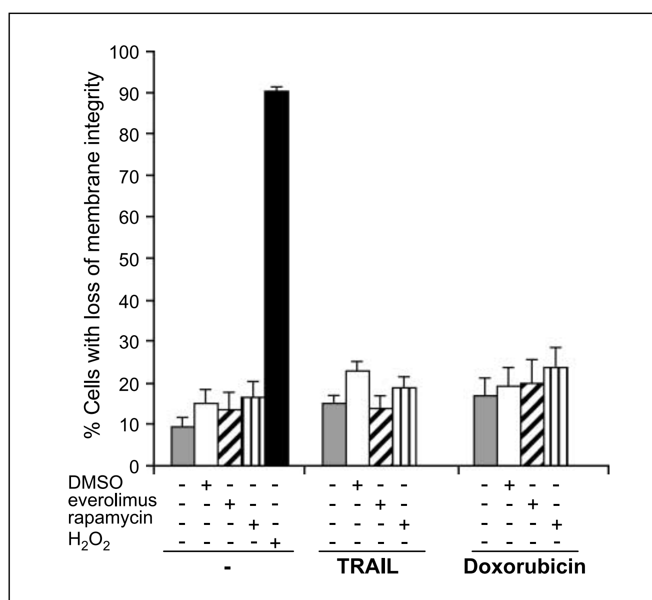

Figure 4. Effect of mTOR inhibitors on necrotic cell death. U87MG cells were left untreated or were pretreated for 1 h with 100 nmol/L everolimus or 100 nmol/L rapamycin and then incubated for 24 h with 2 ng/mL TRAIL or 0.3 μg/mL doxorubicin without (■) or with 0.001% DMSO (□), 100 nmol/L of everolimus (▨), or rapamycin (▥). As a positive control for necrosis, cells were treated for 12 h with a 6% solution of hydrogen peroxide (■). Cells were analyzed by flow cytometry for loss of cell membrane integrity by propidium iodide uptake. *Columns,* mean of two independent experiments done in triplicate; *bars,* SE.

Inhibition of PI3K sensitizes primary cultured glioblastoma cells to TRAIL- or doxorubicin-induced apoptosis. Finally, to validate the results obtained in established cell lines, we extended our studies to primary cultured glioblastoma samples obtained from surgical specimens. Control experiments showed that PI3K inhibition by LY294002 caused inhibition of Akt phosphorylation (Fig. 6*A, top*). Importantly, LY294002 significantly increased TRAIL- and doxorubicin-induced apoptosis and also cooperated with TRAIL and doxorubicin to reduce viability of primary cultured glioblastoma cells (Fig. 6*B* and *C*; Supplementary Fig. S5*C* and *D*). By comparison, the mTOR inhibitors everolimus and rapamycin did not significantly increase TRAIL- and doxorubicin-induced apoptosis (Fig. 6*D*), although they inhibited the phosphorylation of S6 ribosomal protein (Fig. 6*A, bottom*). This shows that PI3K inhibition, but not mTOR inhibition, primes primary cultured, patient-derived glioblastoma cells for TRAIL- or chemotherapy-induced apoptosis.

Discussion

Despite aggressive treatment strategies, patients with glioblastoma still have a dismal prognosis, which highlights the need for novel treatment approaches (1). Aberrant signaling of PI3K, a key mediator of cell survival signals, has been associated with increasing tumor grade, decreased levels of apoptosis, and adverse clinical outcome in human gliomas (20). Because most anticancer therapies primarily act by inducing apoptosis in cancer cells,

resistance to current treatment protocols may be caused by defects in apoptosis programs (e.g., abnormal activation of the PI3K pathway; ref. 3). We therefore asked whether targeting this survival cascade could be exploited to enhance the apoptosis sensitivity of glioblastoma.

