GRP78/BiP/HSPA5/Dna K is a Universal Therapeutic Target for Human Disease

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The chaperone GRP78/Dna K is conserved throughout evolution down to prokaryotes. The GRP78 inhibitor OSU-03012 (AR-12) interacted with sildenafil (Viagra) or tadalafil (Cialis) to rapidly reduce GRP78 levels in eukaryotes and as a single agent reduce Dna K levels in prokaryotes. Similar data with the drug combination were obtained for: HSP70, HSP90, GRP94, GRP58, HSP27, HSP40 and HSP60. OSU-03012/sildenafil treatment killed brain cancer stem cells and decreased the expression of: NPC1 and TIM1; LAMP1; and NTCP1, receptors for Ebola/Marburg/Hepatitis A, Lassa fever, and Hepatitis B viruses, respectively. Pre-treatment with OSU-03012/sildenafil reduced expression of the cox sakie and adenovirus receptor in parallel with it also reducing the ability of a serotype 5 adenovirus or cox sakie virus B4 to infect and to reproduce. Similar data were obtained using Chikungunya, Mumps, Measles, Rubella, RSV, CMV, and Influenza viruses. OSU-03012 as a single agent at clinically relevant concentrations killed laboratory generated antibiotic resistant *E. coli* and clinical isolate multi-drug resistant *N. gonorrhoeae* and *MRSE* which was in bacteria associated with reduced Dna K and Rec A expression. The PDE5 inhibitors sildenafil or tadalafil enhanced OSU-03012 killing in *N. gonorrhoeae* and *MRSE* and low marginally toxic doses of OSU-03012 could restore bacterial sensitivity in *N. gonorrhoeae* to multiple antibiotics. