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# OSU-03012 Promotes Caspase-Independent but PERK-, Cathepsin B-, BID-, and AIF-Dependent Killing of Transformed Cells

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## ABSTRACT

We determined one mechanism by which the putative phosphoinositide-dependent kinase (PDK)-1 inhibitor 2-amino-*N*-{4-[5-(2-phenanthrenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-phenyl}acetamide (OSU-03012) killed primary human glioma and other transformed cells. OSU-03012 caused a dose-dependent induction of cell death that was not altered by p53 mutation, expression of ERBB1 vIII, or loss of phosphatase and tensin homolog deleted on chromosome 10 function. OSU-03012 promoted cell killing to a greater extent in glioma cells than in nontransformed astrocytes. OSU-03012 and ionizing radiation caused an additive, caspase-independent elevation in cell killing in 96-h viability assays and true radiosensitization in colony formation assays. In a cell type-specific manner, combined exposure to OSU-03012 with a mitogen-activated protein kinase kinase 1/2 inhibitor, phosphoinositide 3-kinase/AKT inhibitors, or parallel molecular interventions resulted in a greater than additive induction of cell killing that was independent of AKT activity and caspase function. OSU-03012 lethality as a single agent or when combined with signaling modulators was not modified in cells lacking expression of BIM or of BAX/BAK. OSU-03012 promoted the release of cathepsin B from the lysosomal compartment and release of AIF from mitochondria. Loss of BH3-interacting domain (BID) function, overexpression of $BCL_{XL}$, and inhibition of cathepsin B function suppressed cell killing and apoptosis-inducing factor (AIF) release from mitochondria. In protein kinase R-like endoplasmic reticulum kinase−/− cells, the lethality of OSU-03012 was attenuated which correlated with reduced cleavage of BID and with suppression of cathepsin B and AIF release into the cytosol. Our data demonstrate that OSU-03012 promotes glioma cell killing that is dependent on endoplasmic reticulum stress, lysosomal dysfunction, and BID-dependent release of AIF from mitochondria, and whose lethality is enhanced by irradiation or by inhibition of protective signaling pathways.

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Inhibitors of cyclooxygenase (COX) 2 were originally developed to inhibit inflammatory immune responses, with a primary intention to use such agents clinically in the treatment of chronic diseases (e.g., rheumatoid arthritis) (Kiefer and Dannhardt, 2004; Hawkey and Fortun, 2005). It was also noted that COX2 was overexpressed in many tumor cells and that agents that inhibited COX2 [e.g., celecoxib (Celebrex)] could suppress tumor cell growth in vitro and when grown as

**ABBREVIATIONS:** COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinase; PDK, phosphoinositide-dependent kinase; GBM, glioblastoma multiforme; PTEN, phosphatase and tensin homolog deleted on chromosome 10; JNK, c-Jun $NH_2$-terminal kinase; MAPK, mitogen-activated protein kinase; eIF, eukaryotic initiation factor; HRP, horseradish peroxidase; PERK, protein kinase regulated by RNA-like endoplasmic reticulum kinase; AIF, apoptosis-inducing factor; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; HEH, vehicle; CMV, cytomegalovirus; ca, constitutively active; MEK, mitogen-activated extracellular-regulated kinase; siRNA, small interfering RNA; vIII, variant 3; DCF, dichlorodihydrofluorescein; ROS, reactive oxygen species; ER, endoplasmic reticulum; CHOP, GADD153; MEF, mouse embryonic fibroblast; WT, wild-type; PD184352, 2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3,4-difluoro-benzamide; zVAD, *N*-benzyloxycarbonyl-Val-Ala-Asp; SV40; simian virus 40; OSU-03012, 2-amino-*N*{4-[5-(2-phenanthrenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-phenyl}acetamide; PX-866, acetic acid (1*S*,4*E*,10*R*,11*R*,13*S*,14*R*)-[4-diallylaminomethylene-6-hydroxy-1-methoxymethyl-10,13-dimethyl-3,7,17-trioxo-1,3,4,7,10,11,12,13,14,15,16,17-dodecahydro-2-oxa-cyclopenta[*a*]phenanthren-11-yl ester; PD184352, 2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3,4-difluorobenzamide.

xenografts in animals (Koehne and Dubois, 2004; Cui et al., 2005; Kang et al., 2006; Klenke et al., 2006). Studies in patients demonstrated that individuals with prolonged exposure to COX2 inhibitors as part of an anti-inflammatory therapeutic regimen also had a lower incidence of developing cancer, suggestive that COX2 inhibitors were cancer preventative (Kashfi and Rigas, 2005; Narayanan et al., 2006). However, as the sensitivity of tumor cells to COX2 inhibitors was investigated in greater detail, it became apparent that expression of COX2 did not per se correlate with tumor cell sensitivity to COX2 inhibitor treatment (Kulp et al., 2004; Patel et al., 2005). The agent OSU-03012 was developed as an anticancer agent, with celecoxib as the chemical backbone (Zhu et al., 2004). In vitro, OSU-03012 has an order of magnitude greater antitumor activity than celecoxib, but it lacks COX2 inhibitory activity. Based on these exciting preliminary observations, OSU-03012 has been approved for development by the National Cancer Institute Rapid Access to Interventions Development program, with likely initiation of a phase I drug trial in late 2006.

In the last 5 to 10 years, multiple growth factor receptors and downstream signal transduction pathways have been linked to the advantage tumor cells have over nontransformed cells in terms of increased rates of proliferation and cell survival after exposure to toxic stresses. Signaling by ERBB family and insulin-like growth factor-1 receptors downstream into the ERK1/2, nuclear factor-$\kappa$B, and in particular the PI3K/PDK-1/AKT pathways have been linked to the promotion of genomic instability, tumorigenesis, proliferation, and tumor cell resistance to cytotoxic therapeutic interventions (for review, see Dent et al., 2003a,b). It has been argued that the toxicity of OSU-03012 in tumor cells is due to inhibition of the enzyme PDK-1, part of the PI3K pathway, in as much as OSU-03012 can suppress AKT phosphorylation and showed measurable inhibition of PDK-1 activity in the 5 to 50 $\mu$M range (Zhu et al., 2004). OSU-03012 has also been shown to interact in a synergistic manner with BCR-ABL inhibitors to suppress tumor cell viability and to kill in a manner that is partially caspase-independent (Johnson et al., 2005; Tseng et al., 2005): these studies follow the novel concept that simultaneous inhibition of two or more closely inter-related protective signaling pathways, to which a tumor cell is addicted for survival, leads to profound cell killing (Dai et al., 2001; McKinstry et al., 2002).

However, although OSU-03012 has been noted to suppress PDK-1 function and AKT activity, other data have also strongly argued that OSU-03012 toxicity, and its radiosensitizing effects, could not be closely correlated to suppression of AKT signaling (Caron et al., 2005). Thus, OSU-03012 may have additional PDK-1-independent targets that may mediate, in part, many of its observed cytotoxic properties in tumor cells. In this regard, nonsteroidal anti-inflammatory drugs (including celecoxib) have been shown to promote tumor cell death by multiple mechanisms, including transcriptional dysregulation and inhibition of protein kinases.

In the United States, glioblastoma multiforme (GBM) is diagnosed in ~20,000 patients each year. High-grade tumors such as anaplastic astrocytoma and GBM account for the majority of astrocytic tumors (Greenlee et al., 2000). Radiation therapy is typically used in the management of gliomas, either after surgical resection or as primary therapy in patients who are not surgical candidates. Radiotherapy is also often used to treat many other malignancies, including colorectal cancer, breast cancer, and head and neck cancer. Even under the best of circumstances, in which essentially all of the tumor can be surgically removed and the patients are fully treated with radiation and chemotherapy, the mean length of survival of this disease is extended only from 2 to 3 months to 1 year (Nieder et al., 2000). Because therapeutic strategies combining traditional cytotoxic chemotherapy and radiation have failed to produce the additive or synergistic effects often observed in other tumor cell types, new approaches that enhance the effectiveness of standard treatments in glioma are needed. With regard to this approach, based on in vitro analyses using established glioma cells, many groups are proposing and/or performing patient studies using cell signaling modulators or toxic cytokines to suppress the growth of this malignancy (Yacoub et al., 2003; Li et al., 2004).

In the present study, we have used nonestablished human glioma cells and expanded and passaged these cells in the flanks of athymic mice (Giannini et al., 2005; Rao et al., 2005). Several diverse primary human GBM isolates, which maintain invasive growth in vivo, in contrast to established noninvasive glioma cells (e.g., U87-MG and U251-MG) were selected for study (Table 1). Many of the isolates lack PTEN function, which would a priori be predicted to make these cells more sensitive to growth inhibition/cell killing by a PDK-1 inhibitor. Using these isolates as well as established glioma, colon, and cervical cancer cell lines and transformed fibroblasts lacking expression of proapoptotic proteins, we examined the impact of OSU-03012 on cell viability and radiosensitivity and then defined the molecular mechanisms by which OSU-03012 enhances tumor cell death.

TABLE 1

Primary and established human GBM isolates selected for study

Other alterations: PDGFRA is amplified is GBM 5. The cell line GBM 5 has a low ERK1/2:AKT activity ratio. GBM 6 cells have a high ERK1/2:AKT activity ratio. GBM14 cells have a high ERK1/2:AKT activity ratio.

| GBM Isolate | ERBB1 | Amp. CDK4/MDM2 | PTEN | p53 Mutant | p16 Deletion |
|---|---|---|---|---|---|
| 5 | No | Yes/Yes | WT | WT | No |
| 6 | Yes (vIII) | No/No | WT | Mutant | Yes |
| 10 | No | No/No | +/− | WT | Yes |
| 12 | Yes (WT) | No/No | WT | Mutant | Yes |
| 14 | No | No/No | Mutant | WT | Yes |
| 28 | No | No/No | Mutant | Mutant | No |
| U251 | Yes (WT) | No/No | Homol. del. | Mutant | Yes |
| U87 | Yes (WT) | No/No | Mutant | WT | No |

Amp, amplified; Homol. del., homologous deletion; +/−, variable expression.

