

OSU-03012 and Viagra Treatment Inhibits the Activity of Multiple Chaperone Proteins and Disrupts the Blood–Brain Barrier: Implications for Anti-Cancer Therapies

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We examined the interaction between OSU-03012 (also called AR-12) with phosphodiesterase 5 (PDE5) inhibitors to determine the role of the chaperone glucose-regulated protein (GRP78)/BiP/HSPA5 in the cellular response. Sildenafil (Viagra) interacted in a greater than additive fashion with OSU-03012 to kill stem-like GBM cells. Treatment of cells with OSU-03012/sildenafil: abolished the expression of multiple oncogenic growth factor receptors and plasma membrane drug efflux pumps and caused a rapid degradation of GRP78 and other HSP70 and HSP90 family chaperone proteins. Decreased expression of plasma membrane receptors and drug efflux pumps was dependent upon enhanced PERK-eIF2 α -ATF4-CHOP signaling and was blocked by GRP78 over-expression. In vivo OSU-03012/sildenafil was more efficacious than treatment with celecoxib and sildenafil at killing tumor cells without damaging normal tissues and in parallel reduced expression of ABCB1 and ABCG2 in the normal brain. The combination of OSU-03012/sildenafil synergized with low concentrations of sorafenib to kill tumor cells, and with lapatinib to kill ERBB1 over-expressing tumor cells. In multiplex assays on plasma and human tumor tissue from an OSU-03012/sildenafil treated mouse, we noted a profound reduction in uPA signaling and identified FGF and JAK1/2 as response biomarkers for potentially suppressing the killing response. Inhibition of FGFR signaling and to a lesser extent JAK1/2 signaling profoundly enhanced OSU-03012/sildenafil lethality.

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OSU-03012, is a derivative of the drug celecoxib (Celebrex), and lacks cyclooxygenase (COX2) inhibitory activity (Zhu et al., 2004; Johnson et al., 2005). COX2 is over-expressed in several tumor types and drugs that inhibit COX2, that is, celecoxib have been shown to cause tumor cell-specific increases in cell death, and that are also associated with a lower rate of growth (Koehe and Dubois, 2004; Cui et al., 2005; Kang et al., 2006; Klenke et al., 2006). Prolonged treatment with COX2 inhibitors can reduce the incidence of developing cancer, which, in addition, argues that COX2 inhibitors have cancer preventative effects (Kashfi and Rigas, 2005; Narayanan et al., 2006). Expression levels of COX2 do not simplistically correlate with tumor cell sensitivity to COX2 inhibitors (Kulp et al., 2004; Patel et al., 2005). Thus, COX2 inhibitors must have additional cellular targets to explain their anti-tumor biological actions.

Compared to the parent drug celecoxib, OSU-03012 (developed by Dr. Ching-Shih Chen at Ohio State University in 2004 and also known as AR-12, under licence from Ohio State University to Arno Therapeutics, NJ) has a greater level of bio-availability in pre-clinical large animal models to the parent compound and has an order of magnitude greater efficacy at killing tumor cells (Yacoub et al., 2006; Park et al., 2008; Booth et al., 2012a). Based on encouraging pre-clinical data OSU-03012 underwent Phase I evaluation in patients with solid and liquid tumors. Studies from the initial Phase I trial noted that

Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; CEL, celecoxib also called Celebrex; OSU, OSU-03012 also called AR-12; SIL, sildenafil also called Viagra; VAR, vardenafil also called Levitra; COX, cyclooxygenase; P, phospho-; ca, constitutively active; WT, wild type; PERK, PKR like endoplasmic reticulum kinase; HSP, heat shock protein; GRP, glucose-regulated protein.

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the “C max after single dose was dose-proportional but high PK variability was observed, likely due to inadequate disintegration and dissolution of the formulation in the stomach” (ASCO 2013 meeting. <http://meetinglibrary.asco.org/content/115148-132>) The C max of OSU-03012 in plasma after 1 day at the MTD of 800 mg BID was ~ 1 to $2 \mu\text{M}$. After 28 days of treatment, the C max was ~ 2 to $3 \mu\text{M}$ with the peak C max in some patients being $\sim 8 \mu\text{M}$. Thus, even considering the problems associated with differential OSU-03012 drug absorption in different patients, our use of OSU-03012 in prior in vitro studies and in the present manuscript of ~ 1.0 to $8.0 \mu\text{M}$ of the drug is clinically relevant.

Initially, the tumoricidal effects of OSU-03012 in transformed cells were argued to be via direct inhibition of the enzyme PDK-1, within the PI3K pathway (Zhu et al., 2004). And, in the low micro-molar range in cells, it has been shown that OSU-03012 can lower AKT phosphorylation, presumably by PDK-1 inhibition. In our previous studies, inhibition of either ERK1/2 or phosphatidylinositol 3 kinase signaling enhanced the toxicity of OSU-03012 (Yacoub et al., 2006; Park et al., 2008; Booth et al., 2012a,b). However, our data has also strongly argued that OSU-03012 toxicity, and in addition its radiosensitizing effects, could not simplistically be attributed to suppression of AKT signaling (Yacoub et al., 2006; Park et al., 2008; Booth et al., 2012a,b). Specifically, our prior studies have argued that OSU-03012 killed tumor cells through mechanisms, which involved enhanced endoplasmic reticulum (ER) stress signaling through activation of PKR-like endoplasmic reticulum kinase (PERK), down-regulation/reduced half-life of the ER and plasma membrane localized HSP70 family chaperone GRP78/BiP/HSPA5, and a caspase-independent, cathepsin-dependent autophagy-dependent form of tumor cell death (Yacoub et al., 2006; Park et al., 2008; Booth et al., 2012a,b). One of the hallmarks of any potentially useful anti-cancer drug is that it is found to be relatively non-toxic to “normal” cells/tissues and we previously noted that OSU-03012, alone or in combination with other modalities had an excellent therapeutic window comparing toxicity in normal non-transformed cells to tumor cells both in vitro and in vivo.

ER stress signaling is mediated by three proximal sensors, PERK, the IRE1 (inositol-requiring protein 1 α)/X-box binding protein 1 (XBPI) system and activating transcription factor 6 (ATF6). GRP78 plays a key role in regulating the ER stress response; under resting conditions the majority of GRP78 is associated with PERK and IRE1 and keeps these proteins in an inactive state (Rao et al., 2012; Gorbatyuk and Gorbatyuk, 2013; Roller and Maddalo, 2013). GRP78, as a chaperone, also plays an important role in the protein folding processes that occur in the ER including during cancer, liver disease, and virus replication (Roux, 1990; Earl et al., 1991; Anderson et al., 1992; Hogue and Nayak, 1992; Carleton and Brown, 1997; Xu et al., 1997, 1998; Bolt, 2001; Shen et al., 2002; Dimcheff et al., 2004; He, 2006; Spurgers et al., 2010; Moreno and Tiffany-Castiglioni, 2014; Reid et al., 2014). When high levels of unfolded protein are present in the ER, for example, in a tumor cell or in a virally infected cell, GRP78 disassociates from PERK and IRE1 resulting in their activation, and GRP78 binds to the unfolded proteins in the ER as a chaperone (Lee, 2007; Luo and Lee, 2013; Chen et al., 2014, and references therein). Activation of PERK-eIF2 α signaling acts to prevent the majority of cellular proteins from being synthesized and IRE1 signaling enhances the expression of additional GRP78 protein. Virus infection can cause a profound ER stress response, which could be cyto-toxic or prevent virus protein synthesis, thus some viruses make targeting proteins, similar to mammalian GADD34 and Nck1, that relocate protein phosphatase 1 with eIF2 α thereby preventing eIF2 α phosphorylation and high levels of toxic ER stress signaling (Rathore et al., 2013). As GRP78 chaperones the unfolded protein(s), free GRP78

eventually becomes available to re-associate with PERK and IRE1 thereby shutting off the signaling system (Lee, 2007; Luo and Lee, 2013; Chen et al., 2014). Of note, however, is that prolonged ER stress signaling downstream of PERK and IRE1 can facilitate transformed cell killing, though in our laboratory not primary cell killing (see above), arguing that a prolonged reduction of GRP78 in transformed cells reduces cell viability (Booth et al., 2014a,b).

We have published several manuscripts showing that knock down/inhibition of PERK signaling suppressed OSU-03012 toxicity but that this effect appeared to be only partially eIF2 α dependent as the ability of OSU-03012 to cause high PERK phosphorylation was not reflected in the more modest phosphorylation increase of eIF2 α . The induction of PERK activity by OSU-03012 was associated with reduced GRP78 and HSP90 expression and increased HSP70 expression. More recently, however, we have noted that the ability of sildenafil to strongly enhance OSU-03012 or celecoxib lethality was dependent on an even greater increase in PERK activity, which was now associated with very high levels of eIF2 α phosphorylation.

Tumors of the brain are difficult to control. Untreated adult glioblastoma (GBM) patients have a mean survival of several months that is only prolonged up to 12–16 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 CNS tumors (Booth et al., 2014c,d; Roberts et al., 2014). In two additional even more recent studies, we then noted that celecoxib or OSU-03012 lethality is also enhanced by PDE5 inhibitors (Booth et al., 2014a,b). As celecoxib, OSU-03012 and PDE5 inhibitors all readily cross the blood–brain barrier, the present studies have attempted to further understand how celecoxib and OSU-03012 lethality is regulated by PDE5 inhibitors in malignant brain tumor cells, with specific attention to the regulation of GRP78 expression and activity of other HSP90 and HSP70 family chaperones.

Materials and Methods

Materials

Phospho-/total-antibodies were purchased from Cell Signaling Technologies (Danvers, MA) and Santa Cruz Biotech. (Santa Cruz, CA). All drugs, including OSU-03012, were purchased from Selleckchem (Houston, TX). Commercially available validated short hairpin RNA molecules to knock down RNA/protein levels were from Qiagen (Valencia, CA). At least two different validated siRNA molecules were independently used to confirm the effects observed were not due to non-specific effects. Antibody reagents, other kinase inhibitors, caspase inhibitors cell culture reagents, and non-commercial recombinant adenoviruses have been previously described (Booth et al., 2014a,b,c,d). The plasmid to express GRP78/BiP/HSPA5 was kindly provided to the Dent Laboratory by Dr. A.S. Lee (University of Southern California, Los Angeles, CA). Previously characterized semi-established GBM5/GBM6/GBM12/GBM14 GBM cells were supplied by Dr. C.D. James (University of California, San Francisco) and Dr. J.N. Sarkaria (Mayo Clinic, Rochester MN) and were not further characterized by ourselves (Giannini et al., 2005).

