Regulation of OSU-03012 Toxicity by ER Stress Proteins and ER Stress–Inducing Drugs

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Abstract

The present studies examined the toxic interaction between the non-coxib celecoxib derivative OSU-03012 and phosphodiesterase 5 (PDE5) inhibitors, and also determined the roles of endoplasmic reticulum stress response regulators in cell survival. PDE5 inhibitors interacted in a greater than additive fashion with OSU-03012 to kill parental glioma and stem-like glioma cells. Knockdown of the endoplasmic reticulum stress response proteins IRE1 or XBP1 enhanced the lethality of OSU-03012, and of [OSU-03012 + PDE5 inhibitor] treatment. Pan-caspase and caspase-9 inhibition did not alter OSU-03012 lethality but did abolish enhanced killing in the absence of IRE1 or XBP1. Expression of the mitochondrial protective protein BCL-XL or the caspase-8 inhibitor c-FLIP-s, or knockdown of death receptor CD95 or the death receptor caspase-8 linker protein FADD, suppressed killing by [OSU-03012 + PDE5 inhibitor] treatment. CD95 activation was blocked by the nitric oxide synthase inhibitor L-NAME. Knockdown of the autophagy regulatory proteins Beclin1 or ATG5 protected the cells from OSU-03012 and from [OSU-03012 + PDE5 inhibitor] toxicity. Knockdown of IRE1 enhanced OSU-03012/[OSU-03012 + PDE5 inhibitor]–induced JNK activation, and inhibition of JNK suppressed the elevated killing caused by IRE1 knockdown. Knockdown of CD95 blunted JNK activation. Collectively, our data demonstrate that PDE5 inhibitors recruit death receptor signaling to enhance OSU-03012 toxicity in glioblastoma multiforme (GBM) cells. Mol Cancer Ther; 13(10); 2384–98. ©2014 AACR.
ER stress signaling is mediated by three proximal sensors, PERK, the inositol-requiring protein 1α (IRE1)/XBP1 (X-box binding protein 1) system, and activating transcription factor 6 (ATF6). Previously, we demonstrated that knockdown of PERK suppressed OSU-03012 toxicity; however, this effect appeared to be only partially if2α dependent (12, 13). IRE1 is the oldest and most conserved branch of the unfolded protein response (UPR)/ER stress response in metazoans (16). IRE1 contains both a kinase domain and an endoribonuclease activity. Dimerization and autophosphorylation of IRE1 results in activation of the endonuclease activity, causing alternative splicing of XBP1 mRNA and expression of the active XBP1 transcription factor (17). It has also been noted that IRE1 can have a more relaxed specificity for mRNA substrates, with evolutionary similarity to the actions of RNase L, and that in an XBP1-independent fashion reduces mRNA levels (18, 19). This form of IRE1 biology was called regulated IRE1-dependent degradation (or RIDD; refs. 18, 19). In theory, signaling by PERK and by RIDD should mediate the survival of stressed cells, by reducing protein levels. But, alternatively, RIDD could also possibly facilitate death of ER-stressed cells (20). The IRE1-like endonuclease RNase L is a highly active interferon-inducible RNase and the level to which RNase L is expressed regulates survival/death decisions in ER-stressed cells (21). Thus, low amounts of IRE1 activity could promote survival, with higher levels of activity promoting cell death.

Tumors of the brain are notoriously difficult to control. Untreated adult glioblastoma multiforme (GBM) patients have a mean survival of several months that is only prolonged up to 12 to 16 months by aggressive therapeutic intervention. New therapeutic approaches that could translate to the clinic for this malignancy are urgently required. In two recently published studies, we demonstrated that phosphodiesterase 5 (PDE5) inhibitors enhanced the toxicity of standard-of-care chemotherapies in bladder and pediatric central nervous system (CNS) tumors (22, 23). In the present studies, we determined whether PDE5 inhibitors interacted with OSU-03012 to kill glioma cells and we defined the molecular mechanisms by which the ER stress effector pathways regulated OSU-03012 toxicity.

Materials and Methods

Materials

Phospho-/total antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology. The Anti-CD95 antibody (DX3; ab25638) was from Abcam. All drugs, including OSU-03012, were purchased from Selleckchem; Fig. 1A). Commercially available validated
short hairpin RNA molecules to knockdown RNA/protein levels were from Qiagen. At least two different validated siRNA molecules were independently used to confirm that the effects observed were not due to non-specific effects. Antibody reagents, other kinase inhibitors, caspase inhibitor cell culture reagents, and noncommercial recombinant adenoviruses have been previously described. Previously characterized semi-established GBM5/GBM6/GBM12/GBM14 glioblastoma cells were supplied by Dr. C.D. James (University of California, San Francisco, San Francisco, CA) and Dr. J.N. Sarkaria (Mayo Clinic, Rochester, MN) and were not further characterized by us (24). The primary human GBM isolates (patient 1; patient 2; and patient 3) were obtained/isolated from discarded tumor tissue after standard-of-care surgery. Patients had previously given informed consent under an IRB protocol to the use of tumor tissue. Tumor samples were made anonymous of all patient identifiers by the VCU Tissue and Data Acquisition and Analysis Core (TD AAC) before hand-over to the Dent laboratory.

Methods

Cell culture and in vitro exposure of cells to drugs. All fully established cancer lines were cultured at 37 °C (5% v/v CO²) in vitro using RPMI-1640 supplemented with 10% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids. All primary human GBM cells were cultured at 37 °C (5% v/v CO²) in vitro using RPMI-1640 supplemented with 2% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids at 37 °C (5% v/v CO²). GBM5/6/12/14 stem cells were cultured in growth factor–free media during any study. Cells were transfected with various drug concentrations, as indicated. In vitro small-molecule inhibitor treatments were from a 100 mmol/L stock solution of each drug and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). Cells were not cultured in growth factor–free media during any study.

Cell treatments, SDS-PAGE, and Western blot analysis. Cells were treated with various drug concentrations, as indicated in the Figure legends. Samples were isolated at the indicated times and SDS-PAGE and immunoblotting was performed as described in refs. 11–13, 15. Blots were observed by using an Odyssey IR imaging system (LI-COR Biosciences).

Recombinant adenoviral vectors; infection in vitro. We generated and purchased previously noted recombinant adenoviruses as per refs. 11–13, 15. Cells were infected with these adenoviruses at an approximate multiplicity of infection (m.o.i.) as indicated in the Figure/legend (usually an m.o.i. of 50). Cells were incubated for 24 hours to ensure adequate expression of transduced gene products before drug exposures.

Detection of cell death by Trypan Blue assay. Cells were harvested by trypsinization with Trypsin/EDTA for approximately 10 minutes at 37 °C. Harvested cells were combined with the culture media containing unattached cells and the mixture was centrifuged (800 rpm, 5 minutes). Cell pellets were resuspended in PBS and mixed with Trypan Blue agent. Viability was determined microscopically using a hemocytometer (11–13, 15). Five hundred cells from randomly chosen fields were counted and the number of dead cells was counted and expressed as a percentage of the total number of cells counted. Cell killing was confirmed using the Sceptor instrument (Millipore), which measured tumor cell size/sub-G1 DNA as an indication of tumor cell viability.

Soft-agar colony formation assay. Free-floating GBM5/6/12/14 stem cells were triturated to form single cells and were plated into soft agar in sextuplicate using established procedures (500–1,000 cells per 60-mm dish; ref. 25). Cells were treated with vehicle (DMSO), OSU-03012 (1.0 nmol/L), sildenafil (2 μmol/L), or the drugs combined. Twenty-four hours after drug treatment, the plates were washed with drug-free media and the cover media were replaced with drug-free media. Colonies were permitted to form over the following 20 days, after which they were stained, and counted.

Colony formation assay. Tumor cells plated as single cells (250–4,000 cells per well) in sextuplicate were treated with OSU-03012 (OSU, 0.5–2.0 nmol/L) and/or sildenafil (SIL, 0.5–2.0 μmol/L) at a fixed concentration ratio for 24 hours, after which the media were removed and replaced with drug-free media. Colonies were permitted to form for the following 10 to 14 days. Colonies were fixed, stained, and counted (>50 cells per colony).

Assessment of autophagy. Cells were transfected with a plasmid to express a green fluorescent protein (GFP)– and red fluorescent protein (RFP)–tagged form of LC3 (ATG8). For analysis of cells transfected with the GFP-RFP-LC3 construct, the GFP/RFP-positive vesicularized cells were examined under the 40× objective of a Zeiss Axiosvert fluorescent microscope.