## Materials and Methods

### Materials

Phospho-/total- (ERK1/2, JNK1/2, and p38 MAPK) antibodies, phospho-/total-AKT (T308 and S473), and the total and cleaved caspase 3 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-PERK, anti-eIF2$\alpha$, and anti-eIF2$\alpha$ S51 antibodies were purchased from Cell Signaling Technology Inc. All the secondary antibodies (anti-rabbit HRP, anti-mouse HRP, and anti-goat HRP) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence and terminal deoxynucleotidyl transferase dUTP nick-end labeling kits were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA) and Roche Diagnostics (Mannheim, Germany), respectively. Trypsin-EDTA, Dulbecco's modified Eagle's medium, RPMI 1640 medium, and penicillin-streptomycin were purchased Invitrogen (Carlsbad, CA). PD184352 was chemically synthesized in house based on the published structure of the drug and stored as a powder in a nitrogen atmosphere under light-protected conditions at −80°C. HCT116 cells were purchased from the American Type Culture Collection (Manassas, VA) (Caron et al., 2005). BIM null fibroblasts were provided by Dr. H. Harada (Virginia Commonwealth University, Richmond, VA). BAX/BAK−/− and BID−/− fibroblasts were kindly provided by Dr. S. Korsmeyer (Harvard University, Boston, MA). Transformed protein kinase regulated by RNA-like endoplasmic reticulum (PERK)−/− cells were a kind gift from the Ron laboratory (Skirball Institute, New York University School of Medicine, New York City, NY). HeLa cells stably expressing wild-type or mutant thioredoxin reductase were a kind gift from Dr. David Guis (National Cancer Institute, Bethesda, MD). OSU-03012 was supplied by author C.-S.C. PX-866 was supplied by Dr. G. Powis (M.D. Anderson Cancer Center, Houston, TX), and AKT inhibitor 15B was a very gracious gift supplied by Dr. P. Houghton (St. Jude's Children's Hospital, Memphis, TN). Primary human GBM cells and information on the genetic background of such cells were very kindly supplied for our use by Dr. C. David James (Mayo Clinic, Rochester, MN). Other reagents and cell lines were as described in McKinstry et al. (2002), Dent et al. (2003b), Yacoub et al. (2003, 2004), Bi et al. (2005), Caron et al. (2005), Ihle et al. (2004), and Thimmaiah et al. (2005).

### Methods

**Culture and in Vitro Exposure of Cells to Drugs.** All established cell lines were cultured at 37°C [5% (v/v) $CO_2$] in vitro using RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids. Primary human glioma cells were cultured in 2% (v/v) fetal calf serum to prevent growth of contaminating rodent fibroblasts during in vitro analyses. For short-term cell killing assays, immunoblotting and apoptosis-inducing factor (AIF)/cathepsin release studies, cells were plated at a density of $8 \times 10^4/cm^2$ ($\sim 2 \times 10^5$ cells/well of a 12-well plate), and 48 h after plating they were treated with various drugs, as indicated. In vitro OSU-03012/PD184352/wortmannin/PX-866/AKT inhibitor 15B treatment was from a 100 mM stock solution of each drug, and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). Radiation exposure was from a Picker $^{60}Co$ source with a dose rate of $\sim$2 Gy/min. Cells were not cultured in reduced serum media during any experiment described in this article.

**In Vitro Cell Treatments, SDS-PAGE, and Western Blot Analysis.** For in vitro analyses of short-term cell death effects, cells were treated with vehicle, OSU-03012/PD184352/wortmannin/PX-866/AKT inhibitor 15B or their combination for the indicated times in the figure legends. For apoptosis assays, cells were pretreated with vehicle (DMSO), 50 $\mu$M zVAD, or cathepsin B inhibitor [L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-l-proline methyl ester) (1 $\mu$M). Cells were isolated and either subjected to trypan blue cell viability assay by counting in a light microscope or fixed to slides and stained using a commercially available Diff-Quik (Giemsa) assay kit (Dade Behring, Inc., Deerfield, IL) (McKinstry et al., 2002; Yacoub et al., 2004).VWR.

Cells for in vitro or ex vivo colony formation assays were plated at 250 to 4000 cells/well in sextuplicate and for in vitro assays 14 h after plating cells were treated with either vehicle (DMSO), OSU-03012, PD184352, wortmannin, PX-866, AKT inhibitor 15B, or the drug combinations as indicated for 48 h followed by drug removal, washing of the cells, and continued culture in media lacking any drug. Twenty-four days after exposure, plates were washed in phosphate-buffered saline, fixed with methanol, and stained with a filtered solution of crystal violet [5% (w/v)]. After washing with tap water, the colonies were counted both manually (by eye) and digitally using a ColCount plate reader (Oxford Optronics, Oxford, UK). Data presented are the arithmetic mean ± S.E.M. from both counting methods from multiple studies. Colony formation was defined as a colony of 50 cells or greater.

For SDS-PAGE and immunoblotting, cells were plated at $5 \times 10^5$ cells/cm$^2$ and treated with drugs at the indicated concentrations. After the indicated time of treatment, cells were lysed in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% $\beta$-mercaptoethanol, and 0.02% bromphenol blue), and the samples were boiled for 30 min. The boiled samples were loaded onto 10 to 14% SDS-PAGE, and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22-$\mu$m nitrocellulose, and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized by enhanced chemiluminescence. For presentation, immunoblots were digitally scanned at 600 dpi using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA), their color was removed, and figures were generated in PowerPoint (Microsoft, Redmond, WA).

**Infection of Cells with Recombinant Adenoviruses.** Cells were plated at $2 \times 10^5$ cells/well of a 12-well plate. After plating (24 h), cells were infected (at a multiplicity of infection of 50) with a control empty vector virus (CMV) and adenoviruses to express $BCL_{XL}$, constitutively active (ca) MEK1 EE, and commercially available viruses to express myristoylated [constitutively active (ca)] AKT1, dominant-negative AKT1 (T308A and S473A), or dominant-negative MEK1 (K97M) (Vector Biolabs, Philadelphia, PA). Twenty-four hours after infection, cells were treated with the indicated concentrations of OSU-03012 and/or wortmannin, and cell survival was determined 48 h after drug treatment by trypan blue assay (McKinstry et al., 2002; Yacoub et al., 2004).

**Transfection of Cells with siRNA Molecules to Suppress AIF Expression.** HCT116 cells were plated as described above, and 24 h after plating, they were transfected. RNA interference or gene silencing for down-regulating the expression of PDK1 and AIF was performed using validated target sequences designed by Ambion (Austin, TX). For transfection, 10 nmol annealed siRNA targeting PDK1 or AIF, the positive sense control doubled-stranded siRNA targeting glyceraldehyde-3-phosphate dehydrogenase, or the negative control (a "scrambled" sequence with no significant homology to any known gene sequences from mouse, rat, or human cell lines) was used. The small RNA sequences were transfected by electroporation at 600 V during 60 $\mu$s. Forty-eight or 72 h after transfection, the cells were treated with drugs, as noted above (Caron et al., 2005).

**Manipulation of Drug-Treated Cells to Isolate a Crude Cytosolic Fraction.** A crude membrane fraction was prepared from treated cells as described in Cirman et al. (2004). In brief, cells were washed twice in ice-cold isotonic HEPES buffer (10 mM HEPES, pH 7.5, 200 mM mannitol, 70 mM sucrose, 1 $\mu$M EGTA, and 10 $\mu$M protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO). Cells on ice were scraped into isotonic HEPES buffer and lysed by passing 20 times through a 25-gauge needle. Large membrane pieces, organelles, and cells that did not lyse were removed from the suspension by centrifugation for 5 min at 120*g*. The crude granular fraction and cytosolic fraction were obtained from by centrifugation for 30 min at 10,000*g*, leaving the cytosol as supernatant.

**Assessment of Reactive Oxygen/Nitrogen Species Generation.** Fibroblasts were plated in 96-well plates. Cells were treated with 1 $\mu$M OSU-03012 for the indicated periods. Fifteen minutes before isolation, cells were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (dihydro-DCF; Invitrogen) (5 $\mu$M), which is nonfluorescent in its dihydro form but upon reaction with reactive oxygen (ROS)/nitrogen species becomes highly fluorescent. Dihydro-DCF is sensitive to oxidation by hydroxyl radicals and peroxynitrite directly and hydrogen peroxide in the presence of a peroxidase. Fluorescence measurements were obtained 15 min after OSU-03012 addition with a Packard FluoroCount plate reader. Data are presented corrected for basal fluorescence of dye at each time point. Each time point represents the mean of six data points (Leach et al., 2001).

**Data Analysis.** Comparison of the effects of various treatments was performed using the Student's $t$ test. Differences with a $p$ value of $<0.05$ were considered statistically significant. Experiments shown are the means ± S.E.M. of multiple individual points.

## Results

**OSU-03012 Lethality Does Not Closely Correlate with Inhibition of PDK-1 or with Activation of the PI3K Pathway.** OSU-03012 was initially reported to be an inhibitor of PDK-1, and inhibition of this enzyme was proposed to account for the majority of OSU-03012 toxicity in tumor cells. However, in HCT116 cells, which are easily transfected, OSU-03012 caused considerably more radiosensitization than did transient siRNA suppression of PDK-1 expression, but it caused significantly less inhibition of basal activity levels or radiation-induced activation of AKT (Fig. 1). These findings argue that OSU-03012 must have additional targets, besides PDK-1, in tumor cells that mediate a portion of its cytotoxic actions.

Initial studies in primary human glioma cells examined the dose dependence of OSU-03012-induced killing in short-term (96-h) trypan blue assays and the potential for OSU-03012 to act as a radiosensitizer in both short-term and long-term colony formation assays. OSU-03012 caused a dose-dependent induction in GBM cell death in which the dose response from 1 $\mu$M (modest cell killing) to 5 $\mu$M (high levels of cell death) was evident in all cell isolates (Fig. 2, A and B). It is noteworthy that, at a concentration of 1 $\mu$M, OSU-03012 caused a modest but significant amount of cell killing (~10% above basal levels) in our GBM cells, but it caused a >40% reduction in total cell numbers for all primary GBM isolates, arguing that a primary action of the drug at low doses is to cause growth arrest. Some cell isolates (e.g., GBM12 and the established U251 line) were significantly more sensitive to OSU-03012 exposure. U251 cells lack expression of PTEN, which would be a priori predicted to make these cells more sensitive to the putative PDK-1 inhibitor OSU-03012, although other GBM cells lacking PTEN function (e.g., GBM14 and U87) and those having high basal levels of activated AKT (e.g., GBM 5) were not noted to be hypersensitive to the drug.