Methods

Mammalian 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 supplemented with 10% (v/v) fetal calf serum and 10% (v/v) non-essential amino acids. All primary human GBM cells were cultured at 37°C (5% (v/v) CO₂)

in vitro using RPMI supplemented with 2% (v/v) fetal calf serum and 10% (v/v) non-essential amino acids at 37°C (5% (v/v) CO₂). GBM5/6/12/14 stem cells were cultured in StemCell Technologies NeuroCult NS-A Basal Medium supplemented with 20 µg/ml bFGF, 20 µg/ml epidermal growth factor (EGF), and 2 mM heparin. CD133+ glioma cells from this population were isolated by fluorescence-activated cell sorting analysis. Cells, for example, GBM12, grew as neurospheres and were characterized for multiple stem cell markers, including CD44, SOX2, CD133, CD15, CD36, Integrin B6, and MAP2. Neurosphere GBM cells had an approximate 10-fold greater tumorigenicity in vivo than parental wild type (WT) GBM cells (data not shown). For short-term cell killing assays and immunoblotting, cells were plated at a density of 3 × 10³ per cm² and 24 h after plating were treated with various drugs, as indicated. In vitro small molecule inhibitor treatments were from a 100 mM 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 Booth et al. (2014a,b,c,d). Immunoblots were observed by using an Odyssey IR imaging system (LI-COR Biosciences, Lincoln, NE).

Recombinant adenoviral and other virus vectors; infection in vitro. We generated and purchased previously noted recombinant adenoviruses as per Booth et al. (2014a,b,c,d). Cells were infected with these adenoviruses at an approximate m.o.i. as indicated in the figure/legend (usually an m.o.i. of 0.1–50). Cells were incubated for 24 h to ensure adequate expression of transduced gene products prior to drug exposures. Viruses were purchased from Research BioLabs (Philadelphia, PA).

Detection of cell death by trypan blue assay. Cells were harvested by trypsinization with Trypsin/EDTA for ~10 min at 37°C. Harvested cells were combined with the culture media containing unattached cells and the mixture centrifuged (800 rpm, 5 min). Cell pellets were resuspended in PBS and mixed with trypan blue agent. Viability was determined microscopically using a hemocytometer (a,b,c,d). 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, Billerica, MA), which measured tumor cell size/sub G1 DNA as an indication of tumor cell viability.

Cell death measurements by live/dead assay. Cells were grown in 96-well plates with each well containing ~10,000 cells in 200 µl of media. Cells were treated with the indicated concentrations of drugs for the indicated amounts of time in each panel. Plates were then centrifuged (500 rpm, 5 min) to re-adhere floating dead cells to the base of each well. The media was removed and live/dead assay reagent added and cells incubated for 10 min before the reagent was removed. Cells were imaged in a Hermes WiScan instrument under 10× magnification. Green cells = viable; yellow/red cells = dieing/dead. The numbers of viable and dead cells were counted manually from several images taken from one well together with images from another two wells.

Plasmid transfection.

Plasmids. Cells were plated as described above and 24 h after plating, transfected. Plasmids (0.5 µg) expressing a specific mRNA or appropriate vector control plasmid DNA was diluted in 50 µl serum-free and antibiotic-free medium (1 portion for each sample). Concurrently, 2 µl 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 min. This mixture was added to each well/dish of cells containing 200 µl serum-free and antibiotic-free medium for a total volume of 300 µl and the cells were incubated for 4 h at 37°C. An equal volume of

2× medium was then added to each well. Cells were incubated for 48 h, then treated with drugs. To assess 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 h after plating transfected. Prior to transfection, the medium was aspirated and 1 ml serum-free medium was added to each plate. For transfection, 10 nM 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) were used (predominantly Qiagen; occasional alternate siRNA molecules were purchased from Ambion, Inc., Austin, TX). At least two different validated siRNA molecules were independently used to confirm the effects observed were not due to non-specific effects. Ten nanomolar of siRNA (scrambled or experimental) was diluted in serum-free media. Four microliters 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 temperature for 10 min, then added drop-wise to each dish. The medium in each dish was swirled gently to mix, then incubated at 37°C for 2 h. One milliliter of 10% (v/v) serum-containing medium was added to each plate, and cells were incubated at 37°C for 24–48 h before re-plating (50 × 10³ cells each) onto 12-well plates. Cells were allowed to attach overnight, then treated with drugs (0–48 h). Trypan blue exclusion assays and SDS-PAGE/immunoblotting analyses were then performed at the indicated time points.

Animal studies

Athymic mice were treated by oral gavage with vehicle (cremophore); celecoxib (50 mg/kg) + sildenafil (10 mg/kg); OSU-03012 (50 mg/kg) + sildenafil (10 mg/kg) for 14 days QD after which animals were humanely sacrificed and normal tissue/organs obtained. Organs were fixed and 10 µm sections taken, de-paraffinized and H&E stained (special thanks to Dr. Hope Richards, VCU Department of Pathology). Color images were taken at 10× magnification (special thanks to Dr. Steven Grant, VCU, Department of Hematology/Oncology). Athymic nude mice (~20 g) were injected with 1 × 10⁷ BT474 cells into their fourth mammary fat pad. Seven days after injection, animals with ~50 mm³ tumors were treated by oral gavage with vehicle (cremophore); celecoxib (10 mg/kg) + sildenafil (5 mg/kg); OSU-03012 (10 mg/kg) + sildenafil (5 mg/kg) for 5 days after which normal tissues were taken for IHC (GRP78, ABCB1, and ABCG2) and the tumors and blood isolated for multiplex assays.

Data analysis

Comparison of the effects between various in vitro drug treatments was performed after analysis of variance using the Student's *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).

Results

GBM stem cells compared to parental non-selected GBM cells exhibited higher basal levels of phosphorylated eIF2α, but stem cells nevertheless also expressed more GRP78/BiP/HSPA5 protein (Fig. 1A). Stem cell-like GBM cells expressed considerably more MCL-1 and sphingosine-1-phosphate receptor 1 (EDG-1) than parental WT cells. Of note, the intensity of each image was normalized to the most intense staining image for each antibody probe; WT cells did express MCL-1, EDG-1, and GRP78 albeit at lower levels than stem

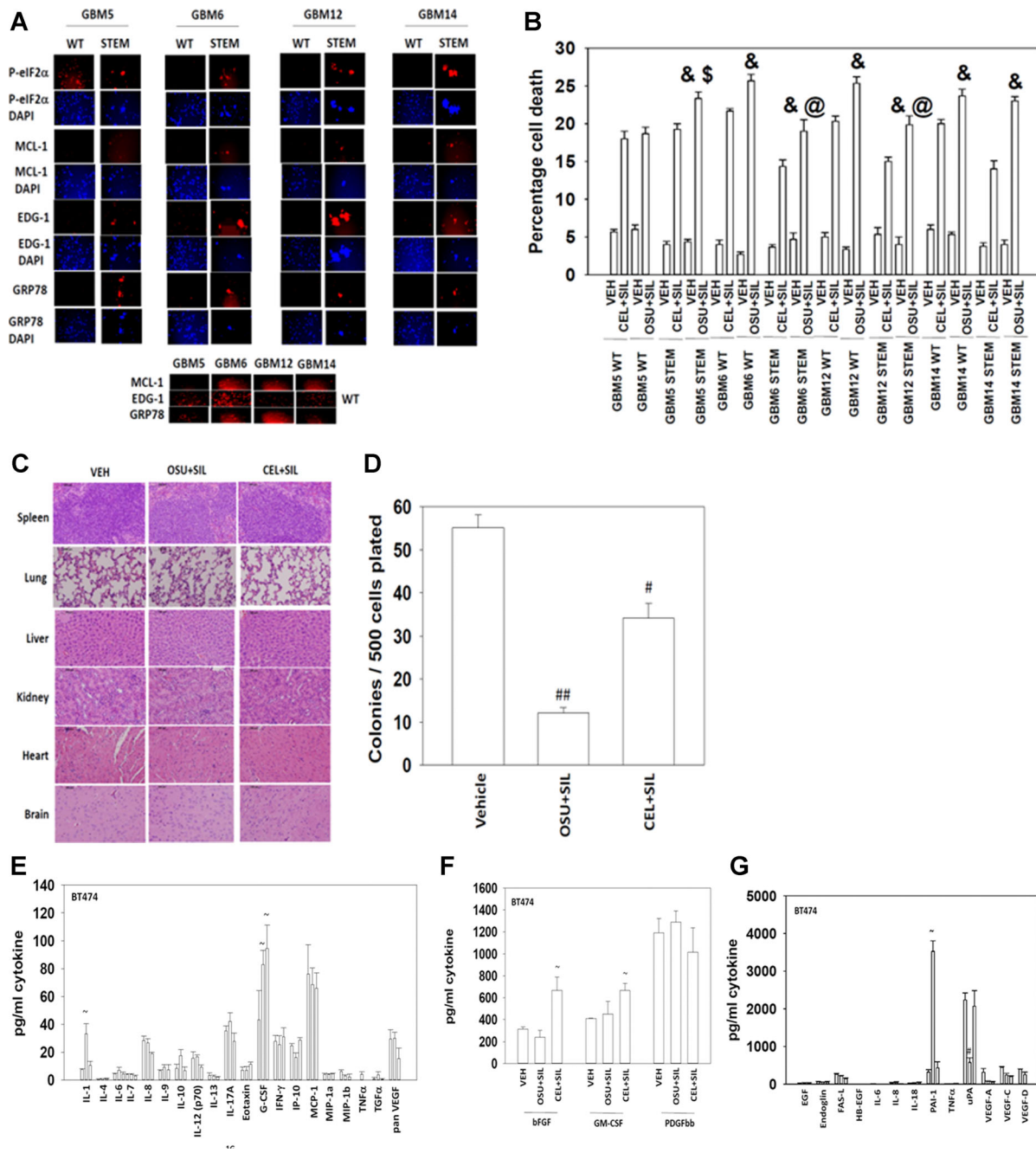


Fig. 1. OSU-03012 and sildenafil interact to kill tumor cells regardless of stem-like or anoikis-like properties, and that the tumor response to this combination provides biomarkers for the addition of a third agent. A: GBM5/6/12/14 cells or stem-like selected variants of these cells were plated in 96-well plates and 24 h after plating cells were cyto-centrifuged to sediment cells to the plate, and cells were fixed with 2% paraformaldehyde containing Triton X100 for permeabilization. Immunofluorescence was performed to determine the expression of GRP78/MCL-1/P-eIF2α, EDG-1, and counter-staining was with DAPI and detected using a Hermes Wiscan machine. The brightness/contrast for each IF image was set to show the most intense staining and all other images were presented with the identical same brightness/contrast setting. **B:** GBM cells or stem-like variants of these cells were treated with vehicle; celecoxib (5 μM) + sildenafil (2 μM); or OSU-03012 (1 μM) + sildenafil and the viability of cells determined by trypan blue exclusion assay after 24 h. (n = 3, ±SEM) and P < 0.05 greater than corresponding value in celecoxib treated cells; §P < 0.05 greater than corresponding value in wild type cells; @P < 0.05 less than corresponding value in wild type cells. **C:** Athymic mice were treated with vehicle (Cremophore); OSU-03012 (50 mg/kg) + sildenafil (10 mg/kg); celecoxib (50 mg/kg) + sildenafil (10 mg/kg) for 14 days (nota bene: below in section (D) that anti-tumor effects were observed using half the doses of these drugs and for half as long). Animals were sacrificed, the normal tissue organs collected and fixed. Sections (10 μm) were taken and H&E stained by The Department of Pathology, VCU. **D:** Athymic nude mice were injected with 1 × 10⁷ BT474 cells into their fourth mammary fat pad. Seven days after injection, animals with ~50 mm³ tumors were treated by oral gavage with vehicle (cremophore); celecoxib (10 mg/kg) + sildenafil (5 mg/kg); OSU-03012 (10 mg/kg) + sildenafil (5 mg/kg) for 5 days after which tumors were isolated, and plated as single cells to determine the ex vivo colony formation ability of in vivo treated tumors (n = 3 ± SEM). ##P < 0.05 less than vehicle control; ###P < 0.05 less than [CEL + SIL] value. **E–J:** Tumors treated for 5 days in section (D) were isolated as was mouse blood and plasma collected. Multiplex assays were performed on tumor lysates and on clarified mouse plasma to detect changes in signal transduction parameters and on plasma cytokine levels (n = 4 tumors ± SEM). The treatments are presented in groups of three bars, with bars on the left being vehicle control; bars in the center being [OSU + SIL]; and bars on the right being [CEL + SIL]. ~P < 0.05 greater than vehicle control value; *P < 0.05 less than vehicle control value. **K:** BT474 cells were treated with vehicle control; OSU-03012 (1 μM) and and sildenafil (2 μM); the FGFR inhibitor BGJ398 (1 μM); the JAK1/2 inhibitor ruxolitinib (1 μM) as indicated for 12 h. Cells viability was determined using a live/dead assay in a Hermes Wiscan machine where red/yellow cells = dead; green cells = alive (n = 3 ± SEM). *P < 0.05 greater than corresponding value in vehicle control treated cells.