Plasmid transfection

Plasmids. Cells were plated as described above and 24 hours after plating, transfected. Plasmids (0.5 μg) expressing a specific mRNA or appropriate vector control plasmid DNA were diluted in 50 μL of serum-free and antibiotic-free medium (1 portion for each sample). Concurrently, 2 μL of Lipofectamine 2000 (Invitrogen) was diluted into 50 μL of serum-free and antibiotic-free medium. Diluted DNA was added to the diluted Lipofectamine 2000 for each sample and incubated at room temperature for 30 minutes. This mixture was added to each well/dish of cells containing 200 μL of serum-free and antibiotic-free media.
medium for a total volume of 300 μL, and the cells were incubated for 4 hours at 37°C. An equal volume of 2× medium was then added to each well. Cells were incubated for 48 hours, then treated with drugs. To assess the transfection efficiency of plasmids, we used a plasmid to express GFP and defined the percentage of cells being infected as the percentage of GFP+ cells. For all cell lines, the infection efficiency was >70%.

siRNA. Cells were plated in 60-mm dishes from a fresh culture growing in log phase as described above, and 24 hours after plating, transfected. Before transfection, the medium was aspirated and 1 mL serum-free medium was added to each plate. For transfection, 10 nmol/L of the annealed siRNA, the positive sense control double-stranded siRNA targeting GAPDH 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 (predominantly Qiagen; occasional alternate siRNA molecules were purchased from Ambion, Inc.). At least two different validated siRNA molecules were independently used to confirm that the effects observed were not due to nonspecific effects. Ten nmol/L siRNA (scrambled or experimental) was diluted in serum-free media. Four μL HiPerFect (Qiagen) was added to this mixture and the solution was mixed by pipetting up and down several times. This solution was incubated at room temp for 10 minutes, then added drop-wise to each dish. The medium in each dish was swirled gently to mix, then incubated at 37°C for 2 hours. One milliliter of 10% (v/v) serum-containing medium was added to each plate, and cells were incubated at 37°C for 24 to 48 hours before replating (50 × 10^3 cells/plate) onto 12-well plates. Cells were allowed to attach overnight, then treated with drugs (0–48 hours). Trypan blue exclusion assays and SDS-PAGE/immunoblotting analyses were then performed at the indicated time points.

Data analysis
Comparison of the effects between various in vitro drug treatments was performed after analysis of variance using the Student t test. Differences with a P value of <0.05 were considered statistically significant. Experiments shown are the means of multiple individual points from multiple studies (±SEM). Median dose-effect isobologram colony-formation analyses to determine synergism of drug interaction were performed according to the methods of Chou and Talalay using the CalcuSyn program for Windows (Biosoft). Cells were treated with agents at an escalating fixed concentration drug dose. A combination index (CI) of <1.00 indicates synergy of interaction between the two drugs; a CI of approximately 1.00 indicates an additive interaction; and a CI value of >1.00 indicates antagonism of action between the agents.

Results
Initial studies examined the interaction between OSU-03012 and the PDE5 inhibitors sildenafil (Viagra) and tadalafil (Cialis) in parental glioma cells and glioma cells selected for stemness growing without attachment in the serum-free media containing growth factors. In a dose-dependent fashion, OSU-03012 interacted with sildenafil to kill glioma cells (Fig. 2A). OSU-03012 interacted with PDE5 inhibitors in an apparent greater than additive fashion to kill both parental and stem-like glioma cells (Fig. 2B–E). This was confirmed using median dose-effect colony formation assays (Table 1). Data very similar to those generated with OSU-03012 were obtained using the FDA-approved parent drug celecoxib at clinically relevant concentrations (Fig. 2F, unpublished data).

Of note was that the amount of killing caused by the drug combination differed little comparing parental and stem-like cell populations. Similar drug interaction data were obtained using stem-like cells in colony formation assays (Fig. 3A). Glioma cells freshly isolated from patient tumors also exhibited a greater than additive killing effect when treated with OSU-03012 and PDE5 inhibitors (Fig. 3B and C). We next determined whether PDE5 represented a key target for PDE5 inhibitors in our drug combination studies. Knockdown of PDE5 in GBM cells enhanced OSU-03012 lethality to a similar extent as did tadalafil (Fig. 3D–F). Multiple PDE5 siRNA molecules gave similar effects, as we have observed before (22, 23). In the tonic regulation vascular smooth muscle relaxation, PDE5 inhibitors are thought to act through increasing levels of cyclic GMP (cGMP), resulting in increased levels of nitric oxide (NO; ref. 26, and references therein). Incubation of GBM cells with the NO synthase inhibitor L-NG-Nitroarginine Methyl Ester (L-NAME) suppressed cell killing by the OSU-03012 and sildenafil drug combination (Fig. 3G).