**OSU-03012 Radiosensitizes Primary Human Glioma Cells in Vitro.** Additional studies then combined OSU-03012 with ionizing radiation exposure to determine whether this agent was capable of radiosensitizing primary human glioma cells in vitro using short-term trypan blue viability assays and long-term colony formation analyses. OSU-03012 interacted with ionizing radiation in an additive manner in GBM5, GBM6, GBM12, and GBM14 cells to cause death within 96 h of exposure (Fig. 2C). Inhibition of caspase function using the pan-caspase inhibitor zVAD largely abolished radiation-induced cell killing but only weakly suppressed the radiosensitizing effects of OSU-03012 in GBM6 cells, suggesting OSU-03012 enhances GBM cell killing after radiation exposure via a caspase-independent process (Fig. 2D). In colony formation assays, GBM5 and GBM12 cell survival after radiation exposure (4 Gy) was 0.75 ± 0.06 and 0.88 ± 0.10 (12 separate data points from two experiments ± S.E.M.), respectively, compared with unirradiated cells. With respect to other cell types previously studied by this group (e.g., DU145 and HCT116), GBM5 and GBM12 cells seem to be relatively radioresistant (Dent et al., 2003; Caron et al., 2005). In the presence of increasing concentrations of OSU-03012, cell survival after radiation exposure was decreased in a greater than additive manner below irradiated cells

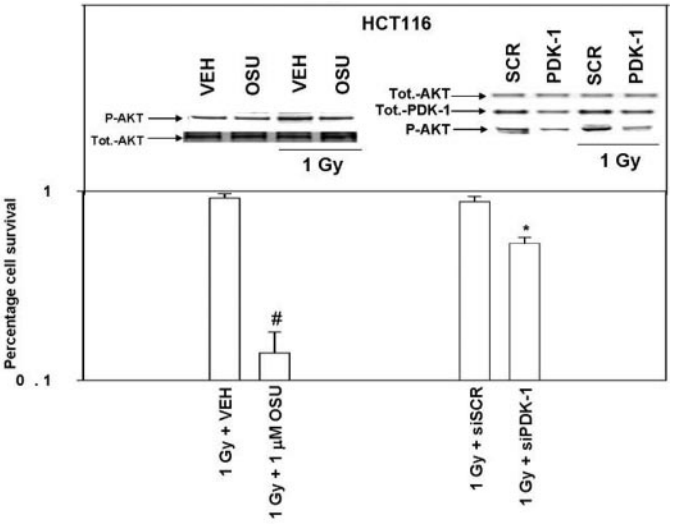


**Fig. 1.** Small interfering RNA-mediated inhibition of PDK-1 expression radiosensitizes HCT116 cells to a lesser extent than 1 $\mu$M OSU-03012. Bottom left, HCT116 cells expressing H-RAS V12 were plated, and 6 h after plating they were serum-starved for 24 h before radiation exposure (1 Gy) (see also similar data presented in Caron et al., 2005). Cells were treated 30 min before exposure with vehicle (VEH; DMSO) or 1 $\mu$M OSU-03012 (OSU). Media containing serum was added 24 h after exposure, and 10 to 14 days later, colonies were counted as indicated under *Materials and Methods*. The numbers of colonies were expressed as a fraction of the respective mock-irradiated cells ± S.E.M. ($n$ = 3). [#, $p <$ 0.05 less than corresponding small interfering RNA molecule against PDK-1 (siPDK-1) value]. Bottom right, HCT116 cells expressing H-RAS V12 were plated and transfected with either a siPDK-1 or a scrambled PDK-1 siRNA (siSCR) as described under *Materials and Methods*. After transfection, single cells were replated for colony formation assays, serum-starved for 24 h, and then irradiated (1 Gy). Media containing serum were added 24 h after exposure, and 10 to 14 days later colonies were counted as indicated under *Materials and Methods*. Data are the means of six separate transfection dishes per experiment from two separate experiments ± S.E.M. (*, $p < 0.05$ less than corresponding siSCR value). The numbers of colonies were expressed as a fraction of the respective mock-irradiated cells. Inset, top left, HCT116 cells expressing H-RAS V12 were plated and serum-starved for 24 h. Cells were treated with either vehicle (DMSO) or 1 $\mu$M OSU 30 min before irradiation (1 Gy). Cells were isolated 6 h after irradiation and subjected to SDS-PAGE and immunoblotting to determine AKT S473 phosphorylation, as described under *Materials and Methods*. Inset, top right, HCT116 cells expressing H-RAS V12 were transfected with either a small interfering RNA molecule against PDK-1 or a scrambled PDK-1 siRNA as described under *Materials and Methods*. Twenty-four hours later, cells were serum-starved for 24 h and then irradiated (1 Gy). Six hours after irradiation, cells were processed for immunoblotting to determine the expression and phosphorylation of various proteins. Data are from a representative experiment ($n$ = 3).

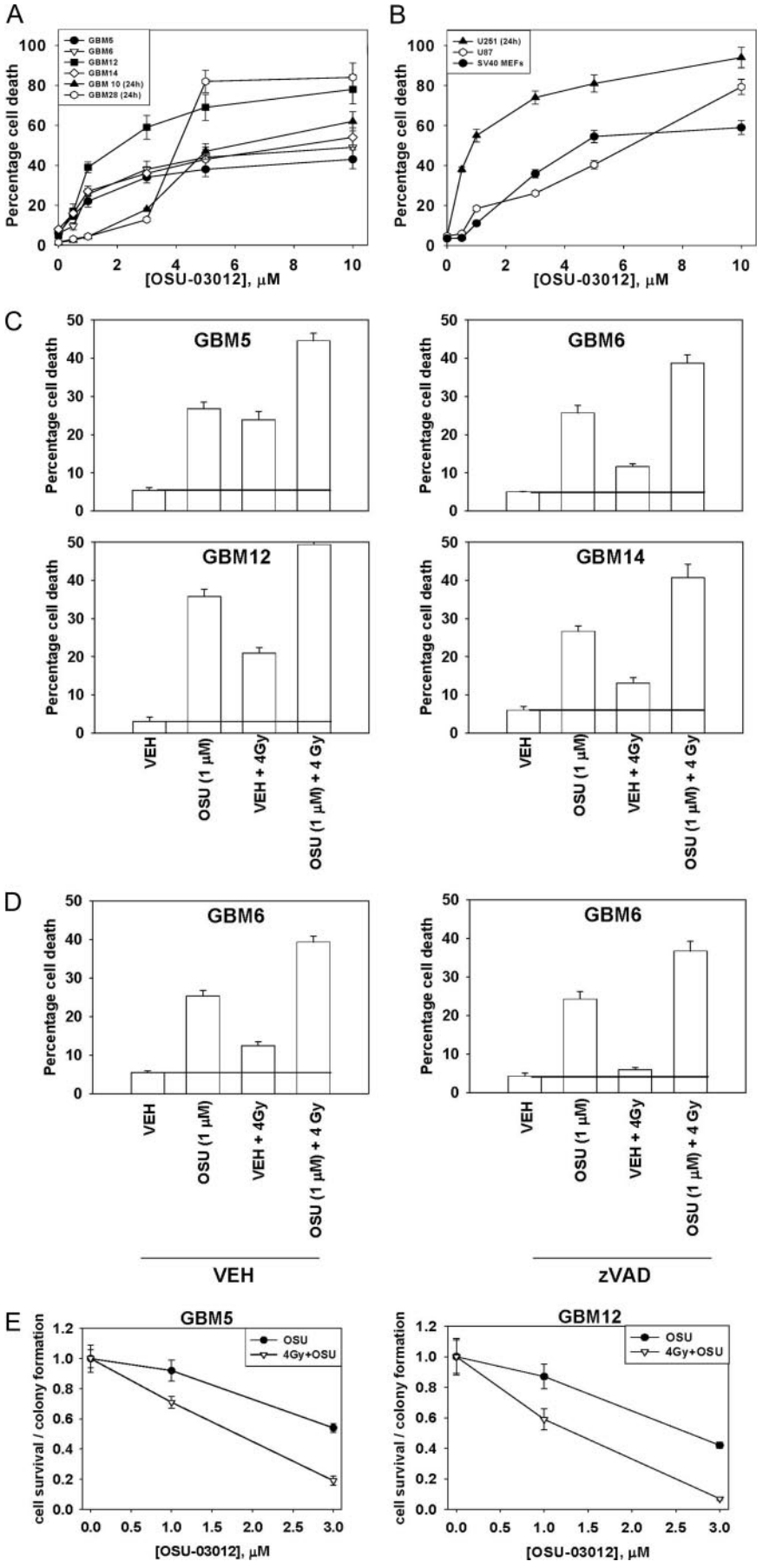


**Fig. 2.** OSU-03012 causes a dose-dependent increase in primary GBM cell death and radiosensitizes cells in a caspase-independent manner. A, primary human GBM cells were plated, and 24 h after plating, cells were treated with increasing concentrations of OSU-03012 (0–10 $\mu$M). Then, cells were isolated by trypsinization 96 h after drug treatment (24 h for GBM10 and GBM28). Cell viability was determined using a trypan blue exclusion assay in triplicate, and a representative experiment is shown from multiple experiments using different primary GBM tumor isolates. B, cells were plated, and 24 h after plating, cells were treated with increasing concentrations of OSU-03012 (0–10 $\mu$M). Then, cells isolated by trypsinization 96 h after drug treatment (24 h for U251). Cell viability was determined using a trypan blue exclusion assay in triplicate, and a representative experiment is shown from multiple experiments. C, primary human GBM cells were plated and cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or 1 $\mu$M OSU, and 30 min later they were irradiated (4 Gy). Cells were isolated by trypsinization 96 h after drug treatment/radiation exposure. Cell viability was determined using a trypan blue exclusion assay in triplicate. A representative experiment is shown from multiple experiments using different isolates of the primary GBM tumors. D, primary human GBM6 cells were plated and cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with 1 $\mu$M OSU and with VEH (DMSO) or the pan caspase inhibitor zVAD (50 $\mu$M, in DMSO), and 30 min later they were irradiated (4 Gy). Media were resupplemented every 24 h with DMSO vehicle or zVAD. Cells were isolated by trypsinization 96 h after drug treatment/radiation exposure. Cell viability was determined using a trypan blue exclusion assay in triplicate. E, primary human GBM5 and GBM12 cells were plated as single cells and cultured as described under *Materials and Methods*. Fourteen hours after plating, cells were treated with vehicle (DMSO) or 1 or 3 $\mu$M OSU. After treatment (30 min), cells were irradiated (4 Gy) or mock-irradiated. After drug treatment (4 days) and irradiation, media were replaced with fresh media lacking drugs. After media change (24 days), cells were fixed and stained, and colonies of >50 cells were counted. The data shown are normalized to vehicle control colony formation under each condition (mock-irradiated; 4-Gy-irradiated) each defined as 1.00. Data are the means ± S.E.M. from two separate experiments with each study performed in sextuplicate.