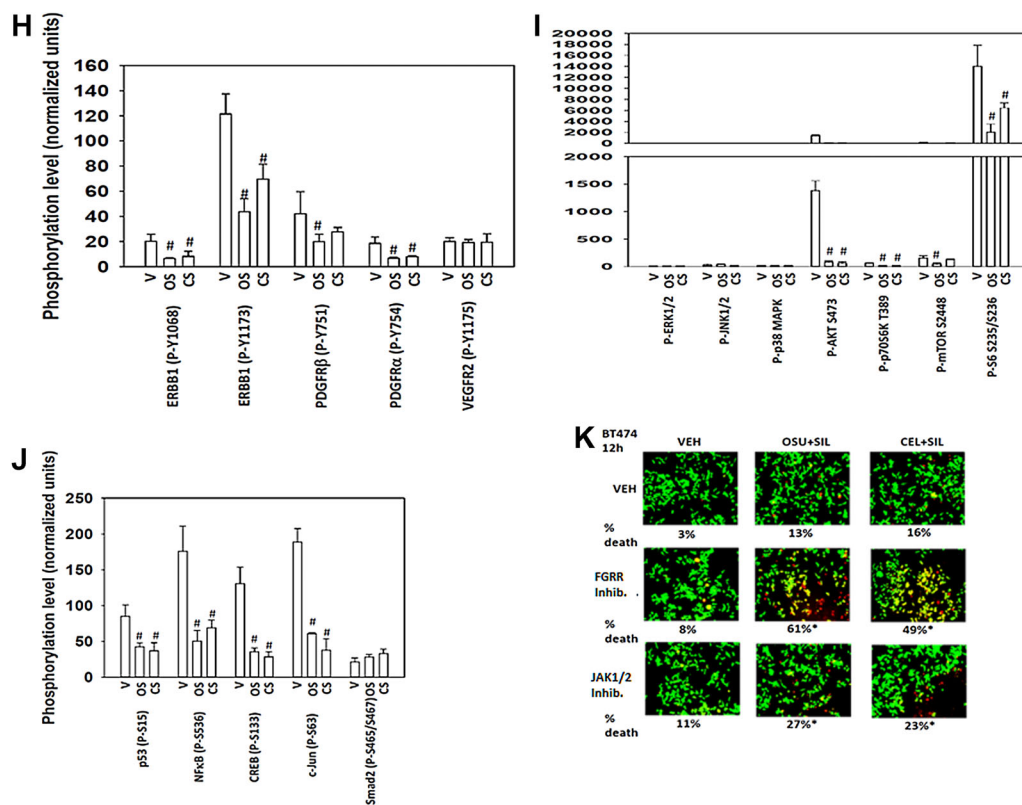


Fig. 1. (Continued)

cells (Fig. 1A, lower images). In GBM cells, it was noted that stem cell selected cells were modestly but significantly more sensitive to OSU + sildenafil also called Viagra (SIL) treatment than parental WT cells. In the majority of GBM cell lines, treatment using 1 μ M OSU-03012 caused a greater toxic response when combined with sildenafil than did treatment with the parental compound 5 μ M celecoxib and sildenafil. Regardless of whether breast cancer cells were parental or generated to be anoikis resistant, the relative ability of celecoxib also called Celebrex (CEL) + SIL treatment to kill parental or anoikis cells preferentially appeared to be more unpredictable than in GBM cells and stochastic in nature (data not shown). Prolonged high concentration dosing of mice with [OSU-03012 + sildenafil] or [celecoxib + sildenafil] did not cause obvious frank damage to normal tissues from an athymic mouse (Fig. 1C). In contrast, treatment of animals with lower doses of [OSU-03012 + sildenafil] or [celecoxib + sildenafil] significantly reduced tumor volumes after 5 days of treatment and reduced the long-term viability of *in vivo* treated cells, as judged using *ex vivo* colony formation assays (Fig. 1D, data not shown).

Based on these findings, we obtained normal brain and liver and obtained tumor material and plasma from [OSU-03012 + sildenafil] and [celecoxib + sildenafil] treated animals carrying human BT474 mammary tumors that had been treated for 5 days with these drug combinations. We discovered that both drug combination treatments increased the levels of G-CSF, and for [CEL + SIL] bFGF and GM-CSF (Fig. 1E,F). Treatment with [OSU + SIL] strongly enhanced expression of

the inhibitor PAI-1 and reduced expression of its cognate cytokine uPA (Fig. 1G). Both drug combinations reduced the phosphorylation of ERBB1 and of the platelet-derived growth factor (PDGF)R α/β , but not VEGFR2 (Fig. 1H). The reduced activity of ERBB1 and PDGFR α/β correlated with reduced AKT, p70 S6K, and mTOR activity in drug treated tumors (Fig. 1I). The reduced activity of signal transduction pathways correlated with reduced activity of the transcription factors p53, NF κ B; CREB; and c-Jun, but not Smad2 (Fig. 1J). Based on our biomarker data, we then determined whether inhibition of specific additional pathways could enhance [OSU + SIL] lethality. Based on data examining G-CSF, bFGF, and GM-CSF levels we determined whether the clinically relevant FGFR pan inhibitor BGJ398 or the FDA approved JAK1/2 inhibitor ruxolitinib enhanced [OSU + SIL] lethality. Inhibition of FGFR signaling profoundly enhanced [OSU + SIL] lethality (Fig. 1K). Inhibition of JAK1/2 signaling more modestly, though significantly, also enhanced [OSU + SIL] lethality.

Expression of ERBB1, ERBB2, and of ERBB3 and ERBB4 in BT474 breast cancer cells was reduced by OSU + SIL treatment (Fig. 2A). Treatment of GBM cells with CEL + SIL and with OSU + SIL rapidly reduced total expression of ERBB1 (GBM12) and ERBB1 vIII (GBM6) within 6 h, whereas expression of c-MET was reduced to a much lesser extent and in a cell-dependent fashion (Fig. 2B). Drug combination exposures rapidly reduced expression of SIPR1 (EDG1) and SIPR5 (EDG5) as they did in a cell type dependent fashion for the IL6R and the IL8R (Fig. 2C,D). Expression of the IGF1R and of TNF α R1 were also reduced by the drug combination

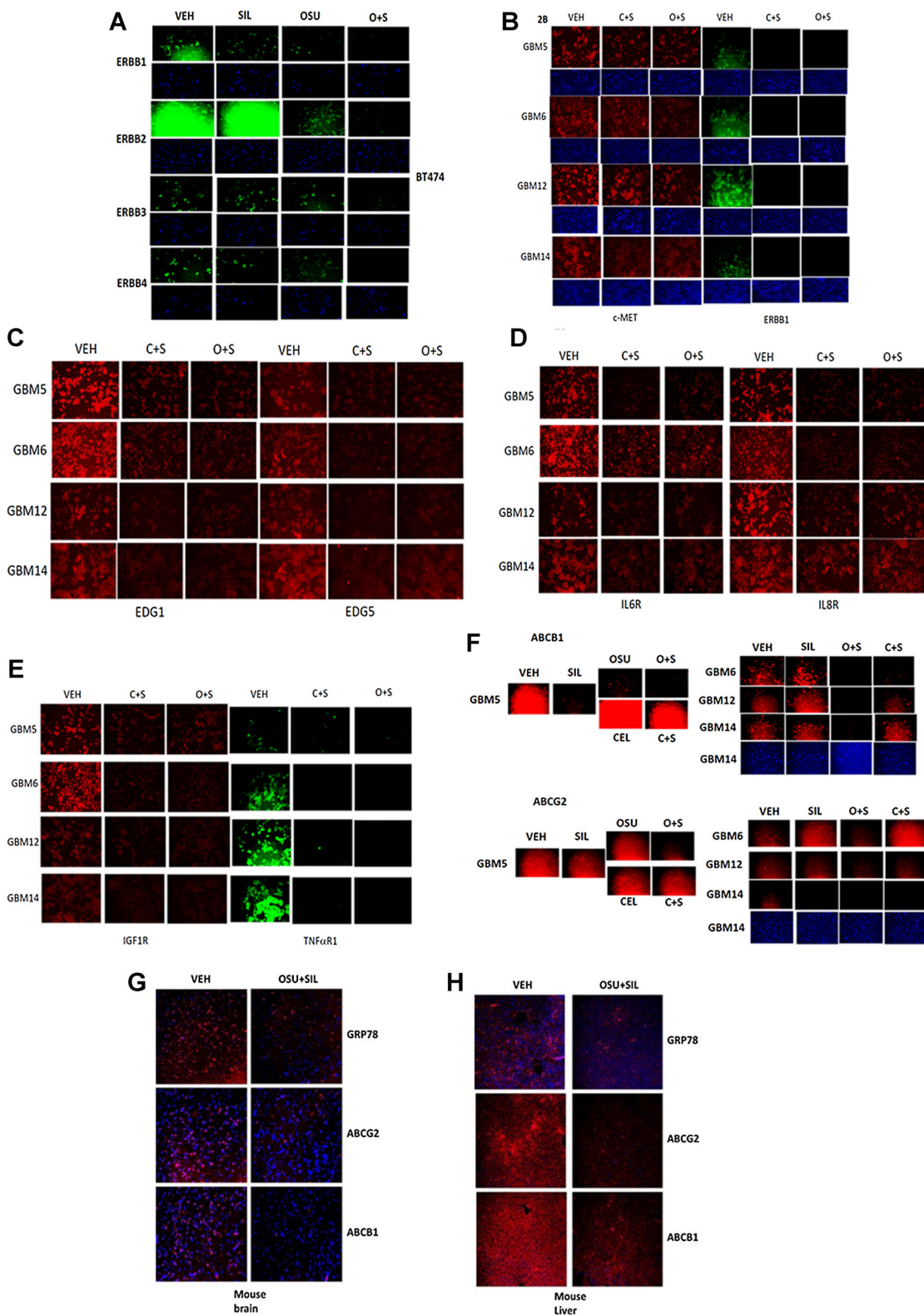


Fig. 2. Treatment of tumor cells with [OSU-03012 + sildenafil] or [celecoxib + sildenafil] rapidly reduces the expression of multiple growth factor receptors. **A:** BT474 mammary carcinoma cells known to strongly over-express ERBB2 and; **B–F:** GBM5/6/12/14 cells; in 96-well plates were treated with vehicle; celecoxib (5 μM) + sildenafil (2 μM); or OSU-03012 (1 μM) + sildenafil and after 6 h treatment were fixed with 2% (v/v) paraformaldehyde containing Triton X100 to permeabilized cells. Immuno-staining of the indicated growth factor receptors and plasma membrane drug transporters was performed using standard techniques and IF images detected using a Hermes Wiscan machine. **G,H:** Normal tissues from mice treated in Figure 1 section (D), brain and liver, were fixed, sectioned and immune-stained to determine the total expression of GRP78, ABCB1, and ABCG2. Images were examined under 10× magnification using a Hermes Wiscan instrument.