In several prior studies, we demonstrated that ER stress signaling by PERK played a major role in mediating the toxic actions of OSU-03012 (12, 13, 15). Our present studies focused on defining the role of the IRE1/XBP1 arm of the ER stress response in OSU-03012/OSU-03012 + sildenafil biology in GBM cells. We found that knockdown of the IRE1/XBP1 arm of the ER stress signaling pathway enhanced OSU-03012 toxicity (Fig. 4A–D). Knockdown of IRE1/XBP1 signaling also enhanced [OSU-03012 + sildenafil] toxicity (Fig. 4E and F).

Previously, we demonstrated that pan-inhibition of caspases using the agent zVAD did not reduce OSU-03012 toxicity, and similar findings were made herein (Fig. 5A and B). However, the ability of IRE1/XBP1 knockdown to enhance OSU-03012 lethality was abolished by use of the pan-caspase inhibitor zVAD. Of note was that the caspase-9 inhibitor, LEHD, but not the caspase 8 inhibitor IETD, protected cells (Fig. 4C, data not shown). This would argue that knockdown of IRE1 is facilitating activation of the intrinsic apoptosis pathway. Unlike treatment with OSU-03012 alone, inhibition of caspase-8 by overexpression of c-FLIP-s reduced [OSU-03012 + sildenafil] lethality (Fig. 5D). As caspase-8 inhibition was protective against [OSU-03012 + sildenafil] lethality, we next determined whether death receptor signaling played a role in drug combination lethality; knockdown of CD95
than OSU-03012 and sildenafil. OSU-03012 and sildenafil interact to kill multiple GBM cell isolates. A, GBM12 cells were treated with vehicle (Veh; DMSO), OSU-03012 (0–2 μmol/L), sildenafil (0–2 μmol/L), or the drugs in combination, as shown. Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); *, P < 0.05 greater than OSU treatment alone; @, P < 0.05 greater than sildenafil treatment alone. B–E, GBM5, GBM6, GBM12, and GBM14 cells as well as selected stem cell-like neurosphere variants of these cells were treated with vehicle (DMSO), OSU-03012 (1 μmol/L), sildenafil (2 μmol/L), tadalafil (1 μmol/L), or the drugs in combination, as shown. Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM): *, P < 0.05 greater than OSU treatment alone. F, GBM6-stem and GBM12-stem cells were treated with vehicle (DMSO), OSU-03012 (1 μmol/L), sildenafil (2 μmol/L), celecoxib (5 μmol/L), or the drugs in combination, as shown. Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM).

or of Fas-associated death domain protein (FADD) suppressed [OSU-03012 + sildenafil] lethality (Fig. 5E). An agonistic anti-CD95 antibody was more effective at killing than [OSU-03012 + sildenafil] treatment and enhanced [OSU-03012 + sildenafil] lethality. Incubation of GBM cells with the NO synthase inhibitor L-NAME suppressed CD95 activation by the OSU-03012 and sildenafil drug combination (Fig. 5F).

Prior studies from our laboratory have shown that increased PERK-dependent autophagy was causal in OSU-03012 toxicity as a single agent (12, 13, 15). Hence, we next determined the induction of autophagy after knockdown of IRE1 and after exposure of GBM cells to OSU-03012 and sildenafil. OSU-03012 and sildenafil interacted in a greater than additive fashion to induce autophagy, and in a time-dependent fashion increased the numbers of GFP+ vesicles (early autophagy, autophagosomes) followed by increased numbers of RFP+ vesicles (late autophagy, autolysosomes; Fig. 6A). Knockdown of IRE1 or of XBP1 enhanced the numbers of OSU-03012–induced GFP+ vesicles (Fig. 6B and C). Although cell killing following IRE1/XBP1 knockdown was inhibited by the pan-caspase inhibitor zVAD (see data in Fig. 5), we also found that knockdown of Beclin1 suppressed the enhancement of autophagy and of cell killing caused by IRE1 knockdown (Fig. 6D). Inhibition of NOS enzymes/NO generation using L-NAME suppressed OSU-03012– and sildenafil-induced GFP+ vesicle formation (Fig. 6E).
Knockdown of the autophagy regulatory proteins Beclin1 or of ATG5 suppressed OSU-03012 and sildenafil toxicity arguing autophagy was a toxic event (Fig. 6F).

We next defined the roles of signaling pathways in the survival/killing of cells treated with OSU-03012 and sildenafil. Initially, we made use of HCT116 colon cancer cells expressing in wild-type cells mutant active K-RAS D13 or mutant forms of H-RAS V12 that specifically activated RAF-1 or PI3K (Fig. 7A; ref. 27). Expression of H-RAS V12 protected cells from OSU-03012 and sildenafil toxicity to a greater extent than did the expression of K-RAS D13. The protective effect of H-RAS V12 was lost in cells expressing a mutant H-RAS V12 protein that specifically activated RAF-1 but not a H-RAS V12 protein that specifically activated PI3K (Fig. 7A; ref. 27). Expression of phosphatase and tensin homolog (PTEN) specifically activated PI3K but not RAF-1 and PDE5 inhibitors interacted with OSU-03012 to kill tumor cells. In multiple primary human GBM cell types, as well as in freshly isolated human GBM cells, sildenafil enhanced OSU-03012 lethality. Furthermore, in primary human GBM cell types selected for CD133+ and stem cell–like growth as neurospheres in serum-free media, tumor cell killing was as effective as that observed in parental cells. Furthermore, the parent compound of OSU-03012, celecoxib, also interacted with PDE5 inhibitors to kill GBM cells (and mammary carcinoma cells, unpublished observations). As discussed below, our data argue for a complex series of signaling events that regulate drug combination toxicity (Fig. 9).

Our prior studies have linked OSU-03012–induced ER stress signaling by PERK as being causal in the toxicity of the drug (12, 13, 15). The present studies demonstrated that the IRE1/XBP-1 arm of the ER stress response was protective against OSU-03012, and that the ATF6 arm seemed not to significantly alter the response. Knockdown of IRE1/XBP-1 also enhanced OSU-03012 and sildenafil combination toxicity. We have previously shown that OSU-03012 as a single agent enhances cell death through a caspase-independent mechanism; however, the enhanced levels of killing caused by IRE1/XBP-1 knockdown was caspase dependent. Inhibition of caspase-9, but not caspase-8, blunted enhanced killing by IRE1/XBP-1 knockdown, which correlated with reduced BCL-XL and MCL-1 expression. Thus, IRE1/XBP-1 knockdown was facilitating activation of the intrinsic apoptosis pathway. In contrast with data with OSU-03012 and IRE1/XBP-1 knockdown, enhanced cell killing by the combination of OSU-03012 and sildenafil was blocked by inhibition of caspase-8 or knockdown of CD95/FADD, demonstrating that the extrinsic pathway was being induced by the drug combination.

Discussion

The present studies were initiated to determine whether PDE5 inhibitors interacted with OSU-03012 to kill tumor cells. In multiple primary human GBM cell types, as well as in freshly isolated human GBM cells, sildenafil enhanced OSU-03012 lethality. Furthermore, in primary human GBM cell types selected for CD133+ and stem cell–like growth as neurospheres in serum-free media, tumor cell killing was as effective as that observed in parental cells. Furthermore, the parent compound of OSU-03012, celecoxib, also interacted with PDE5 inhibitors to kill GBM cells (and mammary carcinoma cells, unpublished observations). As discussed below, our data argue for a complex series of signaling events that regulate drug combination toxicity (Fig. 9).

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Table 1. OSU-03012 and sildenafil synergize to kill glioblastoma cells

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NOTE: GBM6 and GBM12 cells (250–1,500 cells per well) were plated in sextuplicate and allowed to attach for 12 hours. Cells were treated with OSU-03012 (OSU, 0.5–2.0 μmol/L), sildenafil (SIL, 0.5–2.0 μmol/L), or the drugs combined for 24 hours. Media were removed, plates washed, and cells cultured for the next 10 to 14 days in media lacking drugs. After approximately 10 days, media were removed, plates washed, and cells fixed and stained. The number of colonies per plate (≥50 cells per colony) was counted, plating efficiencies determined, and the relative changes in colony formation entered into the CalcuSyn for Windows program to determine synergy (n = 3). A CI of less than 0.70 indicates a strong level of synergy.
Figure 3. OSU-03012 and sildenafil interact to kill multiple GBM cell isolates. A, free-floating GBM5/6/12/14 neurosphere stem cells were triturated to form single cells and were plated into soft agar in sextuplicate (500–1,000 cells per dish). Cells were treated with vehicle (DMSO), OSU-03012 (1.0 μmol/L), sildenafil (2 μmol/L), or the drugs combined. Twenty-four hours after drug treatment, the cover media were replaced with drug-free media. Colonies were permitted to form over the following 20 days, after which they were stained and counted (n = 3 ± SEM); #, P < 0.05 less than vehicle control; ##, P < 0.05 greater than corresponding value in siSCR treatment.