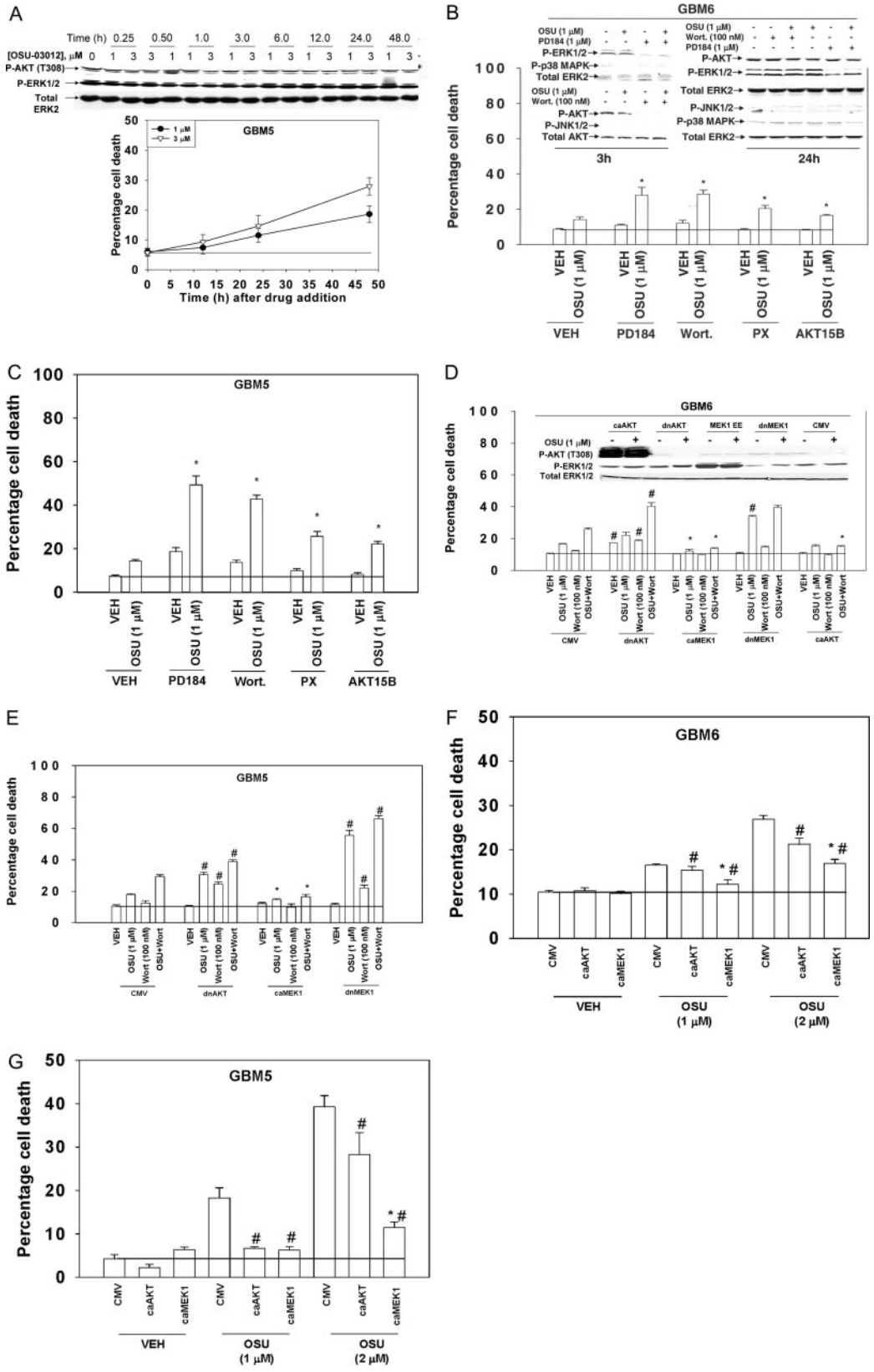


**Fig. 3.** Low concentrations of OSU-03012 that are modestly toxic weakly inhibit AKT phosphorylation; potentiation of OSU-03012 toxicity by inhibition of MEK1/2, PI3K, and AKT. A, top, GBM5 cells were plated, and 24 h later they were treated with vehicle (DMSO) or with 1 or 3 $\mu$M OSU-03012; all time points were isolated at 48 h after drug/vehicle exposure. Cells were prepared for SDS-PAGE. Immunoblotting after SDS-PAGE was performed to determine the phosphorylation of AKT (T308) (the PDK-1 phosphorylation site) and phosphorylation of ERK1/2. Data are from a representative experiment ($n$ = 3). Bottom, GBM5 cells were plated, and 24 h later they were treated with vehicle (DMSO) or with 1 or 3 $\mu$M OSU-03012 (drug). Cells were isolated by trypsinization 24 and 48 h after drug treatment. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n$ = 3). B and C, GBM6 cells (B) and GBM5 cells (C) were plated, and 24 h later they

treated with vehicle control, indicating that OSU-03012 had radiosensitized GBM5 and GBM12 cells (Fig. 2E).

**OSU-03012 Lethality Is Enhanced by Inhibition of Multiple Cytoprotective Signal Transduction Pathways.** Based on the findings presented in Figs. 1 and 2, parallel signaling studies characterized the ability of OSU-03012 to modulate the phosphorylation (activity) of ERK1/2, AKT (T308), JNK1/2, and p38 MAPK in GBM5 cells. At a concentration that promoted a modest level of toxicity and significant growth inhibition (1 $\mu$M) in GBM5 cells, OSU-03012 did not appreciably alter ERK1/2 or AKT (T308) phosphorylation, in contrast to inhibition of AKT phosphorylation by 100 nM wortmannin (Fig. 3, A and B; data not shown). Higher concentrations of OSU-03012 that caused substantial cell killing and largely abolished GBM5 cell growth (3 $\mu$M) correlated with a modest suppression of AKT (T308) phosphorylation. Exposure of GBM5 cells to 1 and 3 $\mu$M OSU-03012 did not seem to significantly alter the phosphorylation levels of either ERK1/2, JNK1/2, or p38 MAPK during the 48-h survival time course (Fig. 3A; data not shown).

Thus, concentrations of OSU-03012 that were modestly toxic and strongly growth suppressive did not seem to profoundly modulate ERK1/2 or AKT activity in GBM5 cells. In multiple prior studies from our laboratories, both the MEK1/2-ERK1/2 and PI3K-PDK-1-AKT pathways have been linked to maintenance of tumor cell survival when cells are exposed to a noxious agent. Hence, we next determined in GBM5 and GBM6 cells whether OSU-03012 interacted with inhibitors of MEK1/2-ERK1/2 signaling and/or of PI3K-AKT signaling to cause glioma cell death. Inhibition of MEK1/2 function enhanced the lethality of OSU-03012 in GBM6 and GBM5 cells 48 h after exposure, as did inhibition of PI3K or AKT function (Fig. 3, B and C). Three hours after combined exposure to OSU-03012 and PD184352 or to OSU-03012 and wortmannin, ERK1/2 and AKT (T308) phosphorylation was suppressed, respectively, whereas 24 h after drug exposure ERK1/2 and AKT phosphorylation were more modestly reduced under conditions of combined drug treatment.

Use of activated and dominant-negative AKT constructs in GBM6 and GBM5 cells demonstrated that dominant-negative AKT promoted OSU-03012 lethality and constitutive AKT activation partially suppressed OSU-03012 lethality but considerably reduced OSU-03012- and wortmannin-induced cell killing (Fig. 3, D–G). In contrast to our findings using activated AKT, constitutively activated MEK1 EE abolished cell death in GBM6 and GBM5 cells with either OSU-03012 treatment as a single agent or when treated with OSU-03012 and wortmannin (Fig. 3, C and D). In both isolates, expression of dominant-negative MEK1 promoted OSU-03012 lethality to a greater extent than caused by expression of dominant-negative AKT. Treatment of nonestablished primary human astrocytes or primary rodent hepatocytes with low doses of OSU-03012 (1–3 $\mu$M) and/or PI3K/MEK1/2 inhibitors did not promote cell death within 48 h (data not shown). Taken together, these findings demonstrate that inhibition of either the MEK1/2-ERK1/2 pathway or the PI3K-AKT pathway enhances the lethality of OSU-03012 in GBM6 and GBM5 cells and that constitutive activation of these pathways, in particular activation of the MER1/2-ERK1/2 pathway, can act to suppress OSU-03012 lethality.

The interaction of OSU-03012 and kinase inhibitors was also investigated in glioma tumor isolates other than GBM5 and GBM6. GBM12 cells overexpress wild-type ERBB1, and we noted that this GBM isolate was very sensitive to treatment with OSU-03012 as a single agent or to individual inhibition of either PI3K or MEK1/2 function. In GBM12 cells inhibition of MEK1/2 but not PI3K signaling modestly enhanced OSU-03012 lethality (Fig. 4A). GBM10, GBM14, and GBM28 cells lack PTEN expression, which would a priori tend to argue that these isolates will be dependent for survival on PI3K signaling and in agreement with this hypothesis, PI3K/AKT inhibitors modestly promoted OSU-03012 lethality (Fig. 4, A and B). MEK1/2 inhibition enhanced OSU-03012 lethality in these isolates to an extent that was at least as great as observed for inhibition of PI3K. Inhibition of PI3K and MEK1/2 enhanced OSU-03012 lethality in U251MG and U87MG cells, respectively (Fig. 4C). In colony formation assays, wortmannin or PD184352 enhanced the lethality of OSU-03012 in a greater than additive manner in both GBM5 and GBM12 cells (Fig. 4D). Taken together, our data argue that in a glioma cell-dependent manner, OSU-03012 lethality can be rapidly potentiated by agents that block either MEK1/2-ERK1/2 signaling, and to a lesser extent PI3K-AKT signaling.

**OSU-03012 Lethality Does Not Involve Activation of Caspases: The Regulation of Cathepsin B, BID, and AIF Function.** The mode and mechanism(s) of cell killing induced by OSU-03012 were investigated using multiple cell types. Based on established methodologies of Wright Geimsa



were treated with 1 $\mu$M PD184352 (PD184), 100 nM wortmannin (wort.), 100 nM PX-866 (PX), or 3 $\mu$M AKT15B followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin or PX-866, drug treatment was for only 1 h before drug removal and replacement with fresh media followed by OSU treatment. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ greater than corresponding value in CMV-infected cells). B, inset, GBM6 cells were plated as per viability assays and treated with the indicated drugs. Cells were isolated 3 and 24 h after treatment, and SDS-PAGE and immunoblotting were performed to determine the phosphorylation status of ERK1/2, AKT, JNK1/2, and p38 MAPK. D and E, GBM6 (D) cells and GBM5 cells (E) were plated, and 24 h later they were infected with recombinant adenoviruses to express activated AKT (caAKT), activated MEK1 (caMEK1), dominant-negative AKT (dnAKT), dominant-negative MEK1 (dnMEK1), or with a control empty vector virus, each at a multiplicity of infection of 50. Twenty-four hours after infection, cells were treated with 100 nM wortmannin, followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin, drug treatment was for only 1 h before drug removal and replacement with fresh media followed by OSU treatment. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in CMV-infected cells; #, $p < 0.05$ greater than corresponding value in CMV-infected cells). D, inset, GBM6 cells infected with virus, isolated 48 h after infection, and 24 h after OSU-03012 treatment. Cell lysates were prepared for SDS-PAGE and immunoblotting performed to determine AKT (T308) and ERK1/2 phosphorylation. F and G, GBM6 cells (F) and GBM5 cells (G) were plated, and 24 h later they were infected with recombinant adenoviruses to express caAKT or caMEK1, or with a control empty vector virus, each at a multiplicity of infection of 50. Twenty-four hours after infection, cells were treated with VEH (DMSO) or with 1 or 2 $\mu$M OSU. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in caAKT-infected cells; #, $p < 0.05$ greater than corresponding value in CMV-infected cells).