(Fig. 2E). Drug combination treatment also reduced the total expression of multiple plasma membrane associated drug efflux pumps; pumps that are generally over-expressed in recurrent patient tumors and are partly responsible for chemotherapy resistance as well as for a functional blood–brain barrier (Fig. 2F). Identical data were obtained *in vivo* examining normal brain and liver tissues (Fig. 2G,H). Similar data showing knock down of ABCB1 and ABCG2 expression were also obtained in stem cell selected variants of GBM5/6/12/14 (data not shown). In previous studies, we have shown that expression of the death receptor CD95 (FAS) does not alter after particular these drug exposures and in fact becomes plasma membrane localized and clustered, observations indicative of CD95 activation (Booth et al., 2014a,b).

As treatment of cells with CEL + SIL or with OSU + SIL weakly reduced expression of c-MET compared to other growth factor receptors, we determined whether a clinically relevant inhibitor of c-MET (crizotinib) could enhance the lethality of CEL + SIL or with OSU + SIL. In a cell type dependent fashion, that did not correlate with basal expression or down-regulation of c-MET protein, crizotinib enhanced CEL + SIL or OSU + SIL lethality (Fig. 3A). The lethality of CEL + SIL was potentially enhanced in all GBM cells by the multi-kinase inhibitor sorafenib (Fig. 3B,C). Unlike [OSU + SIL] treatment in Fig. 2, the JAK1/2 inhibitor ruxolitinib did not significantly enhance CEL + SIL toxicity and the IGF1R inhibitor OSI-906 only enhanced CEL + SIL toxicity in GBM6 and GBM14 cells. In GBM cells that expressed ERBB1 and ERBB2, the ERBB2 inhibitor lapatinib strongly enhanced CEL + SIL lethality. In a previous manuscript, we demonstrated that the histone deacetylase inhibitor and blocker of sphingosine-1-phosphate receptor signaling, FTY720 (multiple sclerosis drug: Gilenya), enhanced CEL + SIL lethality. The more potent HDAC inhibitors AR-42, vorinostat enhanced CEL + SIL lethality in GBM cells with AR-42 being most potent on a molar basis (Fig. 3D). Similar cell killing data were obtained in multiple GI tumor cell types (Fig. 3E). The lethality of the OSU + SIL drug combination was also enhanced by HDAC inhibitors (Fig. 3F,G). The multiple sclerosis drug FT720 as well as another multiple sclerosis drug (mono-methyl fumarate [MMF]) enhanced OSU + SIL lethality (Fig. 3H).

HDAC inhibitors increase the acetylation of HSP90, which reduces its chaperone function. The acetylation of heat shock protein (HSP) 90 was increased and the expression of the HSP90 client protein c-FLIP-s reduced by AR-42 treatment (Fig. 4A, left blots; in agreement with Panner et al., 2007). This collectively argues that the combination of OSU + SIL + AR-42 is inhibiting the functions of both GRP78 and HSP90, which may explain the strong killing interaction between the drugs. Furthermore, over-expression of HSP90 did not significantly protect cells from OSU-03012 toxicity as a single agent, though HSP90 over-expression did modestly reduce the enhancement of cell death caused by IRE1 knock down; this effect was abolished by AR-42 (Fig. 4A, right graph). Sildenafil and OSU-03012 interacted to decrease total protein expression of HSP90 α/β in multiple GBM cell isolates (Fig. 4A, upper IF images). Thus OSU-03012 inhibits HSP90 function as a single agent and combined with sildenafil also considerably reduces HSP90 protein levels. Based on our findings showing the effect of sildenafil and OSU-03012 on HSP90 function, we determined whether a clinically relevant HSP90 inhibitor (SNX2112/SNX5422, 250 nM *in vitro*; patient C max @ 100 mg/m² is \sim 3 μ M) could enhance the lethality of OSU-03012 and of [OSU + SIL] in GBM cells. SNX2112 at its IC₅₀ concentration for HSP90 inhibition enhanced the toxicity of [OSU + SIL] (Fig. 4B).

As GRP78 function appeared to be playing a central role in our studies, we further explored the impact of altered ER

stress signaling on the lethality of our CEL + SIL and OSU + SIL drug combinations. Knock down of PERK expression or expression of a dominant negative (dn) PERK protein suppressed single agent OSU lethality (Fig. 4C). Over-expression of GRP78 protected cells from single agent OSU killing (Fig. 4D, lower graphs). Treatment of cells with OSU + SIL enhanced the phosphorylation of PERK to a greater extent than OSU alone, and profoundly enhanced eIF2 α phosphorylation, which correlated with significantly reduced expression of GADD34, Nck1, and GRP78 (Fig. 4D, upper blots). Of note, OSU-03012 as a single agent modestly increased the expression of GADD34 that was associated with enhanced PERK phosphorylation but not with enhanced eIF2 α phosphorylation. Thus it is probable that GADD34 and Nck1, via the localized actions of protein phosphatase 1, attenuates the ER stress signal from PERK by facilitating dephosphorylation of eIF2 α and that this inhibitory effect on eIF2 α is overcome by the addition of sildenafil which reduces, below basal levels, the expression of GADD34 and Nck1, and *de facto* thus also eIF2 α -associated PPI activity. To test this hypothesis further, cells were treated with OSU + SIL for 12 h and total eIF2 α protein immunoprecipitated; in cells treated with OSU + SIL, the amount of PPI and PPI activity co-precipitating with eIF2 α was reduced (data not shown).

Knock down of IRE1 and XBP-1 enhanced OSU + SIL lethality whereas knock down of ATF6 had no effect (Fig. 4E). Previously, we have shown that OSU-03012 and celecoxib both modestly enhance expression of the HSP70 chaperone protein (Park et al., 2008). OSU-03012 treatment modestly enhanced HSP70 chaperone expression in a cell type dependent fashion, an effect that was abolished by co-exposure of cells with sildenafil resulting in a profound suppression of HSP70 levels (Fig. 4F). Treatment of cells with OSU-03012 and sildenafil or celecoxib and sildenafil reduced expression of the ER-localized chaperone proteins GRP58 and GRP94 (Fig. 4G, H). Treatment of cells with OSU-03012 and sildenafil reduced expression of the ER-localized chaperone proteins HSP27, HSP40, and HSP60 (Fig. 4I).

Treatment of GBM cells with OSU + SIL resulted in a large increase in eIF2 α phosphorylation compared to individual drug treatments (Fig. 5A,B). The reduction in EDG-1 expression (total and cell surface) caused by OSU + SIL was blocked by expression of a dn eIF2 α S51A protein (Fig. 5C–G). Treatment of cells with OSU + SIL increased expression of ATF4 in an eIF2 α -dependent fashion (Fig. 6A,B). The reduction in EDG-1 expression caused by OSU + SIL treatment was prevented by knock down of ATF4 or of CHOP (GADD153) (Fig. 6C–F). The OSU + SIL-induced increase in CHOP expression was prevented by expression of dn eIF2 α and the down-regulation of EDG-1 levels was blocked by over-expression of GRP78 (Fig. 7A–D). Expression of dn eIF2 α also prevented the drug-induced reduction in MCL-1 and BCL-XL expression (Fig. 7E, data not shown). Over-expression of GRP78 prevented the drug-induced increase in eIF2 α phosphorylation arguing that the kinase that phosphorylates eIF2 α is probably inhibited by GRP78 (Fig. 7F). Over-expression of GRP78 also prevented the drug combination-induced rapid declines in the expression of EDG-1, ERBB1, IL6R, IGF1R, and the PDGFR α/β , as well as drug efflux/blood–brain-barrier ABC transporters, suggesting that the reduction in receptor expression and transporter expression is also a result of reduced GRP78 levels and enhanced ER stress signaling (Fig. 7G,H). Knock down of CHOP expression suppressed CEL + SIL and OSU + SIL lethality (Fig. 7I).

In prior studies, we have shown that OSU-03012 reduces the expression of GRP78 through protein destabilization/ lower half-life without significantly altering GRP78 promoter activity and that celecoxib increases GRP78 expression, with