B and C, freshly isolated GBM tumor cells (patients 1–3) were treated with vehicle (Veh; DMSO), OSU-03012 (1 μmol/L), sildenafil (2 μmol/L), tadalafil (1 μmol/L), or the drugs in combination. Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); *P < 0.05 greater than OSU treatment alone. D-F, GBM5/6/12 cells were transfected with either a scrambled siRNA (siSCR) or with a siRNA to knock down the expression of PDE5. Thirty-six hours after transfection, cells were treated with vehicle (Veh; DMSO), OSU-03012 (1 μmol/L), sildenafil (2 μmol/L), tadalafil (1 μmol/L), or the drugs in combination, as indicated. Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); †, P < 0.05 greater than corresponding value in siSCR treatment. G, GBM5/12 cells were pretreated with vehicle (PBS) or L-NAME (1 μmol/L). Cells were then treated with vehicle (DMSO), OSU-03012 (1 μmol/L), sildenafil (2 μmol/L), or the drugs in combination. Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); ‡, P < 0.05 less than OSU + SIL treatment in the absence of L-NAME.

Studies by others have shown that ER stress via IRE1 can signal to activate the JNK pathway; thus, it was to our considerable surprise that loss of IRE1 function facilitated OSU-03012–induced activation of the JNK pathway by OSU-03012 and this enhancement in JNK signaling was causal in cell death (28, 29). We noted that
IRE1 knockdown was enhancing basal levels of PERK phosphorylation and it is possible that our JNK activation was due to this compensatory signaling effect. Expression of an activated form of AKT suppressed drug/knockdown-induced JNK activation and maintained tumor cell viability. Using point mutants of H-RAS V12 that activate specific downstream signaling pathways as well as manipulation of PTEN function and AKT activity, we determined that signaling by the AKT pathway also protected against OSU-03012 and sildenafil drug combination toxicity. In this instance, drug combination–induced JNK pathway activation
Knockdown of IRE1 enhances OSU-03012 toxicity through caspase-dependent pathways. A, GBM6 cells were transfected with either a scrambled siRNA (siSCR) or with a siRNA to knock down the expression of IRE1. Thirty-six hours after transfection, cells were pretreated with the pan-caspase inhibitor zVAD (50 μmol/L) and then treated with vehicle (Veh; DMSO) or OSU-03012 (1.0 μmol/L). Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); *, P < 0.05 greater than corresponding value in siSCR treatment; #, P < 0.05 less than siIRE1 + OSU-03012 value. Inset blots, GBM6 cells were transfected with either a scrambled siRNA (siSCR) or with a siRNA to knock down the expression of IRE1. Thirty-six hours after transfection, cells were pretreated with the pan-caspase inhibitor zVAD (50 μmol/L) and then treated with vehicle (Veh; DMSO) or OSU-03012 (1.0 μmol/L). Six hours after treatment, cells were lysed and immunoblotting performed to determine the expression of MCL-1 and BCL-XL and the phosphorylation of PERK. B, GBM6 cells were transfected with either a scrambled siRNA (siSCR) or with a siRNA to knock down the expression of XBP1. Thirty-six hours after transfection, cells were pretreated with the pan-caspase inhibitor zVAD (50 μmol/L) and then treated with vehicle (DMSO) or OSU-03012 (1.0 μmol/L). Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); *, P < 0.05 greater than corresponding value in siSCR treatment; #, P < 0.05 less than (siXBP1 + OSU-03012) value. C, GBM6 cells were transfected with either a scrambled siRNA (siSCR) or with a siRNA to knock down the expression of IRE1. Thirty-six hours after transfection, cells were pretreated with the caspase-9 inhibitor LEHD (50 μmol/L) and then treated with vehicle (DMSO) or OSU-03012 (1.0 μmol/L). Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); *, P < 0.05 greater than corresponding value in siSCR treatment; #, P < 0.05 less than siIRE1 + OSU-03012 value. D, GBM6 and GBM12 cells were infected at 50 m.o.i. with either an empty vector adenovirus (CMV) or viruses to express dominant-negative caspase-9, BCL-XL, or the caspase-8 inhibitor c-FLIP-s. Twenty-four hours after infection, cells were treated with vehicle (DMSO) or with OSU-03012 (1.0 μmol/L) and sildenafil (2 μmol/L) combined. Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); *, P < 0.05 greater than corresponding value in CMV-infected cells; #, P < 0.05 less than value in BCL-XL–infected cells. Inset, cells were treated with vehicle (DMSO), OSU-03012 (1.0 μmol/L), sildenafil (2 μmol/L), or the drugs combined. Six hours after treatment, cells were lysed and immunoblotting performed to determine the expression of MCL-1, BCL-XL, and c-FLIP-s. (Continued on the following page.)
OSU-03012 and ER Stress