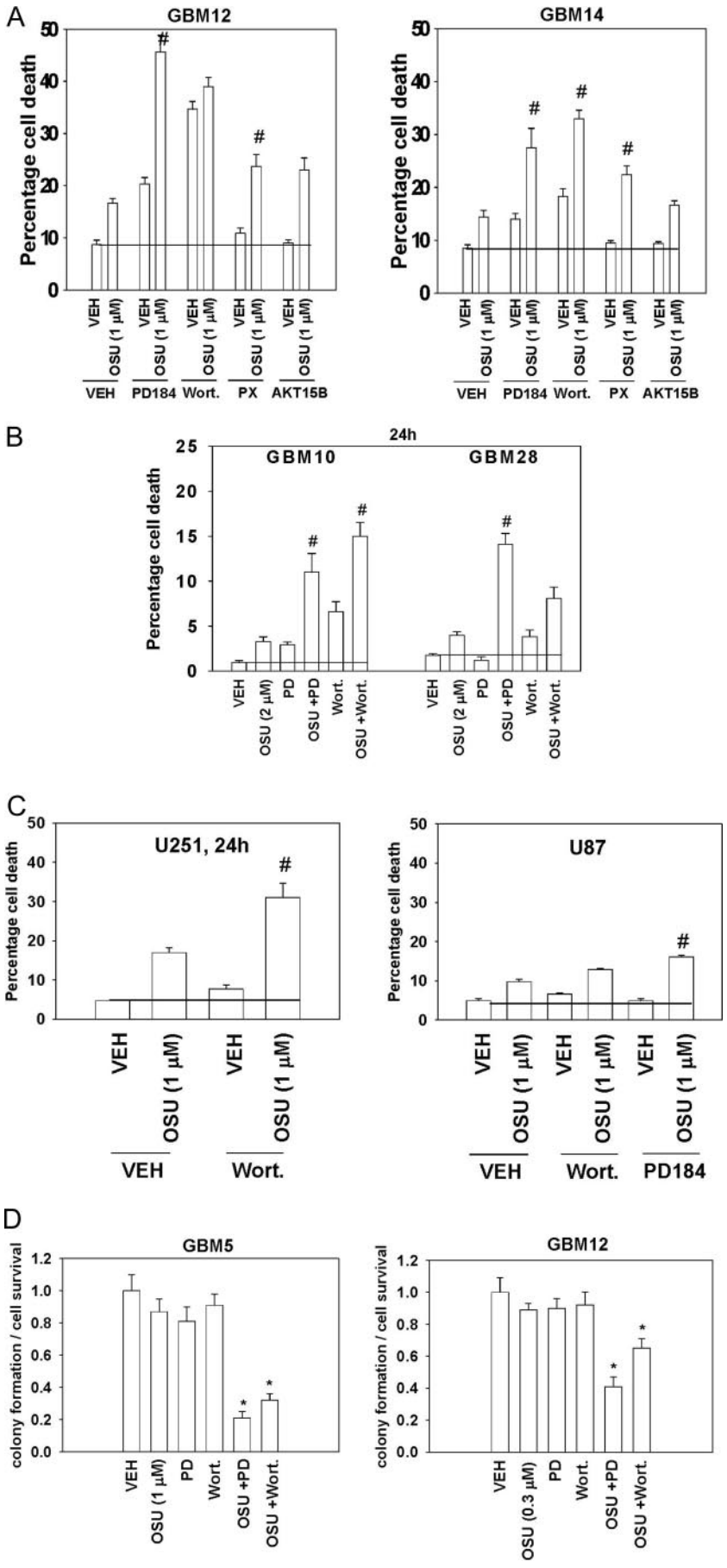


**Fig. 4.** Low concentrations of OSU-03012 that are modestly toxic weakly inhibit AKT phosphorylation; potentiation of OSU-03012 toxicity by inhibition of MEK1/2, PI3K, and AKT. A and B, GBM12 cells, GBM10 cells, GBM28 cells, and GBM14 cells were plated, and 24 h later they were treated with 1 $\mu$M PD184352 (PD184), 100 nM wortmannin (wort.), 100 nM PX, or 3 $\mu$M AKT15B followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin or PX-866, drug treatment was for only 1 h before drug removal and replacement with fresh media followed by OSU treatment. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (#, $p < 0.05$ greater than corresponding value in CMV-infected cells). C, U87 cells and U251 cells were plated, and 24 h later they were treated with 1 $\mu$M PD184, 100 nM wortmannin, 100 nM PX, or 3 $\mu$M AKT15B followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin or PX-866, drug treatment was for only 1 h before drug removal and replacement with fresh media. Cells were isolated by trypsinization 48 h (U87) or 24 h (U251) after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (#, $p < 0.05$ greater than corresponding value in CMV-infected cells). D, GBM5 and GBM12 cells were plated in sextuplicate as single cells for colony formation assays, as described under *Materials and Methods*. Cells were permitted to attach, and 12 h after plating, each well was individually pretreated for 60 min with 100 nM wortmannin; then, wortmannin was removed from the culture media followed by treatment with the indicated concentration of OSU-03012. For 1 $\mu$M PD184352 (PD) treatment, cells were simultaneously treated with OSU-03012 and PD184352 for 48 h. After 48 h of OSU-03012 drug treatment, media were carefully removed, the cells were washed, and fresh media lacking drug were added to the cultures. Colonies were permitted to form over the following 28 days. Cells were fixed, stained, and counted both manually and using a Colcount machine (Oxford Optronics). The mean ± S.E.M. colony numbers from both methods of counting were used to calculate plating efficiency ($n = 6$) for each treatment condition (*, $p < 0.05$ less than corresponding value in CMV-infected cells). The numbers below in parentheses are the corrected values for clonogenic survival based on the survival of wortmannin- or PD184352-treated cells (as a single agent) being defined as 1.00.

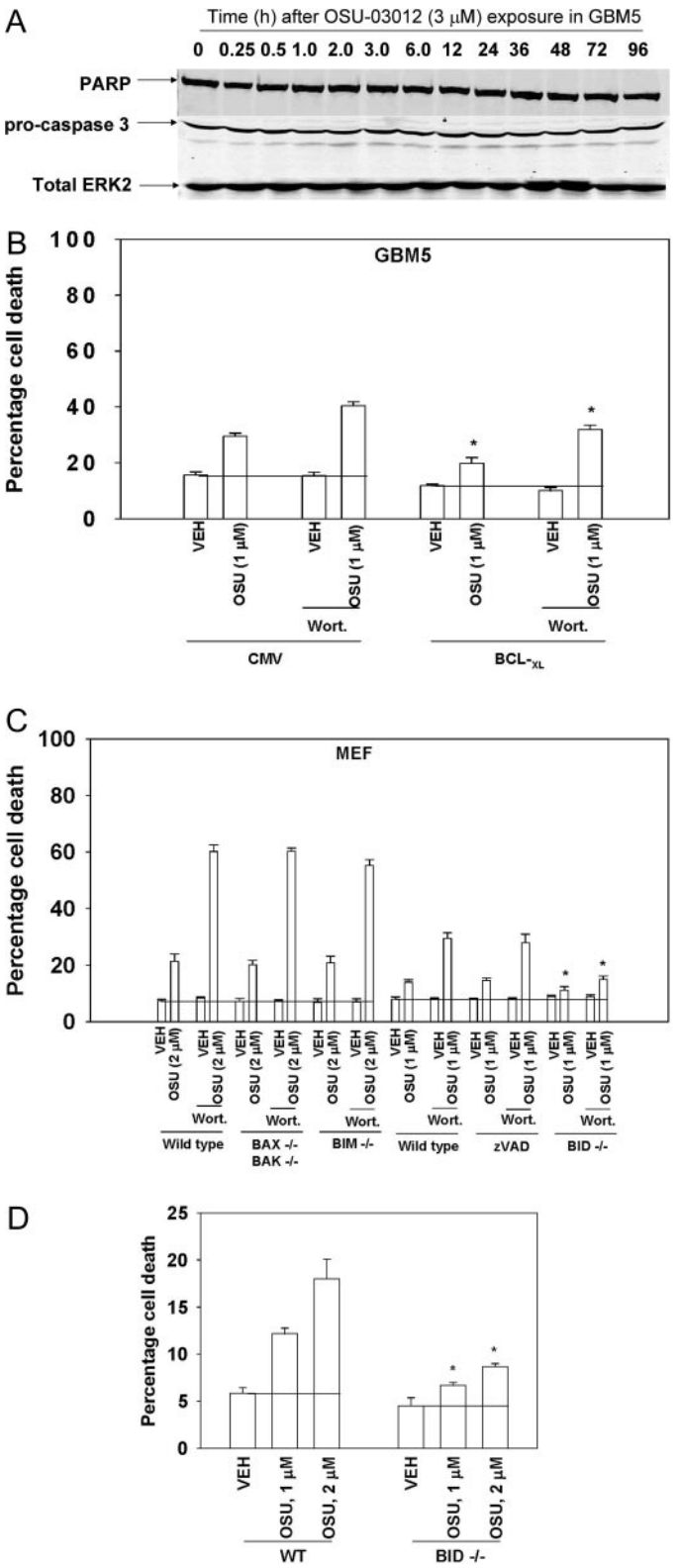


**Fig. 5.** OSU-03012 causes caspase-independent cell death in SV40-transformed mouse embryonic fibroblasts that is also independent of BAX, BAK, or BIM function, but it is dependent on BID. A, GBM5 cells were plated, and 24 h later they were treated with vehicle (DMSO) or with 3 $\mu$M OSU; all time points were isolated at 96 h after drug/vehicle exposure. Cells were prepared for SDS-PAGE and immunoblotting after PAGE was performed to determine the expression/integrity of poly(ADP-ribose) polymerase and pro-caspase 3. Data are from a representative experiment ($n = 3$). B, GBM5 cells were plated, and 24 h later they were infected with recombinant adenoviruses to express $BCL_{XL}$ or with a control empty vector virus, each at a multiplicity of infection of 50. Twenty-four hours after infection, cells were treated with VEH (DMSO) or 100 nM wortmannin (wort.) followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin, drug treatment was for only 1 h before drug removal and replacement with fresh media. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in CMV-infected cells). C, SV40-transformed mouse embryonic fibroblasts (MEFs) either wild type (WT), BAX−/−, and BAK−/−, BIM−/−, or BID−/− were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or 100 nM wortmannin followed 1 h later by VEH (DMSO) or with 1 or 2 $\mu$M OSU as indicated. Note that in the case of wortmannin, drug treatment was for only 1 h before drug removal and replacement with fresh media. In studies using the pan caspase inhibitor zVAD (50 $\mu$M), cells were treated 30 min before wortmannin/OSU-03012 treatment, and media were resupplemented every 24 h with DMSO vehicle or zVAD. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in wild-type fibroblasts). D, SV40 MEFs, either wild type (WT) or BID−/−, were cultured under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or with OSU. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in wild-type fibroblasts).

and terminal deoxynucleotidyl transferase dUTP nick-end labeling staining of glioma cells fixed to glass slides, "classic" nuclear apoptotic morphology was not microscopically evident in GBM5 and GBM6 cells treated with either OSU-03012 alone or in combination with radiation or signaling pathway modulators, despite cells exhibiting trypan blue inclusion, and that correlated with no observed alteration in caspase 3 or PARP expression/integrity (Fig. 5A; data not shown). This is in contrast to our prior findings showing caspase-dependent cell killing of primary human GBM cells using the novel cytokine melanoma differentiation associated gene-7/interleukin-24 (Yacoub et al., 2004). In a similar manner to our present findings with respect to radiation exposure, incubation of cells with a pan caspase inhibitor (50 $\mu$M zVAD) did not modify the death response of GBM5 cells treated with OSU-03012, wortmannin, PD184352, or the inhibitor drugs in combination (data not shown). Overexpression of the mitochondrial- and endoplasmic reticulum-associated protein $BCL_{XL}$ partially protected GBM5 cells from OSU-03012 lethality as a single agent and in combination with wortmannin (Fig. 5B).