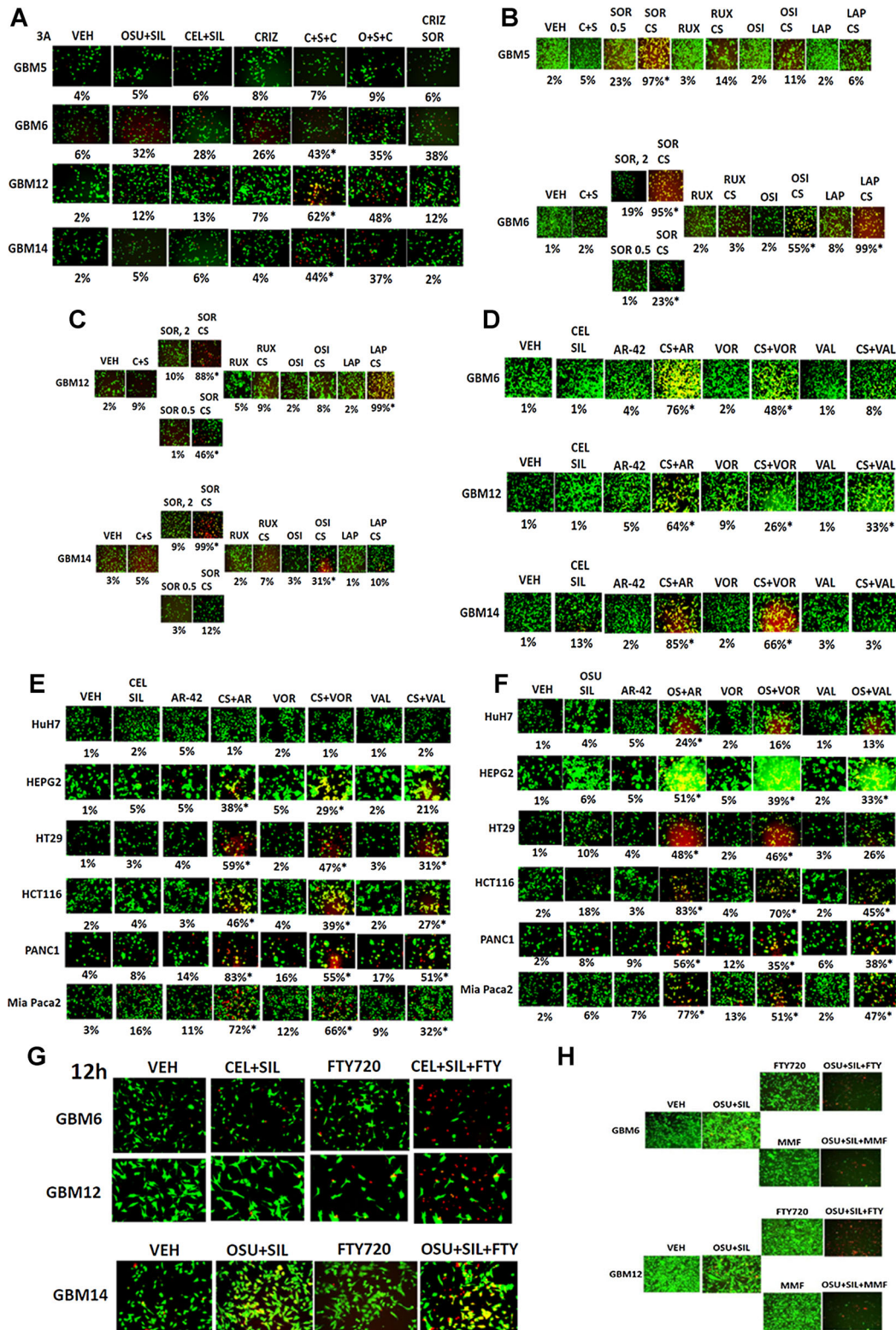


Fig. 3. Third drug combinations with [OSU-03012 + sildenafil] and with [celecoxib + sildenafil] to further enhance tumor cell killing. **A:** GBM5/6/12/14 cells in 96-well plates were treated with vehicle; celecoxib (5 μ M) + sildenafil (2 μ M); or OSU-03012 (1 μ M) + sildenafil, with or without the drug crizotinib (1 μ M) and after 12 h of treatment were subjected to live/dead assays where the percentage cell death is noted below each image ($n = 3 \pm$ SEM). * $P < 0.05$ greater than death value in two drug treated cells. **B,C:** GBM5/6/12/14 cells in 96-well plates were treated with vehicle; celecoxib (5 μ M) + sildenafil (2 μ M), with or without the drugs: sorafenib (0.5, 2.0 μ M); ruxolitinib (0.5 μ M); OSI-994 (1 μ M); lapatinib (1 μ M), as indicated. After 12 h of treatment cells were subjected to live/dead assays where the percentage cell death is noted below each image ($n = 3 \pm$ SEM). * $P < 0.05$ greater than death value in two drug treated cells. **D-F:** GBM5/6/12/14 cells or [HuH7, HEPG2 liver], [HT29, HCT116 colon], [PANC1, Mia Paca2 pancreatic] cells in 96-well plates were treated with vehicle; celecoxib (5 μ M) + sildenafil (2 μ M) or OSU-03012 (1 μ M) + sildenafil with or without the drugs: AR-42 (250 nM); vorinostat (500 nM); valproate (0.1 mM), as indicated. After 12 h of treatment, cells were subjected to live/dead assays where the percentage cell death is noted below each image ($n = 3 \pm$ SEM). * $P < 0.05$ greater than death value in two drug treated cells. **G,H:** GBM cells were treated with vehicle; celecoxib (5 μ M) + sildenafil (2 μ M) or OSU-03012 (1 μ M) + sildenafil with or without FTY720 (50 nM) with or without MMF (5 μ M). After 12 h of treatment, cells were subjected to live/dead assays.

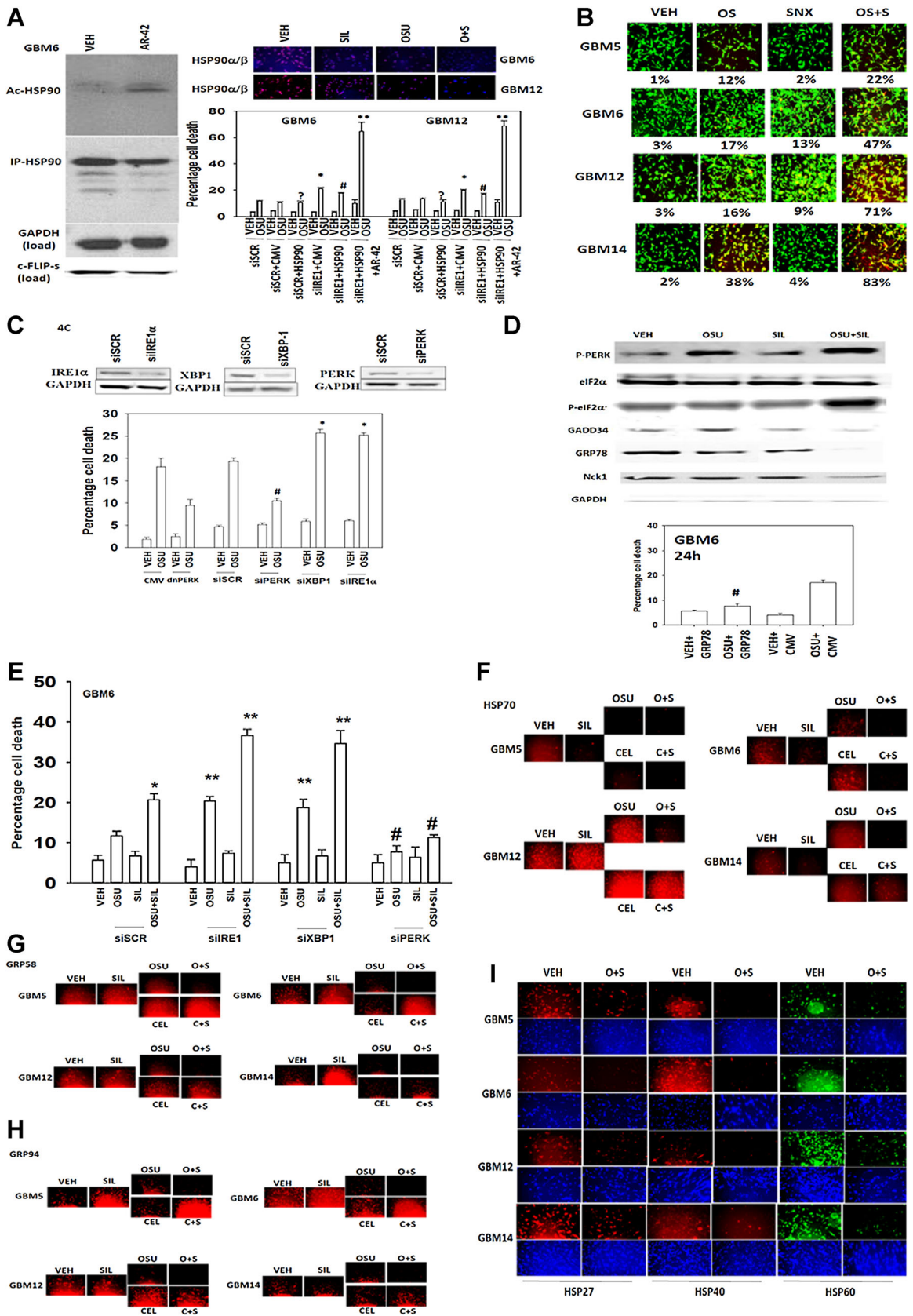


Fig. 4.

both drugs causing similar amounts of PERK phosphorylation. This implies that both drugs, that are very similar in structure, are probably binding to GRP78 with OSU-03012 destabilizing the protein and celecoxib likely inhibiting the chaperone function of the protein, which both collectively result in increased PERK activity. Treatment of GBM cells with celecoxib and sildenafil increased GRP78 expression, that is, sildenafil did not alter the ability of celecoxib to increase GRP78 levels (Fig. 8A). Nevertheless, this increase in GRP78 expression was associated with increased eIF2 α phosphorylation. Treatment of GBM cells with OSU-03012 and sildenafil reduced expression of GRP78 and strongly enhanced eIF2 α phosphorylation. Treatment of GBM cells with OSU-03012 rapidly reduced cell surface and total expression of GRP78, an effect that was further enhanced by sildenafil (Fig. 8B–E). In dose–response studies, we discovered that OSU-03012 + sildenafil treatment more rapidly and completely reduced the expression of GRP78, HSP70, and HSP90 than OSU-03012 treatment alone (Fig. 8F). Similar data were obtained in primary mouse hepatocytes, and of note, as judged by DAPI staining no cell killing was observed (Fig. 8G). There are at least four kinases that phosphorylate eIF2 α : PERK; PKR; GCN2; HRI. The nitric oxide synthase inhibitor L-NAME abolished the ability of OSU-03012 to reduce GRP78 levels (Fig. 8H). However, the cGMP-dependent kinase inhibitor reduced the ability of both OSU-03012 and [OSU-03012 + sildenafil] to lower GRP78 levels arguing that OSU-03012 may mediate some of its biologic effects through modulation of cGMP signaling. Knock down of PERK uniformly reduced the ability of OSU + SIL to increase eIF2 α phosphorylation in all lines tested (Fig. 8I). However, in a cell isolate dependent fashion, we also found that PKR and HRI could contribute to the drug-induced eIF2 α phosphorylation (Fig. 8I).

Discussion

The present studies were initiated to determine the role of the ER chaperone protein GRP78/BiP/HSPA5 in the cell death

response of tumor cells exposed to the drugs celecoxib or OSU-03012 when combined with PDE5 inhibitors. We discovered that sildenafil (and other PDE5 inhibitors, unpublished results) enhanced the ability of OSU-03012 to suppress GRP78 expression and blocked the ability of celecoxib to increase GRP78 expression. This was associated with increased expression of ATF4 and CHOP and elevated cell killing; with decreased expression of multiple growth factor receptors as well as plasma membrane drug transporters, effects that were blocked by GRP78 over-expression. Killing was evident in GBM, breast, and GI tumor cells, and in cells selected for stem-ness and for anoikis-resistance.

In a prior study, we demonstrated that the HDAC inhibitor and SIP signaling inhibitor FTY720 enhanced the toxicity of CEL + SIL in vitro and in vivo (Booth et al., 2014b). The toxicity of CEL + SIL and OSU + SIL treatments were enhanced to a greater extent than FTY720 by high affinity HDAC inhibitors such as the clinically relevant drugs AR-42 and vorinostat. Vorinostat is an FDA approved HDAC inhibitor for lymphoma and AR-42 will in 2015 be re-entering the clinic combined with the multi-kinase inhibitor pazopanib for a phase I trial in sarcoma and renal carcinoma at The Medical College of Virginia Hospitals, Richmond, Virginia (Poklepovic and Dent, unpublished observations). HDAC inhibitors were also shown to cause inactivating acetylation of the HSP90 chaperone, implying that the combination of OSU + SIL + AR-42 will probably reduce the chaperone functions of both GRP78, HSP70, HSC70, and HSP90, resulting in a tumor cell predisposed to the stimulation of cell death processes.