Figure 6. The regulation of autophagy by OSU-03012 and sildenafil. A, GBM6 cells were transfected with a plasmid to express LC3-GFP-RFP. Twenty-four hours after transfection, cells were treated with vehicle (DMSO), OSU-03012 (1.0 μmol/L), sildenafil (2 μmol/L), or the drugs combined. Cells were microscopically examined 6 and 12 hours after drug exposure for the numbers of GFP+ and RFP+ intense staining vesicles (n = 3 ± SEM). B and C, GBM5 and GBM6 cells were transfected with a plasmid to express LC3-GFP and either a scrambled siRNA (siSCR) or with siRNA molecules to knock down the expression of IRE1 or XBP1. Thirty-six hours after transfection, cells were treated with vehicle (DMSO) or OSU-03012 (1.0 μmol/L). Cells were microscopically examined 6 hours after drug exposure for the numbers of GFP+ intense staining vesicles (n = 3 ± SEM). #, P < 0.05 less than corresponding value in siSCR-transfected cells. D, GBM6 cells were transfected with either a scrambled siRNA (siSCR) or with siRNA molecules to knock down the expression of Beclin1 or ATG5. Thirty-six hours after transfection, cells were treated with vehicle (DMSO), OSU-03012 (1.0 μmol/L), sildenafil (2 μmol/L), or the drugs combined. Cells were microscopically examined 12 hours after drug exposure for the numbers of GFP+ and RFP+ intense staining vesicles (n = 3 ± SEM), #, P < 0.05 less than corresponding value in siSCR-transfected cells. E, GBM6 cells were transfected with a plasmid to express LC3-GFP-RFP. Twenty-four hours after transfection, cells were pretreated with vehicle (PBS) or L-NAME (1 μmol/L). Cells were then treated with vehicle (DMSO), OSU-03012 (1.0 μmol/L), sildenafil (2 μmol/L), or the drugs combined. Cells were microscopically examined 12 hours after drug exposure for the numbers of GFP+ and RFP+ intense staining vesicles (n = 3 ± SEM), #, P < 0.05 less than corresponding value in siSCR-transfected cells. F, GBM6 and GBM12 cells were transfected with either a scrambled siRNA (siSCR) or with siRNA molecules to knock down the expression of Beclin1 or ATG5. Thirty-six hours after transfection, cells were treated with vehicle (DMSO), OSU-03012 (1.0 μmol/L), sildenafil (2 μmol/L), or the drugs combined. Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM). #, P < 0.05 less than corresponding value in siSCR-transfected cells.

(Continued.) E, GBM6 and GBM12 cells were transfected with either a scrambled siRNA (siSCR) or with siRNA molecules to knock down the expression of CD95, FADD, or RIP-1. Thirty-six hours after transfection, cells were treated with vehicle (DMSO) or with OSU-03012 (1.0 μmol/L) and sildenafil (2 μmol/L) combined. As a positive control, one set of siSCR-transfected cells were treated with an agonistic azide-free anti-CD95 antibody (2 μg); the use of a control IgG was also performed but is not shown for clarity). Twenty-four hours after drug treatment, cells were isolated and viability determined by Trypan blue exclusion assay (n = 3 ± SEM); #, P < 0.05 less than corresponding value in siSCR-transfected cells. F, GBM6 cells in 96-well plates were pretreated with vehicle or L-NAME (1 μmol/L). Cells were then treated with OSU-03012 (OSU 1.0 μmol/L) and/or sildenafil (SIL, 2.0 μmol/L). Six hours after treatment, cells were fixed to the plate and immunohistochemistry performed to determine the plasma membrane levels of CD95. The intensity of CD95 immunostaining was determined using a Hermes Wiscan instrument with associated Wisoft data analysis package (n = 3 ± SEM). #, P < 0.05 value greater than celecoxib treatment alone; #, P < 0.05 less than corresponding value in vehicle-treated cells.