Based on our findings with $BCL_{XL}$ overexpression, we made use of SV40-transformed mouse embryonic fibroblasts that have been deleted in both alleles for specific pro-cell death BH3 domain containing proteins. In these transformed fibroblasts, the lethality of OSU-03012 was enhanced by inhibition of PI3K but not by inhibition of MEK1/2 (data not shown). The lethality of OSU-03012 and wortmannin was not altered in transformed fibroblasts lacking expression of BH3 domain containing protein molecules such as BAX and BAK or in cells lacking expression of BIM (Fig. 5C). Loss of BID function suppressed the lethality of OSU-03012 as a single agent and in combination with wortmannin, reducing the proapoptotic interaction of OSU-03012 with wortmannin by >80% (Fig. 5, C and D).

Because the induction of cell death by OSU-03012 seemed to be caspase independent but weakly blunted by $BCL_{XL}$ overexpression and significantly reduced by genomic deletion of BID, additional studies examined whether mitochondrial

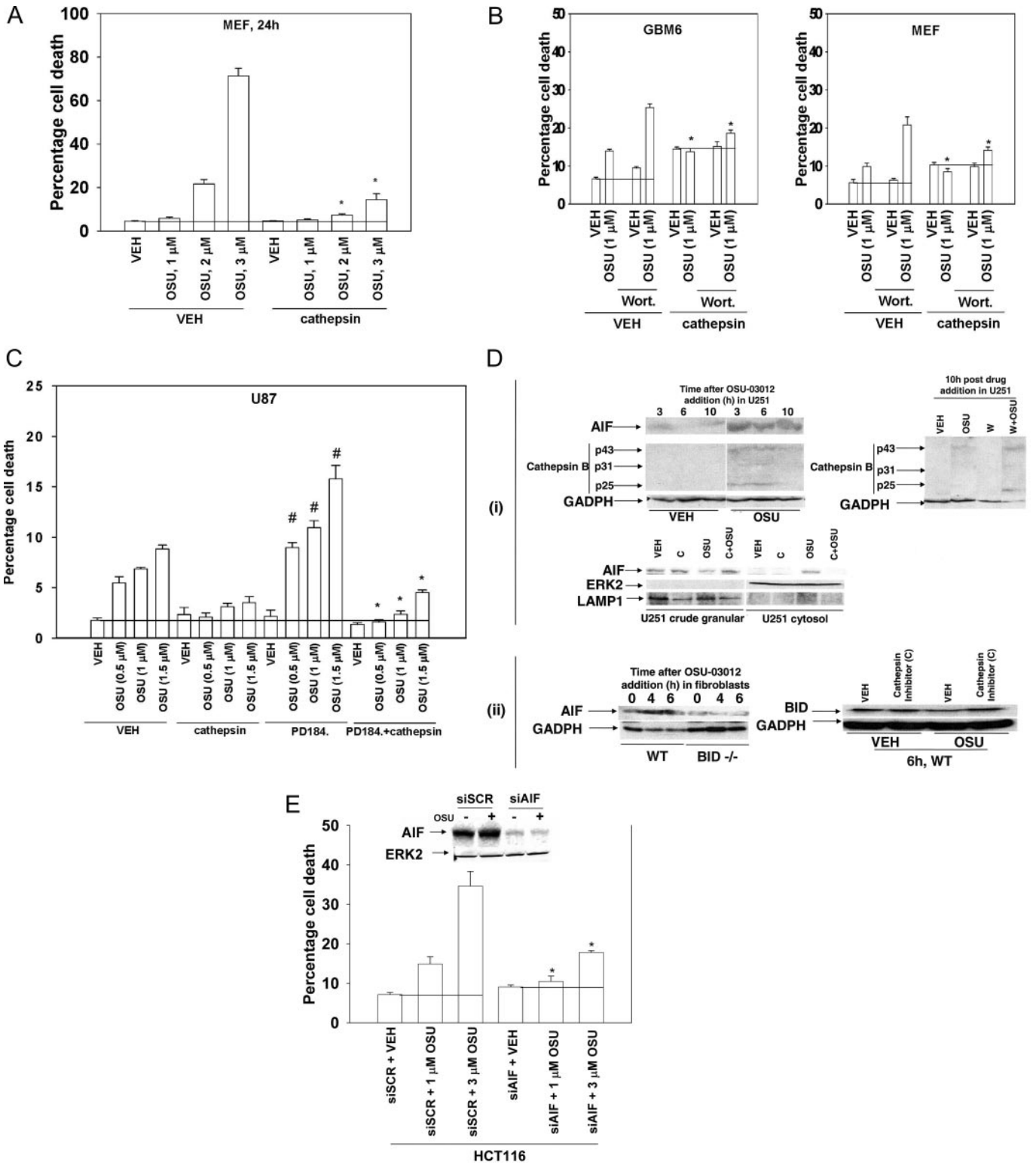


**Fig. 6.** OSU-03012 promotes cell death via activation of cathepsin B, BID cleavage, and AIF release from mitochondria. A, SV40-transformed WT MEFs were cultured as described under *Materials and Methods*. Cells 24 h after plating were treated with DMSO vehicle or with the cathepsin B inhibitor (1 $\mu$M cathepsin). Thirty minutes after cathepsin inhibitor treatment, cells were treated with VEH (DMSO) or with 1 to 3 $\mu$M OSU. Cells were isolated by trypsinization 24 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in the absence of cathepsin B inhibitor). B, SV40-transformed WT MEFs and GBM6 cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating cells were treated with VEH (DMSO) or 100 nM wortmannin (wort.) 100 followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin, drug treatment was for only 1 h before drug removal and replacement with fresh media. In studies treating with either DMSO vehicle or with the cathepsin B inhibitor (1 $\mu$M cathepsin), cells were treated 30 min before wortmannin/OSU-03012 treatment, and media were resupplemented every 24 h with vehicle or cathepsin B inhibitor. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in the absence of cathepsin B

function and other pro-cell death protease enzymes were involved in OSU-03012-induced cell death (e.g., cathepsins). Cathepsins are proteases that have also been reported to use BID as a substrate to initiate cell killing (Stoka et al., 2001; Bidere et al., 2003; Lamparska-Przybysz et al., 2005). Inhibition of cathepsin B function ("cathepsin") suppressed the lethality of OSU-03012 as a single agent and combination with wortmannin in GBM6 and SV40-transformed fibroblasts (Fig. 6, A and B). Inhibition of cathepsin B function suppressed the lethality of OSU-03012 as a single agent and combination with the MEK1/2 inhibitor PD184352 in U87 established glioma cells (Fig. 6C).

Cathepsins have been proposed to cause cell killing in part by promoting BID cleavage leading to mitochondrial dysfunction, including release into the cytosol of proapoptotic proteins such as cytochrome *c* and AIF. Because cytochrome *c* promotes caspase-dependent cell killing, we focused our analyses on changes in AIF subcellular localization into the cytosolic compartment. Exposure of U251 and SV40-transformed fibroblasts to OSU-03012 promoted BID cleavage as well as the release of AIF from mitochondria and cathepsin B from lysosomes, effects that were suppressed in BID−/− fibroblasts and by inhibition of cathepsin B function (Fig. 6D, i and ii). Primary human GBM cells and established U251 cells are difficult to transfect, and no validated siRNA molecules are commercially available to modulate AIF expression in rodent cells; thus, to determine whether AIF played a prominent role in the death process, we made use of HCT116 colon cancer cells transfected with siRNA molecules, in a similar manner to our studies shown in Fig. 1. The ability of OSU-03012 to promote cell killing in HCT116 cells with knocked-down AIF expression was significantly lower compared with scrambled siRNA control-transfected cells (Fig. 6E). Taken together, these findings strongly suggest that OSU-03012 promotes cathepsin-dependent cleavage of BID, which in turn causes AIF release from mitochondria, leading to cell death.

**OSU-03012 Lethality Requires ROS Generation: ROS Effects Are a Secondary Event after AIF Release into the Cytosol.** Overexpression of $BCL_{XL}$ can also suppress the production of ROS generated in mitochondria that, in a cell type and stimulus manner, can promote cell death. ROS production has often been noted to be a primary event in cell signaling and cell death processes. In GBM6 cells and SV40-transformed fibroblasts, the ROS quenching agents *N*-acetyl cysteine and Trolox significantly suppressed OSU-03012 lethality alone or when combined with signaling pathway modulators (Fig. 7A; data not shown). Further studies demonstrated that stable expression of a dominant-negative thioredoxin reductase protein in HeLa cells enhanced OSU-03012 lethality, whereas overexpression of wild-type thioredoxin reductase suppressed OSU-03012-induced cell death (Fig. 7B) (Karimpour et al., 2002).

The impact of OSU-03012 on proliferation was also investigated in these experiments. Treatment of vector transfected or wild type thioredoxin reductase expressing cells with 1 $\mu$M OSU-03012 caused 12.4 ± 2.9 and 5.6 ± 1.4% reductions in total cell numbers, respectively, where expression of dominant-negative thioredoxin reductase permitted OSU-03012 to suppress total cell numbers by 50.2 ± 4.5%. In contrast, apoptosis was enhanced above basal levels in vector, wild-type thioredoxin reductase and mutant wild-type thioredoxin reductase expressing cells by 4.3 ± 1.1, 2.1 ± 0.5, and 11.1 ± 0.8%, respectively. These findings provide further support for the concept that OSU-03012 suppresses cell growth before inducing cell death, an effect that is primarily ROS-dependent. However, OSU-03012 did not enhance intracellular ROS levels within 6 h of OSU-03012 exposure, a time at which AIF was released into the cytosol (Fig. 6B), suggesting that ROS play a secondary but essential role in the lethality of OSU-03012 (Fig. 7C).

**OSU-03012 Lethality Requires Expression of PERK.** Overexpression of either $BCL_{XL}$ or BCL-2 can protect mitochondria as well as the endoplasmic reticulum (ER) from toxic stresses, and the parental compound of OSU-03012, celecoxib, has been proposed to use ER stress to kill malignant cells (Tsutsumi et al., 2006). Thus, to test whether ER stress signaling was involved in OSU-03012-stimulated cell killing, we made use of PERK-null (PERK−/−) fibroblasts. The ability of OSU-03012 to cause cell death alone or when combined with wortmannin was significantly reduced in PERK−/− cells (Fig. 8A). It is surprising, however, that treatment of fibroblasts with low concentrations of OSU-03012 (1 $\mu$M), or when combined with wortmannin, did not significantly alter the phosphorylation of one established PERK substrate, eIF2$\alpha$, at position Serine 51 (Fig. 8A, inset) (Ron, 2002). PERK −/− cells also exhibited a reduced release of AIF and cathepsin B into the cytosol after OSU-03012 exposure compared with wild-type fibroblasts (Fig. 8A, inset). Control studies using the ER stress-inducing agent

inhibitor). The cathepsin B inhibitor in a stochastic manner occasionally enhanced basal levels of cell morbidity for unknown reasons. C, U87 glioma cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or cathepsin B inhibitor (1 $\mu$M cathepsin) followed 1 h later by VEH (DMSO), OSU as indicated, 1 mM PD184, or the drug combination. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in the absence of cathepsin B inhibitor; #, $p < 0.05$ greater than corresponding value in the absence of cathepsin B inhibitor). D, i, OSU-03012 promotes AIF and cathepsin B release from the crude granular fraction. ii, OSU-03012 promotes AIF release in a BID-dependent manner and promotes BID cleavage in a cathepsin B-dependent manner. SV40-transformed wild-type and BID−/− MEFs and U251 cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated where indicated with VEH (DMSO) or 100 nM wortmannin followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin, drug treatment was for only 1 h before drug removal and replacement with fresh media. In studies treating with either DMSO vehicle or with the cathepsin B inhibitor (1 $\mu$M cathepsin; C) cells were treated 30 min before wortmannin/OSU-03012 treatment. Cells were isolated by trypsinization at the indicated times after drug treatments (0–10 h). The cytosolic fraction was isolated as described under *Materials and Methods*. The cytosolic fraction was subjected to SDS-PAGE and immunoblotting was performed to determine the release into the cytosolic fraction of AIF or of cathepsin B (proenzyme and active fragments). Data for OSU/wortmannin treatment examining cathepsin B release 10 h after exposure is a cut and paste from the blot to its left. E, HCT116 cells were transfected as described under *Materials and Methods* 24 h after plating with either a scrambled anti-siRNA against AIF (siSCR) or an siRNA against AIF (siAIF). Forty-eight hours after transfection, cells were treated with VEH (DMSO) or with 1 or 3 $\mu$M OSU. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding siSCR value). Inset, cells were isolated 24 h after vehicle or OSU-03012 treatment, and SDS-PAGE followed by immunoblotting for AIF was performed.