For GBM cells, CEL + SIL treatment only modestly down-regulated c-MET expression in GBM5 and GBM12 cells and in two of our four GBM isoaltes, GBM12 and GBM14, the c-MET inhibitor crizotinib enhanced CEL + SIL and OSU + SIL lethality. However, c-MET expression was effectively down-regulated in GBM14 and not down-regulated in GBM5 arguing that simplistic changes in receptor expression are not a valid

Fig. 4. The regulation of chaperone function and expression by OSU-03012, sildenafil and HDAC inhibitors. **A:** (Left blots) GBM6 cells were treated with AR-42 (250 nM) for 90 min, after which HSP90 was immunoprecipitated and probed for total HSP90 expression and HSP90 acetylation. In parallel tumor, cell lysate was immunoblotted for the total expression of GAPDH and of c-FLIP-s. Right graph: GBM6 and GBM12 cells were infected with an empty vector virus or a virus to express HSP90, and in parallel transfected with either a scrambled siRNA (siSCR) or an siRNA to knock down IRE1 expression. Twenty-four hours after infection, cells were treated with vehicle or OSU-03012 (1 μ M). Cells were isolated after 24 h and viability determined by trypan blue exclusion assay ($n = 3 \pm$ SEM). * $P < 0.05$ greater than siSCR + CMV value; ** $P < 0.05$ greater than siIRE1 + CMV value; # $P < 0.05$ less than siIRE1 + CMV value; † $P > 0.90$ between siSCR + CMV and siSCR + HSP90 values. IF images above graph: GBM6/12 cells were treated with vehicle; OSU-03012 (1 μ M); sildenafil (2 μ M), or the drugs in combination and after 6 h treatment were fixed with 2% (v/v) paraformaldehyde containing Triton X100 to permeabilized cells. Immuno-staining of HSP90 α/β was performed using standard techniques and IF images detected using a Hermes Wiscan machine. **B:** GBM cells were treated for 12 h with vehicle, [OSU-03012 (1 μ M) + sildenafil (2 μ M)], SNX2112 (250 nM), or the three drugs combined. Cell viability under each condition was determined by live/dead assays ($n = 3 \pm$ SEM). **C:** GBM6 cells were transfected with siSCR or siRNA molecules to knock down expression of IRE1, XBPI, and PERK. In parallel sets of plates, cells were transfected with empty vector plasmid (CMV) or to express dominant negative PERK. Twenty-four hours after transfection, cells were treated with vehicle or with OSU-03012 (2 μ M). Twenty-four hours after drug treatment, cell viability was determined by trypan blue exclusion assay ($n = 3 \pm$ SEM). # $P < 0.05$ lower than corresponding value in siSCR cells; * $P < 0.05$ greater than corresponding value in siSCR cells. **D:** (Upper blots) GBM6 cells were treated with OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 6 h after which cells were lysed. Immunoblotting and phospho-immunoblotting was performed to detect the expression/phosphorylation of the indicated proteins. Lower Graphs: GBM6 and GBM12 cells were transfected with empty vector (CMV) or to express GRP78. Twenty-four hours after transfection, cells were treated with 2 μ M OSU-03012 and 24 h later viability determined via trypan blue exclusion assay ($n = 3 \pm$ SEM). # $P < 0.05$ less than corresponding value in CMV transfected cells. **E:** GBM6 cells were transfected with scrambled control (siSCR) or siRNA molecules to knock down IRE1, XBPI, or PERK. Twenty-four hours after transfection, cells were treated with OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 24 h. Cell viability was determined by trypan blue exclusion assay ($n = 3 \pm$ SEM). * $P < 0.05$ greater than OSU-03012 single agent treatment in siSCR cells; ** $P < 0.05$ greater than corresponding values in siSCR cells; # $P < 0.05$ less than corresponding values in siSCR cells. **F–I:** GBM cells were treated with vehicle; celecoxib (5 μ M), sildenafil (2 μ M), OSU-03012 (1 μ M) as single agents or in the indicated combinations for 6 h. Cells were then fixed with 2% (v/v) paraformaldehyde containing Triton X100 to permeabilize cells. Immuno-staining of total HSP70 and HSC70 expression combined (part F) or expression of GRP94 and GRP58 (parts G and H) or expression of HSP27/HSP40/HSP60 (part I) was performed using standard techniques and IF images detected using a Hermes Wiscan machine.

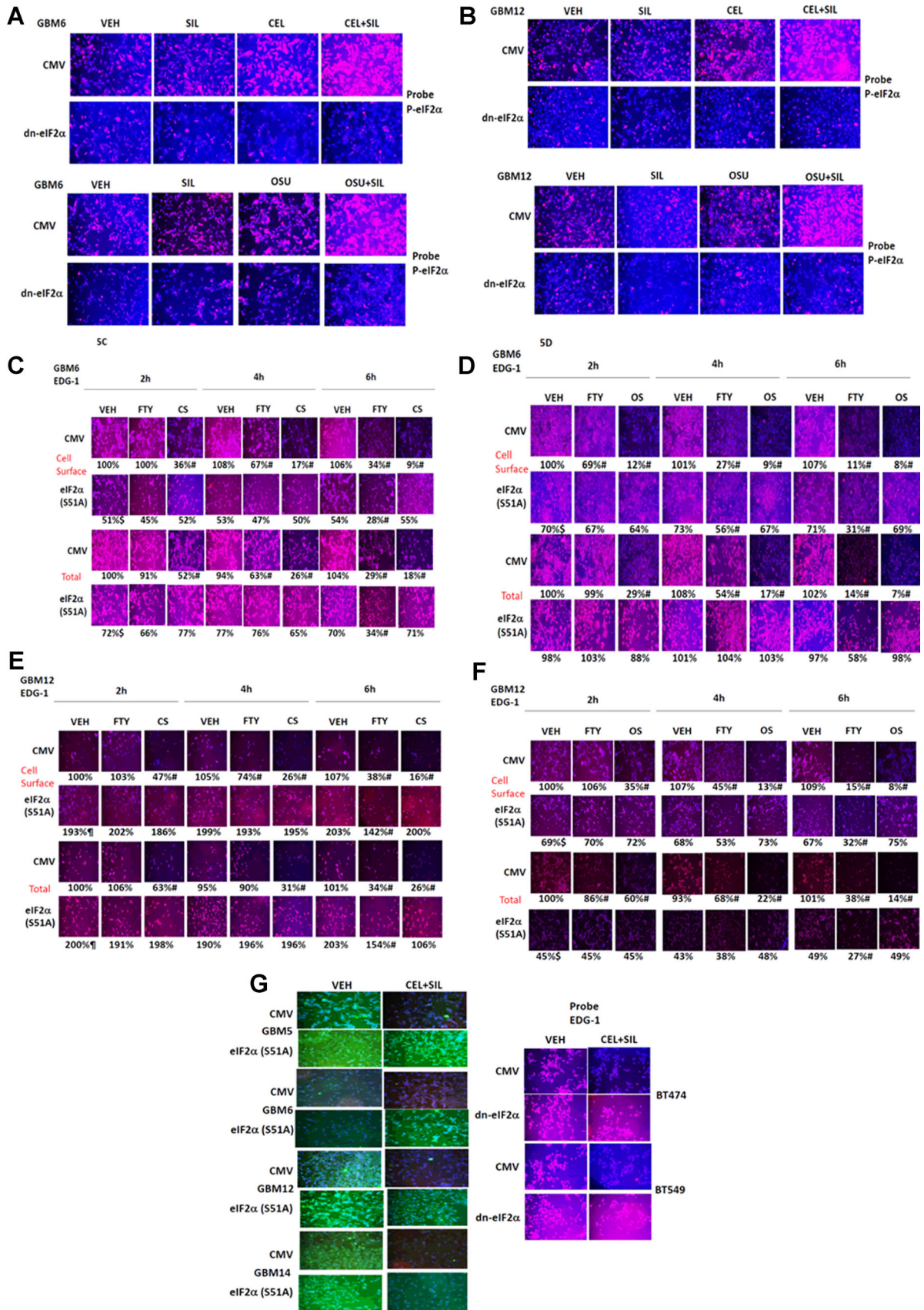


Fig. 5.

biomarkers, and that the phosphorylation level of c-MET may be a much better predictor of whether a tumor cell has any survival addition to signals from this receptor. As the diabetes drug metformin has also been shown to reduce GRP78 expression, we believe this agent could also interact with OSU-03012 and sildenafil to profoundly enhance OSU + SIL lethality.

Our prior studies have linked OSU-03012-induced ER stress signaling by PERK as being causal in the toxicity of OSU-03012. The present studies demonstrated that the drug combination of CEL + SIL or OSU + SIL strongly activated both PERK and eIF2 α that was associated with decreased GADD34 and Nck1 and GRP78 expression. Reduced GADD34/Nck1 expression will facilitate eIF2 α signaling by reducing co-localization of the Ser/Thr phosphatase PPI with eIF2 α . Knock down of CHOP prevented drug combination down-regulation of growth factor receptors and suppressed drug combination lethality, demonstrating that enhanced ER stress signaling distal to PERK and eIF2 α was essential for drug combination toxicity. As published previously, we noted that the IRE1 arm of the ER stress response was protective against OSU-03012 and OSU + SIL treatment, and that the ATF6 arm appeared not to significantly alter the response. Knock down of IRE1/XBP-1 also enhanced OSU-03012 and sildenafil combination toxicity. Further studies will need to understand how and why IRE1 signaling in our system is protective, for example, modulation of RIDD or XBP-1 function (Tong et al., 2011; Donnelly et al., 2013; Sano et al., 2013; Maurel et al., 2014). Preliminary studies suggest the drug combination regulates viability downstream of IRE1 via altered EDEM expression (unpublished observations).

We have previously shown that OSU-03012 as a single agent increases the levels of autophagosomes and autolysosomes and that this effect is significantly enhanced by sildenafil; enhanced autophagy in this system is cytotoxic as judged by the ability of ATG5 or Beclin1 knock down to prevent formation of autophagic vesicles and to prevent tumor cell killing. We also demonstrated that this elevated level of autophagy was suppressed by over-expression of GRP78, and that GRP78 over-expression protected tumor cells. In the present studies, we noted that the drug combination reduced the expression of both MCL-1 and BCL-XL, and as we have previously shown that this down-regulation can lead to increased levels of unbound Beclin1, which stimulate autophagy, the ER stress signaling pathway will likely lead to increased toxic tumor cell autophagy as well as to increased mitochondrial instability. Thus cell death pathways through autophagy and caspase activation will become engaged following OSU + SIL treatment, suggestive that tumor cell resistance mechanisms will be subverted and become ineffective (Fig. 9).