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previously explored (30, 31). As we know from prior studies that OSU-03012 induces autophagy and causes cathepsin B–dependent cleavage of BID, our findings suggest that the induction of autophagy is a primary event that facilitates mitochondrial dysfunction, thereby promoting activation of the intrinsic pathway.

Sildenafil was developed as an inhibitor of PDE5 with cardioprotective effects, and serendipitously became an approved therapeutic for erectile dysfunction (32). PDE5 expression is not confined to the corpus cavernosum in the human penis and is expressed in the wider vasculature, myocardium, and tumor cells (33, 34). PDE5 catalyzes the degradation of cGMP; i.e., thus, PDE5 inhibitors increase cGMP levels (35). NO induces smooth muscle relaxation via the actions of cGMP (36). NO at nanomolar levels binds tightly to a heme group in NO-guanylyl cyclase (GC), also known as soluble guanylyl cyclase, and causes an approximately 150-fold activation of the enzyme (37). Activation of NO-GC elevates cGMP levels, which initiate the cGMP signaling pathway, in part through activation of...
cGMP-dependent protein kinase (PKG; ref. 38). It is known in nontumor cells that cGMP/PKG, through its stimulatory actions upon the ERK, p38 MAPK, JNK, and NFκB pathways can increase the expression of inducible NO synthase (iNOS; refs. 39–41). In our present studies, we noted that sildenafil treatment increased iNOS levels (unpublished observations). Thus, increased levels of NO activate GC and increase cGMP levels, which activates signaling pathways that increase iNOS levels; and increased iNOS levels lead to further increases in cellular NO. Elevation of cGMP or overexpression of constitutively active PKG can result in phosphorylation and activation of the JNK pathway and promote apoptosis (42–46). This is similar to our findings in which sildenafil and OSU-03012 interacted to cause toxic JNK activation. High concentrations of sildenafil and vardenafil induce
A key role in CD95 activation, particularly through ceramide generation, can play another possible regulatory step in the CD95 activation expression of CD95 that promotes apoptosis (52, 55). Others, however, have argued that NO can upregulate NO-induced cardiomyocyte cell killing (58). The ability of PDE5 inhibitors and OSU-03012 to interact in a toxic fashion could also possibly be dose- and schedule-dependent, although prior studies using sildenafil at higher concentrations still argued for sildenafil as a potentiator of chemotherapy toxicity (22, 23). Clearly, much additional experimentation will be required to fully understand how the PDE5 inhibitor/OSU-03012 drug combination activates the extrinsic/CD95 pathway.

OSU-03012 is presently undergoing phase I clinical testing. The C max of the drug was approximately 2 μmol/L at the MTD of 800 mg twice a day, although the C max was variable due to poor adsorption from the stomach (14). The drug is presently being reformulated for additional phase I clinical studies. Our data in the present article demonstrated that the parent drug, celecoxib, also interacted with PDE5 inhibitors to kill GBM cells. Furthermore, in a separate series of studies combining celecoxib with sildenafil, we were able to demonstrate a strong antitumor action in vivo using both HER2+ and triple-negative mammary carcinoma cells (unpublished observations). Although some of the recently developed anticancer drugs have strong single-agent effects due to their targeting oncogene-addicted cells, many drugs will have more modest single-agent effects, and as such will need to be rationally combined with other agents to achieve a better therapeutic index. Our studies combining OSU-03012 and sildenafil are a case in point, that is, modest individual toxicities but together significant toxicity. On the basis of our data in both GBM and mammary carcinoma cells combining celecoxib and sildenafil, a phase I trial will open in 2015 combining these agents for the therapy of solid tumor malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L. Booth, A. Poklepovic, P. Dent
Development of methodology: L. Booth
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Booth, J.L. Roberts, N. Cruickshanks
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Booth, P. Dent
Writing, review, and/or revision of the manuscript: L. Booth, S. Grant, P. Dent
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Booth, P. Dent
Study supervision: L. Booth

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Regulation of OSU-03012 Toxicity by ER Stress Proteins and ER Stress–Inducing Drugs


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