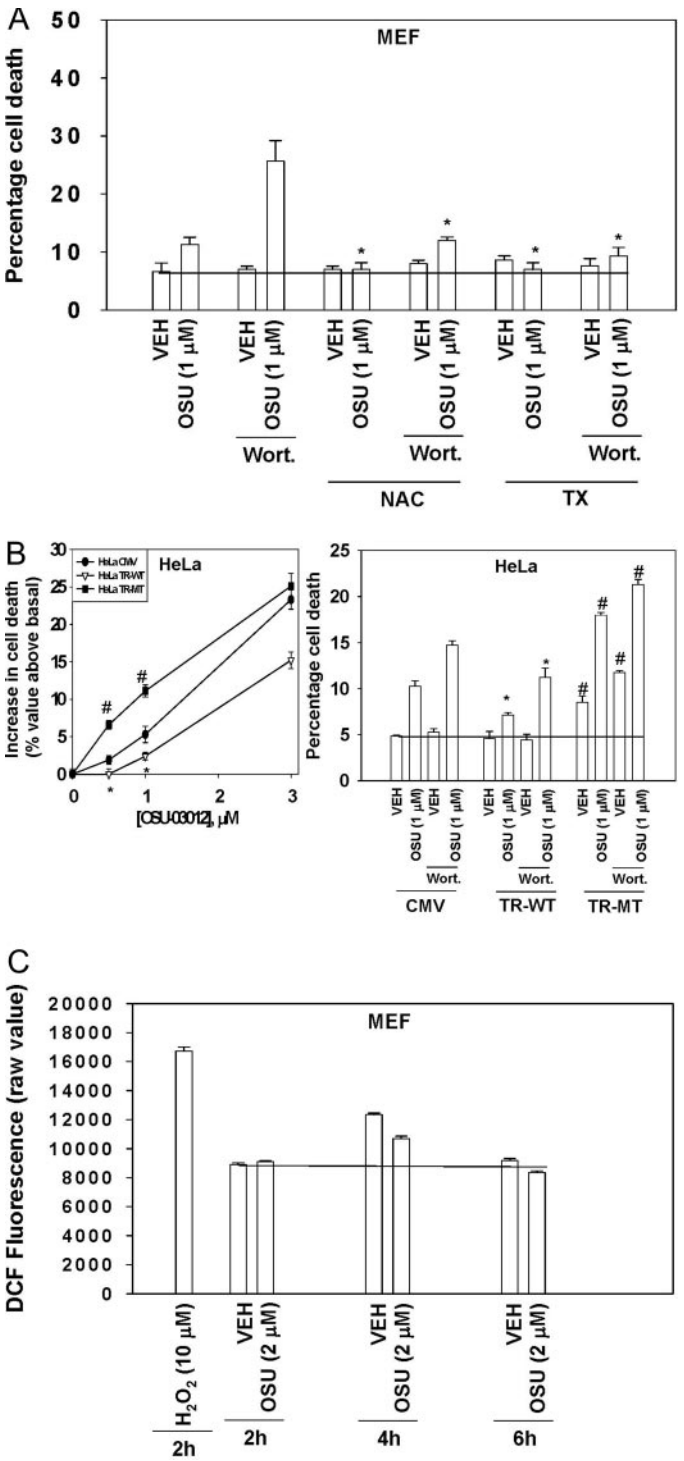


**Fig. 7.** ROS play a secondary but essential role in OSU-03012 lethality. A, SV40-transformed WT mouse MEFs were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (phosphate-buffered saline), with 20 mM *N*-acetylcysteine (NAC), or with 10 $\mu$M Trolox (TX). After 30 min, cells were treated with VEH (DMSO) or 100 nM wortmannin (wort.) followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin, drug treatment was for only 1 h before drug removal and replacement with fresh media. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in the absence of NAC or TX). B, HeLa cells were plated, and 24 h after plating cells were treated as indicated with VEH (DMSO) or 100 mM wortmannin followed 1 h later by VEH (DMSO) or with 0 to 3 $\mu$M OSU as indicated. Note that in the case of wortmannin, drug treatment was for only 1 h before drug removal and replacement with fresh media. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in vector control-transfected cells (#, $p < 0.05$ greater than corresponding value in vector control-transfected cells). C, SV40-transformed wild-type MEFs were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or with 1 $\mu$M OSU. In one set of cells, cells were treated with hydrogen peroxide. Fifteen minutes before each indicated time point, dichlorofluorescein was added to the culture media. After 15 min, media were removed, fresh media were added, and DCF fluorescence was measured in octuplicate in a 96-well plate reader.

thapsigargin demonstrated that unlike OSU-03012, thapsigargin lethality was enhanced in PERK−/− cells compared with wild-type cells (Fig. 8B). In confirmatory dose-response survival analyses, a lack of PERK expression suppressed the lethality of OSU-03012 in short-term trypan blue/cell death assays and in long-term survival/colony formation assays (Fig. 8C).

Based on previous studies arguing that RAS transformation promotes activation of PI3K and that wortmannin enhanced OSU-03012 lethality in SV40-transformed fibroblasts, we determined the impact of activated RAS expression on OSU-03012 lethality in combination with wortmannin in wild-type and PERK−/− cells (Caron et al., 2005; Fig. 1). In both SV40- and RAS-transformed cells, OSU-03012 lethality was attenuated by loss of PERK function (Fig. 8D). However, whereas in SV40-transformed cells the apoptotic response of OSU-03012 and wortmannin treatment was *reduced* by loss of PERK function, the apoptotic response of RAS transformed cells to the same combined drug exposure in PERK−/− cells was *enhanced*. These findings suggest that ER stress signaling is involved in cell killing after OSU-03012 exposure, but that under certain conditions, OSU-03012 lethality may be reduced or enhanced by loss of PERK function.

## Discussion

Previous studies have demonstrated that the novel celecoxib derivative OSU-03012 at concentrations in the low micromolar range killed hematopoietic, lung, and colon cancer cells. The present study was initiated to determine the molecular mechanism by which OSU-03012 kills transformed cells, and in particular, primary human glioma cells, in vitro.

OSU-03012 promoted a dose-dependent induction of primary human glioma cell killing and interacted in an additive manner with radiation exposure in short-term viability assays to promote cell death. This effect was evident in glioma cells overexpressing ERRB1 and ERBB1 vIII, expressing activated PI3K molecules, and lacking PTEN expression. OSU-03012 has been proposed to be a PDK-1 inhibitor and to kill cells primarily via this mechanism. However, OSU-03012 toxicity and the inhibition of AKT function by OSU-03012 did not correlate closely compared with the signaling and cytotoxic effects of siRNA knock-down of PDK-1 expression. In primary GBM cells, concentrations of OSU-03012 that caused a modest level of toxicity weakly and transiently suppressed AKT activity and did not alter ERK1/2, JNK1/2, and p38 MAPK phosphorylation. Although low concentrations of OSU-03012 had modest effects on viability or signal-

ing pathway activities, the same drug levels caused a significant suppression in proliferation. Thus, the primary effect of OSU-03012 on cell behavior was growth suppression with higher concentrations abolishing growth and promoting cell death.

Previous studies by this group have solidified the overall concept that blocking two parallel survival signal transduction pathways leads to tumor cell killing using a variety of small molecule inhibitors of kinases and other enzymes (e.g., MEK1/2 inhibitors and UCN-01) (Dai et al., 2001; McKinstry