Sildenafil was developed as an inhibitor of PDE5 with cardioprotective effects, and serendipitously became an approved

therapeutic for erectile dysfunction (Zusman et al., 1999). PDE5 expression is not confined to the corpus cavernosum in the human penis and is expressed in the wider vasculature, myocardium, and tumor cells (Karami-Tehrani et al., 2012; Shan et al., 2012). We have independently validated that PDE5 is over-expressed in liver, colorectal, and lung cancer cells compared to normal tissues, and that viral infection increases PDE5 expression (unpublished observations). PDE5 catalyzes the degradation of cyclic GMP; that is, thus PDE5 inhibitors increase cGMP levels (Zhang et al., 2012). Nitric oxide (NO) induces smooth muscle relaxation via the actions of cGMP (Kato et al., 2012). NO at nanomolar levels binds tightly to a heme group in NO-guanylyl cyclase (GC), also known as soluble guanylyl cyclase, and causes a \sim 150-fold activation of the enzyme (Potter, 2011). Activation of NO-GC elevates cGMP levels, which initiate the cGMP signaling pathway, in part through activation of cGMP-dependent protein kinase (PKG) (Friebe and Koesling, 2009). It is known in non-tumor cells that cGMP/PKG, through its stimulatory actions upon the ERK, p38 MAPK, JNK, and NF κ B pathways can increase the expression of inducible nitric oxide synthase (iNOS) (Komalavilas et al., 1999; Choi et al., 2007; Das et al., 2008). Thus, it is possible in our system that increased levels of NO activate GC and increase cGMP levels, that activates signaling pathways, which 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 (Thompson et al., 2000; Deguchi et al., 2004; Zhu et al., 2005a, 2009; Zhu and Strada, 2007). Our prior findings showed that OSU + SIL interacted to cause toxic JNK activation (Booth et al., 2012b). High concentrations of sildenafil and vardenafil induce caspase-dependent apoptosis of B-chronic lymphocytic leukemia cells but not in normal B cells, suggesting a tumor selective toxicity of PDE5 inhibitors (Sarfati et al., 2003). PDE5 inhibitors enhance tumor/vasculature permeability and efficacy of chemotherapy in a rat brain tumor model (Black et al., 2008). When transiently expressed in HT29 colon cancer cells, constitutively activated mutants of PKG beta, inhibit colony formation and induce apoptosis (Zhu et al., 2005b). In PC12 cells cGMP signaling, via activation of the AKT pathway prevents apoptosis (Ha et al., 2003). Others have argued that cGMP and NO kills cells through activation of the CD95/FAS-L pathway (Hayden et al., 2001). These latter findings are similar to the data in our most recent manuscript examining this drug where OSU-03012 and sildenafil interacted to kill through CD95 activation.

In conclusion, treatment of cells with OSU + SIL rapidly reduces the expression of the key chaperone protein GRP78, as well as reducing the chaperone functions of multiple other survival factors. The drug combination also largely abolishes the blood-brain barrier.

Fig. 5. Celecoxib and sildenafil interact to increase eIF2 α serine 51 phosphorylation which facilitates reduced expression of SIPRI in GBM cells. A,B: GBM6/12 cells were plated in 96-well plates and transfected with either empty vector or with a plasmid to express eIF2 α S51A. Twenty-four hours after transfection, cells were treated with vehicle, OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 6 h. Cells were fixed and permeabilized and immuno-fluorescence performed to determine the levels of phospho-S51 eIF2 α . C–G: GBM6/12 cells were plated in 96-well plates and transfected with either empty vector or with a plasmid to express eIF2 α S51A. Twenty-four hours after transfection, cells were treated with vehicle, celecoxib (5 μ M)/OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination, or with FTY720 (50 nM), for 2, 4, and 6 h, as indicated. Cells were fixed and permeabilized or not permeabilized as indicated and immuno-fluorescence performed to determine the levels of SIPRI (EDG-1) in the plasma membrane and in the whole permeabilized cell (n = 3 \pm SEM). *P < 0.05 less than vehicle treated value at that time point; [§]P < 0.05 less than corresponding value in CMV transfected cells.

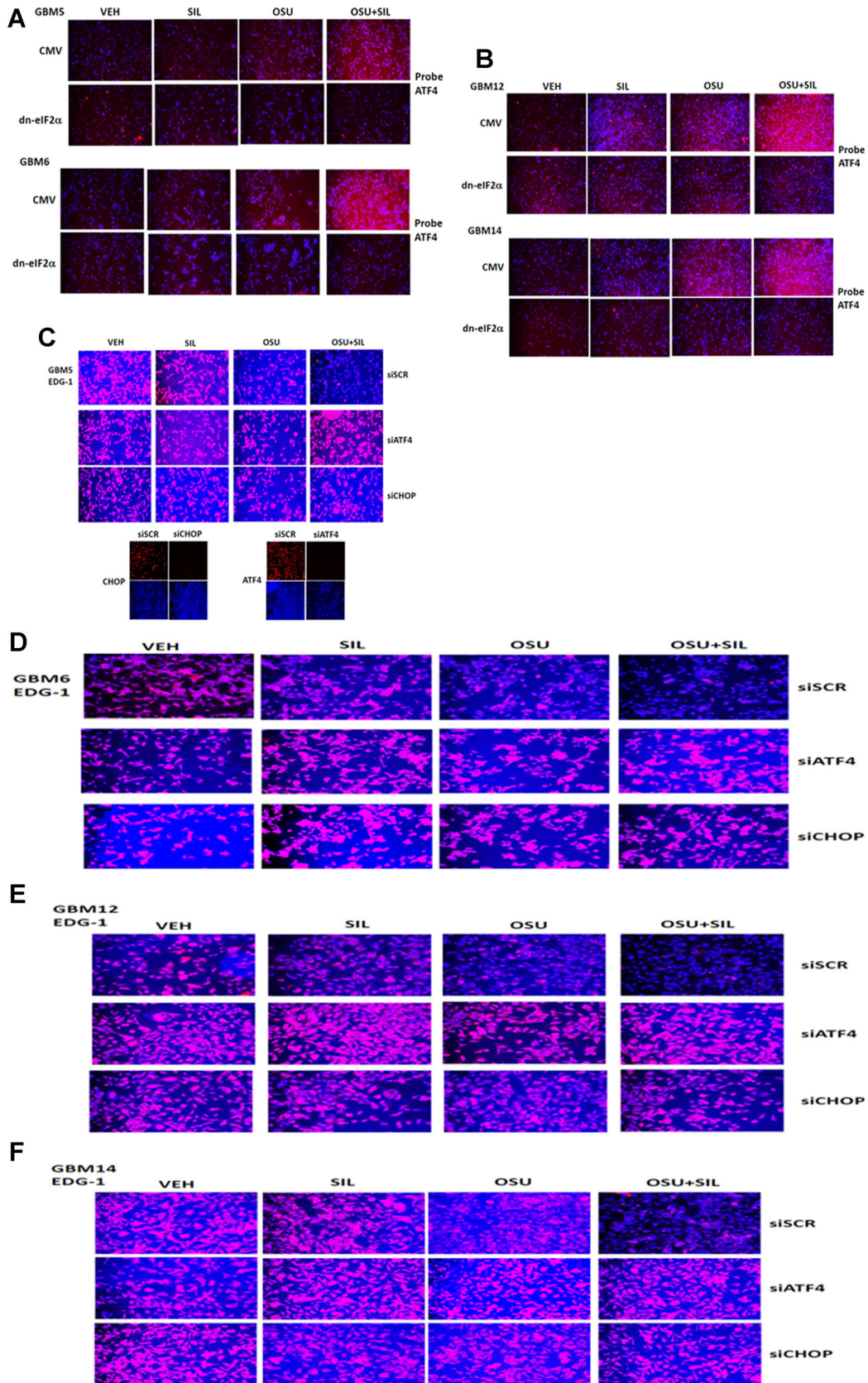


Fig. 6. Increased expression of ATF4 after [OSU-03012 + sildenafil] treatment is eIF2 α dependent and the drug-induced suppression of EDG-1 expression requires ATF4/CHOP signaling. **A,B:** GBM cells were transfected with empty vector CMV or to express dominant negative eIF2 α . Twenty-four hours after transfection, cells were treated with vehicle, OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 6 h. Cells were fixed and permeabilized and immuno-fluorescence performed to determine the levels of ATF4. **C–F:** GBM cells were transfected with a scrambled siRNA (siSCR) or siRNA molecules to knock down the expression of ATF4 or CHOP. Twenty-four hours after transfection, cells were treated with vehicle, OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 6 h. Cells were fixed and permeabilized and immuno-fluorescence performed to determine the total cellular levels of SIPRI (EDG-1).