Targeting PI3K for apoptosis sensitization of glioblastoma. Here, we report that inhibition of PI3K is an efficient strategy to broadly sensitize glioblastoma cells to apoptosis induction, either via the death receptor (extrinsic) apoptosis pathway or via the mitochondrial (intrinsic) apoptosis pathway. This conclusion is supported by several independent lines of evidence. First, PI3K inhibition by LY294002 enhances apoptosis via a synergistic interaction with death receptor stimulation or with chemotherapeutic drugs doxorubicin, etoposide, and vincristine, all of which trigger the mitochondrial pathway. Second, the combination treatment is superior to single agents in suppressing long-term survival in clonogenic assays. Third, genetic silencing of PI3K isoforms by RNAi also significantly enhances TRAIL- or doxorubicin-induced apoptosis similar to pharmacologic inhibitors. In contrast, pharmacologic or genetic inhibition of mTOR fails to

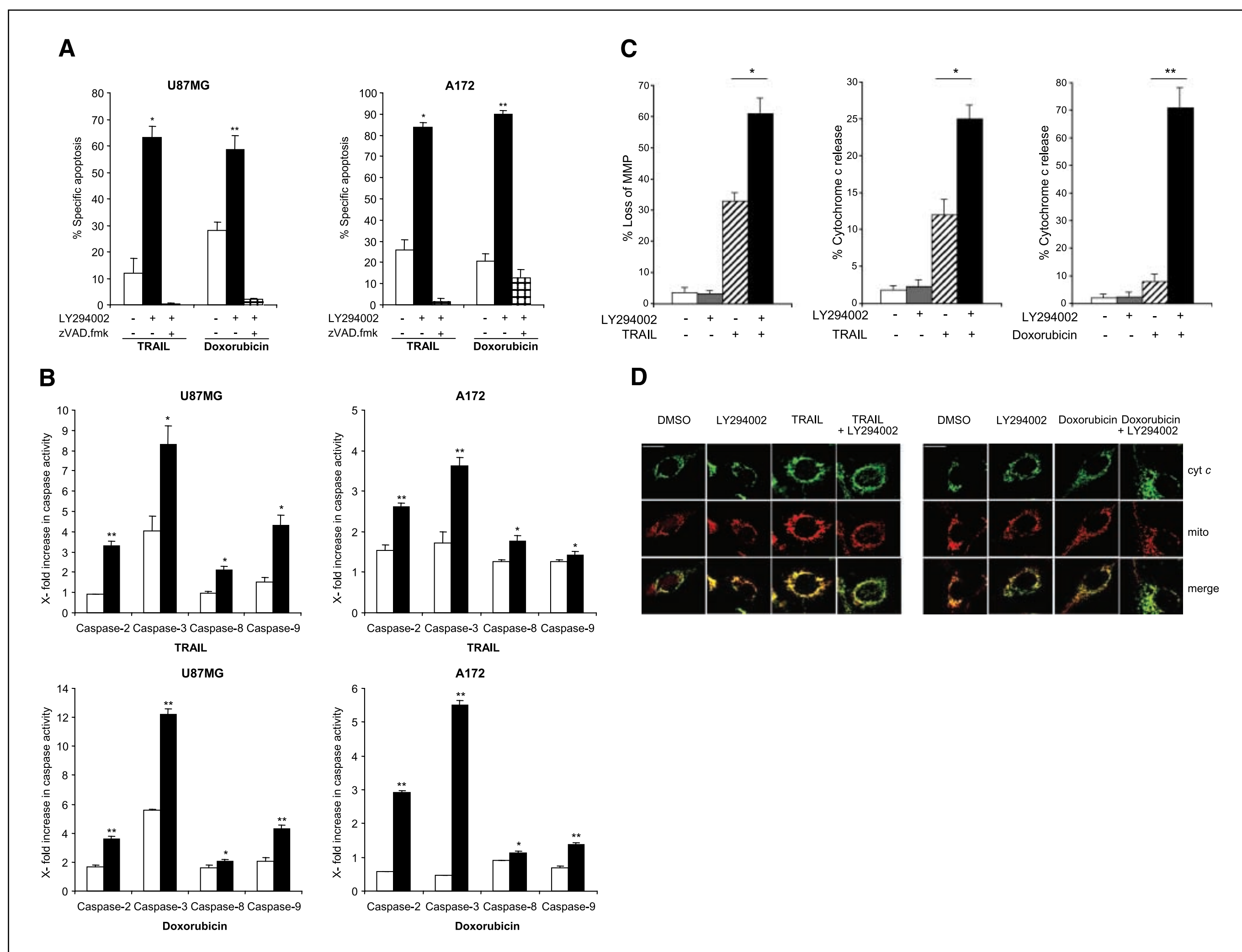

Figure 5. Inhibition of PI3K sensitizes glioblastoma cells to TRAIL- or doxorubicin-induced caspase activation and mitochondrial perturbations. *A,* sensitization for TRAIL- or doxorubicin-induced apoptosis by PI3K inhibition is caspase dependent. Glioblastoma cells were pretreated or not with 20 μmol/L LY294002 for 1 h and then treated with 2 ng/mL TRAIL or 0.3 μg/mL (U87MG) or 0.1 μg/mL (A172) doxorubicin for 48 h with (■) or without (□) 20 μmol/L LY294002 or 50 μmol/L zVAD.fmk plus 20 μmol/L LY294002 (▦). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei; the percentage of specific apoptosis is shown. *Columns,* mean of three independent experiments done in duplicate; *bars,* SE. Apoptosis induced by zVAD.fmk alone at 48 h: U87MG and A172 cells, 5%. Apoptosis induced by LY294002 alone at 48 h: U87MG cells, 21%; A172 cells, 