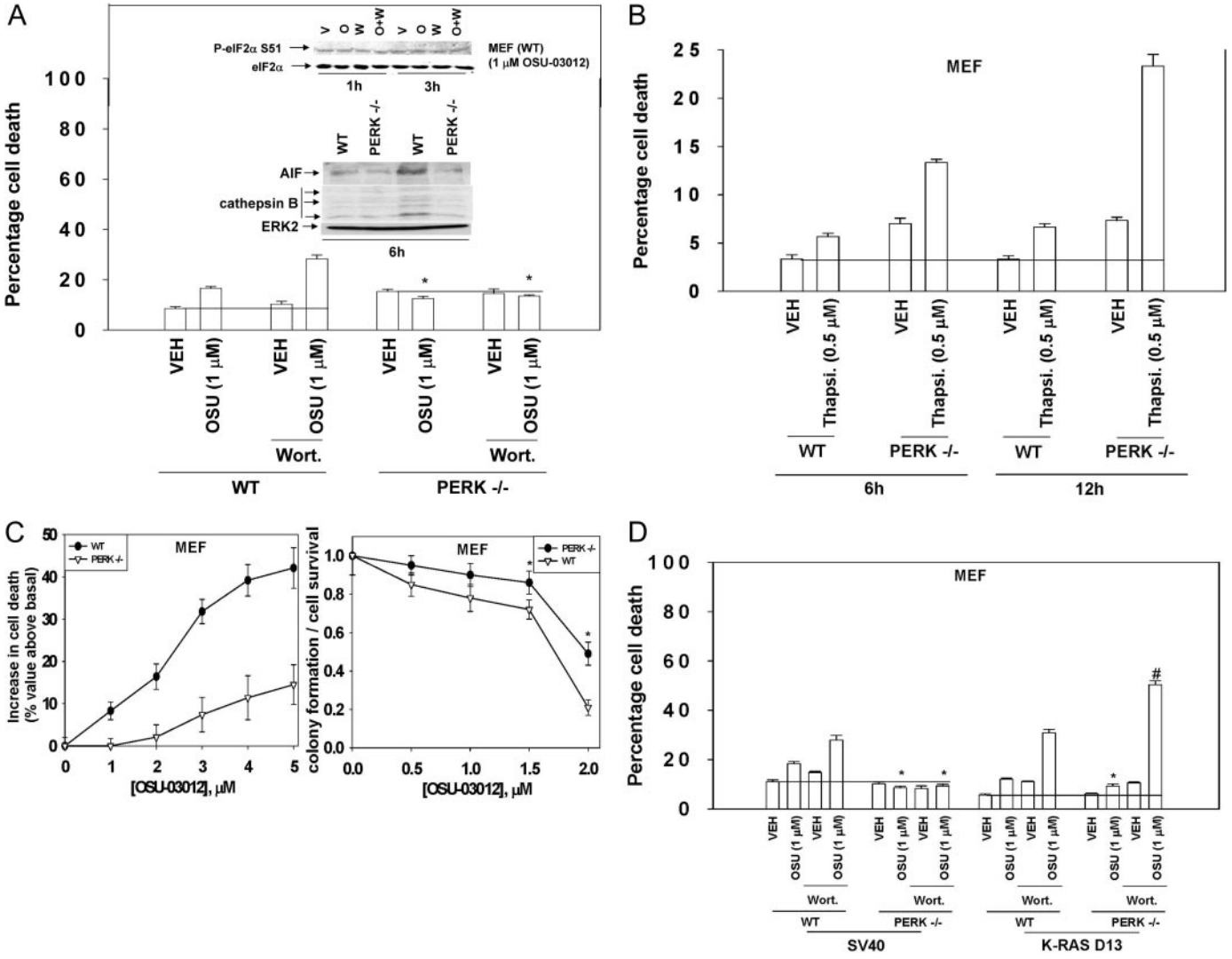


**Fig. 8.** Loss of PERK expression alters sensitivity of cells to OSU-03012 in a transformation-dependent manner. A, SV40-transformed WT and PERK−/− MEFs were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or 100 nM wortmannin (wort.) followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Note that in the case of wortmannin, drug treatment was for only 1 h before drug removal and replacement with fresh media. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$) (*, $p < 0.05$ less than corresponding value in wild-type cells) Inset, top, SV40-transformed wild-type MEFs were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or 100 nM wortmannin followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Cells were isolated 1 and 3 h after OSU-03012 treatment, prepared for SDS-PAGE, and immunoblotted for eIF2$\alpha$ S51 phosphorylation. Inset, bottom, SV40-transformed wild-type and PERK−/− MEFs were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or 100 nM wortmannin followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Six hours after OSU-03012 exposure, cells were isolated and cytosolic fractions obtained. The release of AIF into the cytosol after OSU-03012 exposure was determined by immunoblotting. B, SV40-transformed WT and PERK−/− fibroblasts were cultured as described under *Materials and Methods*, and 24 h after plating they were treated with VEH (DMSO) or with 0.5 $\mu$M thapsigargin (Thapsi.). Cells were isolated by trypsinization 6 and 12 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. C, left, SV40-transformed WT and PERK−/− MEFs were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or with 0 to 5 $\mu$M OSU. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate. Data are from a representative experiment ($n = 3$). Right, SV40-transformed WT and PERK−/− MEFs were plated as single cells. Twenty-four hours after plating, cells were treated with VEH (DMSO) or with 0 to 2 $\mu$M) OSU for 48 h. After 48 h, the drugs were removed from the culture media, and cells were permitted to form colonies over the following 14 days. Each data point is the mean of six separate individual treatments (*, $p < 0.05$ greater survival than corresponding value in wild-type cells). D, SV40-transformed WT and PERK−/− MEFs and K-RAS D13-transformed wild-type and PERK−/− MEFs were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with VEH (DMSO) or 100 nM wortmannin followed 1 h later by VEH (DMSO) or with 1 $\mu$M OSU. Cells were isolated by trypsinization 48 h after drug treatments. Cell viability was determined using a trypan blue exclusion assay in triplicate (*, $p < 0.05$ less than corresponding value in wild-type cells; #, $p < 0.05$ greater than corresponding value in wild-type cells).

et al., 2002). Inhibition of ERK1/2 in GBM cells promoted the lethality of OSU-03012 and, more surprisingly, if PDK-1 was the sole target protein in the biological actions of OSU-03012 did inhibition of PI3K function. Combinatorial cell killing with these drugs was not observed in nontransformed/nonestablished primary cell types, arguing that OSU-03012-induced cell killing was relatively specific for tumor/transformed cells.

In a similar manner to our findings combining OSU-03012 and ionizing radiation, the death-response profile of primary human GBM cells and established GBM cell lines exposed to OSU-03012 in combination with MEK1/2 or PI3K inhibition did not correlate simplistically with the mutational or expression status of proto-oncogenes, including cyclin-dependent kinase 4, platelet-derived growth factor receptor, ERBB1, PI3K, PTEN, p16, or p53. The mutational/expression status of proteins such as ERBB1, PI3K, PTEN, and p53 in tumor cells have also been linked by others to a reduced apoptotic response after irradiation, a response that has generally been believed to be mediated by caspase proteases. However, OSU-03012 promoted cell death, combined with radiation or signaling pathway inhibitors, via a largely caspase-independent mechanism that was also not reliant on the function of BAX, BAK, or BIM, but it was significantly inhibited by loss of BID function and to a lesser extent by overexpression of $BCL_{XL}$. In contrast to our findings in solid malignancies, studies by Johnson et al. (2005) using OSU-03012 in leukemia cells argued that BCL-2 overexpression had no effect on cell survival after drug exposure.

We then determined in more detail the pathway by which cells were induced to die after OSU-03012 treatment. The caspase-independent cell death induced by OSU-03012 correlated with cleavage of BID and release of AIF from mitochondria into the cytosol. Loss of BID function suppressed AIF release into the cytosol, and inhibition of cathepsin B function suppressed BID cleavage and AIF release. OSU-03012 also promoted release of cathepsin B into the cytosolic compartment. These findings are in agreement with the hypothesis that OSU-03012 promotes cathepsin protease translocation into the cytosol, which catalyzes BID cleavage that in turn promotes AIF release into the cytosol, leading to cell death. Loss of PERK function/ER stress signaling blocked the release of cathepsin B and AIF into the cytosol. Inhibition of PI3K signaling and also MEK1/2 signaling promoted a modest further release of cathepsin B into the cytosolic compartment, which correlated with enhanced cell killing, effects that were not observed in cells lacking PERK function or in cells incubated with a specific cathepsin B inhibitor (A. Yacoub, M. A. Park, and P. Dent, unpublished data). Taken together, our data argue that OSU-03012 causes an as yet undefined form of an ER stress response that leads to activation of a cathepsin B/BID/AIF-dependent cell death pathway. Inhibition of survival signal transduction pathways has the potential to enhance cathepsin B release into the cytosol that was dependent on the initial induction of ER stress.

Celecoxib-induced apoptosis in nontransformed endothelial cells has been argued to be ER stress-dependent, with loss of GADD153 (CHOP) function preventing cell killing (Tsutsumi et al., 2004, 2006). In this model, simplistically, Celecoxib-induced apoptosis would thus be impeded in cells lacking PERK expression, which is the opposite to our findings with OSU-03012 treatment in transformed cells, wherein both SV40- and K-RAS D13-transformed fibroblasts were more resistant to drug toxicity when PERK was not expressed. Several possible explanations exist for this difference, including the following: 1) SV40-transformed cells lacking PERK expression were still competent to be killed by OSU-03102, albeit at higher concentrations, arguing that ER stress signaling represents only one portion of the mechanism by which OSU-03012 acts to kill cells; i.e., exposure of wild-type cells to 2 $\mu$M OSU-03012 caused the same level of killing as observed in PERK$-/-$ cells treated with 5 $\mu$M OSU-03012; 2) loss of PERK expression in SV40-transformed cells abolished 1 $\mu$M OSU-03012 lethality, whereas loss of PERK function in K-RAS D13 cells reduced OSU-03012 lethality by only ~40%; and 3) in cells transformed by K-RAS D13 loss of PERK expression *enhanced* the toxicity of combined wortmannin and OSU-03012 exposure. With respect to the actions of OSU-03012, some novel agents [e.g., the proteasome inhibitor bortezomib (Velcade)] have been shown to kill tumor cells via increased expression of ER stress markers such as CHOP and BiP while inhibiting PERK activity and the phosphorylation of eiF2$\alpha$ and in a manner not indicative of classic proteotoxicity (Jiang and Wek, 2005; Nawrocki et al., 2005).

We had noted that nontransformed cells were more resistant to the toxic effects of OSU-03012 as a single agent and when combined with MEK1/2 or PI3K inhibitors, as has been observed for the proteasome inhibitor Velcade, and collectively, our findings argue that the mode of cell transformation may alter the role PERK and ER stress signaling plays in OSU-03012 toxicity. Based on the observation that ER stress signaling can promote a toxic response (e.g., elevated CHOP expression) or a protective response (e.g., enhanced Grp78 expression) and the fact that PERK signaling can enhance or suppress OSU-03012 lethality, additional studies outside the scope of the present manuscript will be required to determine the precise mechanisms upstream and downstream of PERK by which OSU-03012 alters transformed cell survival.

In many studies using chemotherapeutic agents or after radiation exposure, the generation of ROS is a primary event that can lead to altered cell signaling, cell growth, and cell survival responses (Leach et al., 2001). Inhibition of ROS generation using small molecule or molecular reagents suppressed OSU-03012 lethality, 48 h after drug exposure. The ERK1/2 and AKT signaling pathways have been shown by many groups to be activated by ROS generation; however, at a time after exposure where OSU-03012 was promoting AIF release into the cytosol (2–6 h), GBM cells treated with 3 $\mu$M drug had reduced AKT phosphorylation at T308 without any large changes in ERK1/2 phosphorylation. Furthermore, no significant increase in ROS levels after OSU-03012 treatment was detected within this time frame. These findings argue that OSU-03012-induced ROS generation is a secondary event that occurs at some point in time after AIF release but that is essential for cell killing to occur. Our data also argue that ROS play an important role in the antiproliferative effects of OSU-03012. In the majority of the cell types examined in this study, 1 $\mu$M OSU-03012 caused a modest amount of cell killing (~10% increase over basal) but a large suppression of cell growth (~40% reduction below basal). Our studies using HeLa cells with different levels of thioredoxin reductase expression and function, and therefore differential ability to metabolize ROS, strongly suggested that a lower

ability to maintain reduced thioredoxin levels, for ROS detoxification, resulted in a modest enhancement in OSU-03012 lethality (~5% increase) but a large suppression in cell growth (~40% reduction). These findings demonstrate that at low, modestly toxic concentrations OSU-03012 promotes growth arrest in an ROS-dependent manner.

Our studies demonstrated that OSU-03012 was at least additive in its ability to enhance the toxic effects of ionizing radiation, and radiotherapy is typically used in the management of gliomas, either after surgical resection or as a primary therapy in patients who are not surgical candidates. COX2 inhibitors, including celecoxib, have been shown to rapidly penetrate the central nervous system and in GBM the blood-brain barrier is frequently compromised. Together, these findings argue that this class of drugs may have potential in glioma treatment (Dembo et al., 2005; Reardon et al., 2005; Kang et al., 2006). Together with the data presented in this article, these findings provide evidence that the novel agent OSU-03012 warrants further investigation as a therapeutic agent in GBM.

**Acknowledgments**

This article is dedicated to the father of P.D., Lawrence Miller Dent, who died from GBM in October 2005, 10 months after initial disease presentation.

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