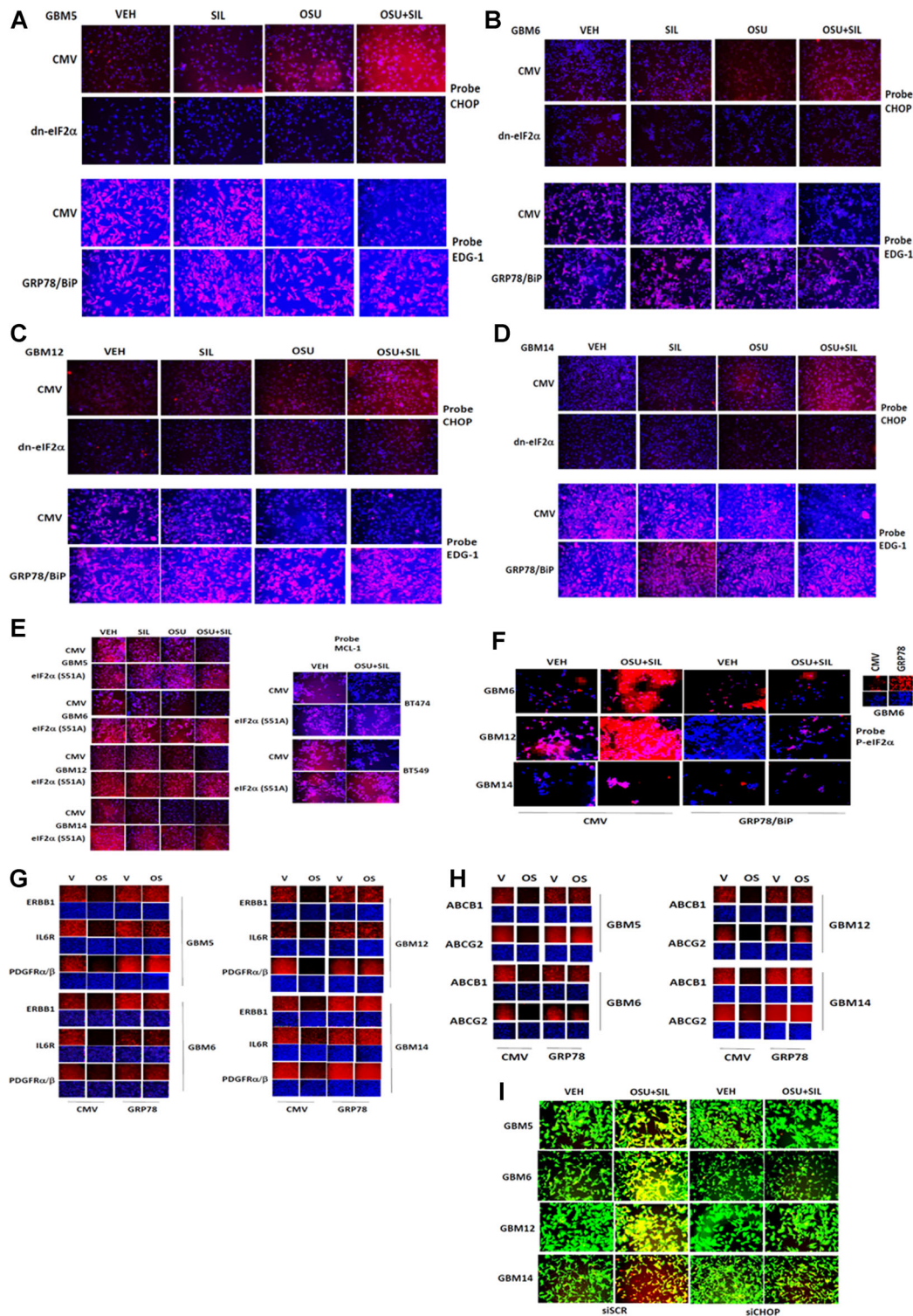


Fig. 7. Activation of eIF2 α signaling promotes expression of CHOP and down-regulation of EDG-1 that is blocked by over-expression of GRP78. **A–D:** GBM cells as indicated were transfected with empty vector (CMV), with a plasmid to express GRP78, or with a plasmid to express dominant negative eIF2 α . Twenty-four hours after transfection, cells were treated with vehicle, OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 6 h. Cells were fixed and permeabilized and immuno-fluorescence performed to determine the total cellular levels of S1PR1 (EDG-1), and CHOP (GADD153). **E:** Tumor cells were transfected with empty vector CMV or to express dominant negative eIF2 α . Twenty-four hours after transfection, cells were treated with vehicle, OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 6 h. Cells were fixed and permeabilized and immuno-fluorescence performed to determine the levels of MCL-1. **F:** Tumor cells were transfected with empty vector CMV or to express GRP78. Twenty-four hours after transfection, cells were treated with vehicle, OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 6 h. Cells were fixed and permeabilized and immuno-fluorescence performed to determine the levels of eIF2 α serine 51 phosphorylation. **G,H:** Tumor cells were transfected with empty vector CMV or to express GRP78. Twenty-four hours after transfection, cells were treated with vehicle or with OSU-03012 (1 μ M) and sildenafil (2 μ M) for 6 h. Cells were fixed and permeabilized and immuno-fluorescence performed to determine the total cellular levels of the indicated growth factor receptors and drug efflux pump proteins. **I:** GBM cells were transfected with a scrambled siRNA (siSCR) or an siRNA molecule to knock down the expression of CHOP. Twenty-four hours after transfection, cells were treated with vehicle or with OSU-03012 (1 μ M) and sildenafil (2 μ M). After 24 h of treatment, cells were subjected to live/dead assays where green cells are alive and yellow/red cells are dead.

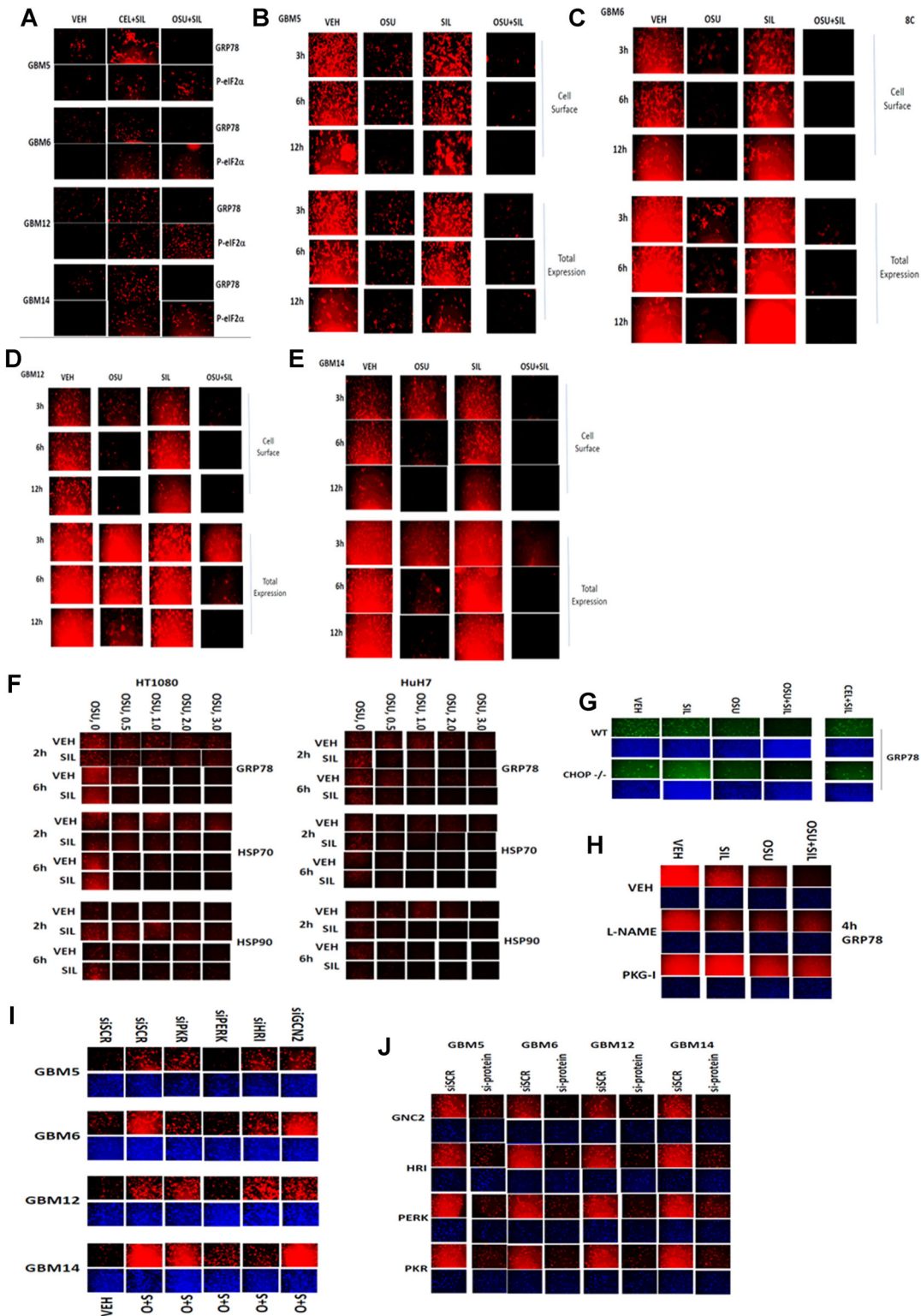


Fig. 8. Sildenafil facilitates a rapid further reduction in GRP78 protein expression levels in cells treated with OSU-03012. **A:** GBM cells were treated with vehicle, celecoxib (5 μ M)/OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for 6 h. Cells were fixed and permeabilized and immuno-fluorescence performed to determine the total cellular levels of GRP78. **B–E:** GBM cells were treated with vehicle, OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination for the indicated amounts of time. At each time point, cells were fixed with paraformaldehyde and portions of fixed cells also being permeabilized with Triton X100. **F:** HuH7 (hepatoma) and HT1080 (sarcoma) were treated with vehicle, OSU-03012 (0–3 μ M), sildenafil (2 μ M), or the drugs in combination. Cells after 2 and 6 h drug exposure were fixed in place and permeabilized. Immuno-fluorescence was performed to determine the total cellular levels of GRP78; HSP90; HSP70. **G:** Primary mouse hepatocytes (wild type C57 black or CHOP / C57 black) were plated and 12 h after plating treated with vehicle, OSU-03012 (1 μ M), sildenafil (2 μ M), or the drugs in combination. Six hours after drug exposure cells were fixed in place and permeabilized. Immuno-fluorescence was performed to determine the total cellular levels of GRP78. **H,I:** GBM cells were transfected with scrambled siRNA or siRNA molecules to knock down the expression of PERK, PKR, GNC2, or HRI. Twenty-four hours after transfection, cells were treated with vehicle or OSU-03012 (1 μ M) + sildenafil and the cells fixed and permeabilized 6 h later. Immuno-fluorescence antibody staining was performed to determine the total levels of phospho-eIF2 α serine 51 under each condition.

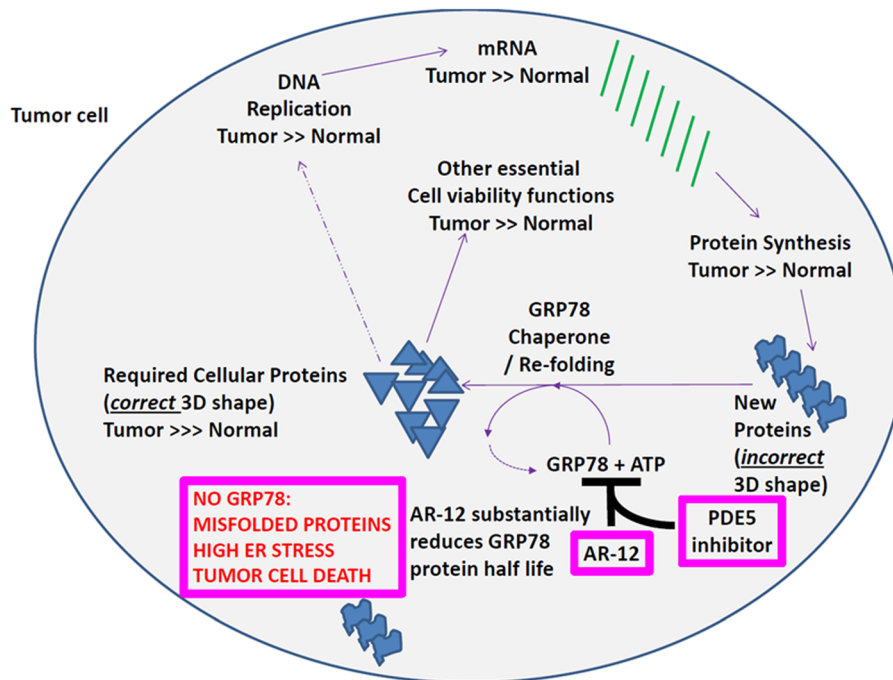


Fig. 9. Possible molecular mechanisms by which OSU-03012 (AR-12) and PDE inhibitors could kill tumor cells. The ability of OSU-03012 (AR-12) alone, or enhanced by a PDE5 inhibitor, to reduce GRP78 expression and the function of multiple other chaperones will prevent the correct folding of essential tumor cell proteins resulting in dead tumor cells. The combination of OSU-03012 and a PDE5 inhibitor will also reduce the expression of plasma membrane signaling receptors whose expression is essential for tumor cell growth and invasion. The loss of GRP78 and other chaperones in tumor cells will result in much lower expression levels of key oncogenic kinases, including PI3K-AKT signaling (Liu et al., 2013, 2015; Tsai et al., 2015). Collectively our data strongly argue that it is a strong possibility that our drug combination will abolish the blood-brain barrier and have profound anti-tumor capabilities.

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