10%. *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without LY294002 (Student's *t* test). *B,* inhibition of PI3K sensitizes for TRAIL- or doxorubicin-induced caspase activation. Glioblastoma cells were pretreated or not with 20 μmol/L LY294002 for 1 h and then treated with 2 ng/mL TRAIL, 0.3 μg/mL doxorubicin (U87MG), or 0.1 μg/mL doxorubicin (A172) with (■) or without (□) 20 μmol/L LY294002. Caspase activity was determined by enzymatic assay using fluorogenic caspase substrates as described in Materials and Methods. *X*-fold increase in caspase activity compared with cells treated with DMSO or LY294002 alone is shown. *Columns,* mean of one representative experiment done in triplicate; *bars,* SD. Similar results were obtained in three independent experiments. *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without LY294002 (Student's *t* test). *C* and *D,* inhibition of PI3K sensitizes for TRAIL-induced loss of mitochondrial membrane potential and cytochrome *c* release. A172 cells were pretreated or not with 20 μmol/L LY294002 for 1 h and then left untreated (□) or treated with 2 ng/mL TRAIL for 12 (*D*) or 24 h (*C*) or with 0.1 μg/mL doxorubicin for 24 h (*D*) or 48 h (*C*), without (▨) or with 20 μmol/L LY294002 (■). In *C,* mitochondrial membrane potential (*MMP*) and cytochrome *c* release were assessed by flow cytometry. *Columns,* mean of three independent experiments done in triplicate; *bars,* SE. *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without LY294002 (Student's *t* test). For immunohistochemical analysis of cytochrome *c* release (*D*), cells were fixed, stained for cytochrome *c* (*cyt c*; *green*) and mitochondria (*mito*; *red*), and analyzed by confocal microscopy. *Bar,* 10 μm. Results of one representative experiment; similar results were obtained in three independent experiments.

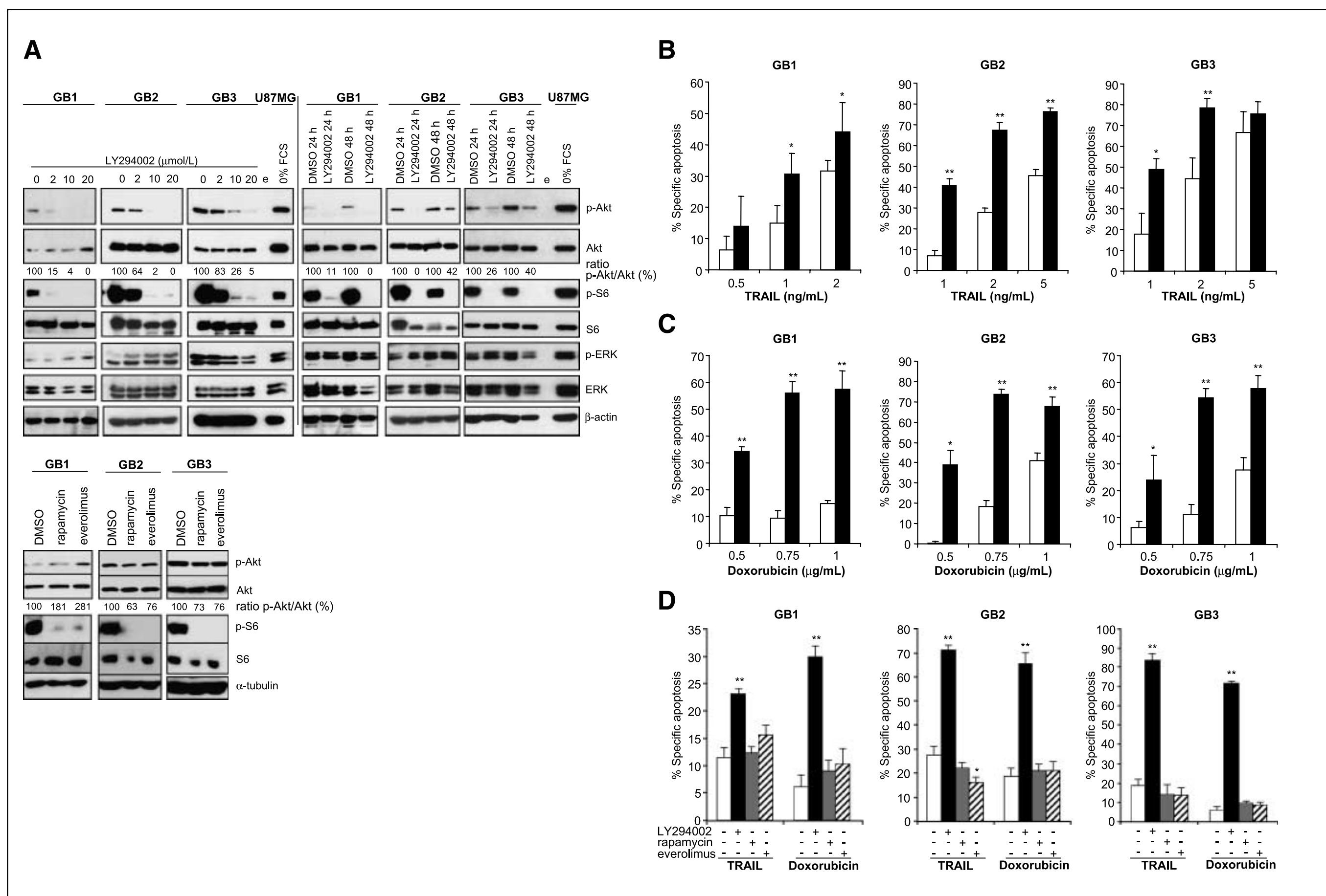

Figure 6. Primary cultured glioblastoma cells can also be sensitized to TRAIL- or doxorubicin-induced apoptosis by PI3K, but not mTOR inhibition. *A,* activation of the PI3K pathway in primary cultured glioblastoma cells. *Top,* primary cultured glioblastoma cells (GB1, GB2, and GB3) were treated for 24 h with LY294002 at the indicated concentrations (*left*) or with 20 μmol/L LY294002 for the indicated time periods (*right*). *Bottom,* primary cultured glioblastoma cells were treated for 24 h with 100 nmol/L everolimus or 100 nmol/L rapamycin. Protein expression levels and phosphorylation status of Akt or S6 ribosomal protein were assessed by Western blotting. β-Actin or α-tubulin was used as a loading control. The relative phosphorylation of Akt was densitometrically determined and is expressed in percent as phospho-Akt/Akt ratio, with the DMSO control set at 100%. *B* and *C,* inhibition of PI3K sensitizes primary cultured glioblastoma cells to TRAIL- or doxorubicin-induced apoptosis. Primary cultured glioblastoma cells were pretreated or not with 20 μmol/L LY294002 for 1 h and then treated for 24 h (GB1 and GB2) or 48 h (GB3) with TRAIL (*B*) or for 24 h with doxorubicin (*C*) at the indicated concentrations in the presence (■) or absence (□) of 20 μmol/L LY294002. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei; the percentage of specific apoptosis is shown. *Columns,* mean of two independent experiments done in triplicate; *bars,* SE. Apoptosis induced by LY294002 alone: GB1, 40% (24 h); GB2, 32% (24 h); GB3, 30% (24 h) and 40% (48 h). *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without LY294002 (Student's *t* test). *D,* inhibition of PI3K, rather than of mTOR, sensitizes primary cultured glioblastoma cells to TRAIL- or doxorubicin-induced apoptosis. Primary cultured glioblastoma cells were pretreated or not with 20 μmol/L LY294002, 100 nmol/L rapamycin, or 100 nmol/L everolimus for 1 h and then treated for 24 h with 1 ng/mL TRAIL or with 0.75 μg/mL doxorubicin in the presence (■) or absence (□) of 20 μmol/L LY294002, 100 nmol/L rapamycin (▥), or 100 nmol/L everolimus (▨). Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei; the percentage of specific apoptosis is shown. *Columns,* mean of two independent experiments done in triplicate; *bars,* SE. Apoptosis induced by LY294002 alone: GB1, 40%; GB2, 32%; GB3, 30%. Apoptosis induced by rapamycin alone: GB1, 17%; GB2, 14%; GB3, 11%. Apoptosis induced by everolimus alone: GB1, 16%; GB2, 13%; GB3, 10%. *, $P < 0.05$; **, $P < 0.001$, comparing treatment with and without kinase inhibitors (Student's *t* test).

enhance sensitivity to TRAIL- or doxorubicin-mediated apoptosis despite target inhibition. Most importantly, inhibition of PI3K primes primary cultured glioblastoma cells for TRAIL- or chemotherapy-induced apoptosis. Together, these findings provide convincing evidence that inhibition of PI3K is a promising approach to lower the threshold for apoptosis induction by death receptor triggering or cytotoxic drugs in glioblastoma.

There is currently much interest in therapeutic intervention of aberrant PI3K signaling in glioblastoma because increased activity of the PI3K pathway has been shown to correlate with its poor prognosis (20). In neuroblastoma, we previously identified activation of Akt as a novel indicator of poor outcome, which is associated with apoptosis resistance (34). PI3K inhibitors as single agents have recently been reported to exert cytostatic activity against malignant glioma and to cooperate with blockade of epidermal growth factor receptor in *PTEN*-mutant glioma (32, 35). Although combinations with cytotoxic agents are of particular interest because PI3K inhibitors were found to be primarily cytostatic (35), there are thus far only few studies evaluating PI3K inhibitors in combination protocols in glioblastoma. Our study is the first to show that PI3K inhibition broadly sensitizes glioblastoma cells, established cell lines as well as primary cultured tumor samples, to apoptosis induction via the extrinsic and intrinsic apoptosis pathways. Furthermore, we show that this effect is not restricted to *PTEN*-mutant cells because wild-type *PTEN* glioblastoma cells could be equally sensitized. Synergistic interaction of PI3K inhibitor occurs with several different chemotherapeutic drugs and is not restricted to antimicrotubule agents as previously suggested (36). Thus, our findings have important implications for the design of protocols combining PI3K inhibitors

together with conventional anticancer agents or experimental therapeutics, such as TRAIL, to enhance the efficacy of TRAIL or chemotherapy in glioblastoma. Moreover, restoration of sensitivity toward TRAIL- or CD95-triggered apoptosis by PI3K inhibition may contribute to effective tumor elimination via the innate or acquired immune system because TRAIL is an effector molecule of the innate immune response and an intact CD95 signaling system is pivotal for effective killing of tumor cells by CTLs (37, 38).

Inhibition of PI3K is superior over mTOR inhibition for apoptosis sensitization of glioblastoma. Another important implication of our study relates to the question of which component of the PI3K network presents the most suitable target for apoptosis sensitization of glioblastoma. Our results, the first to incorporate data from established glioblastoma cell lines and primary patient material, show that PI3K inhibition is superior over mTOR inhibition in increasing apoptosis sensitivity of glioblastoma cells. In contrast to upstream inhibition of PI3K, blockade of the mTOR arm by pharmacologic inhibitors or genetic silencing consistently fails to augment apoptosis sensitivity on treatment with TRAIL or doxorubicin despite mTOR inhibition. The failure of mTOR inhibitors to efficiently prime glioblastoma cells for apoptosis is unlikely to be simply due to feedback activation of Akt by mTOR inhibitors, as previously reported for these agents (29–31), because no additional sensitization for TRAIL- or doxorubicin-induced apoptosis is seen with the concomitant use of everolimus or rapamycin together with LY294002 compared with treatment with LY294002 alone. In addition, we found no evidence that mTOR inhibitors enhance necrotic cell death on treatment with TRAIL or doxorubicin in the glioblastoma cell lines investigated. Cell line or cell type, stimulus, and the microenvironment may influence the susceptibility to mTOR inhibitor–induced apoptosis because everolimus has been shown to increase the therapeutic effects of irradiation or TRAIL in glioblastoma cell lines or of cytotoxic drugs in non–small cell lung carcinoma, head and neck cancer, or neuroblastoma (39–43). The differential effect of PI3K versus mTOR inhibition on apoptosis sensitivity found in our study may indicate that other arms of the PI3K network, possibly, but not necessarily, independent of mTOR-mediated signaling, are involved in the control of apoptosis in glioblastoma cells (e.g., via phosphorylation or transcriptional modulation of cell death proteins). Furthermore, conceptually, inhibition of the signaling apex, here the PI3K protein, may be a more promising target to inhibit than a signal side arm of the network. It will be the object of our further investigations to identify the molecules mediating the sensitization effect of PI3K inhibitors for apoptosis.

By showing that PI3K inhibition synergizes with chemotherapy or death-inducing ligands to trigger apoptosis in glioblastoma cells, our findings have important implication for apoptosis-based therapy of glioblastoma. The antitumor activity of TRAIL may be curtailed in many tumors, including glioblastoma, by primary or acquired resistance to TRAIL, despite expression of both apoptosis-inducing TRAIL receptors (44–46). Our findings argue for combining PI3K inhibitors with TRAIL receptor agonists or conventional chemotherapeutic agents to prime glioblastoma cells for death receptor– or chemotherapy-induced cell death. Clinically, resistance to apoptosis is a major cause of treatment failure in glioblastoma (47). In terms of a clinical perspective, our findings provide a basis for further investigation of PI3K inhibitors in combination with TRAIL or chemotherapy to overcome apoptosis resistance in glioblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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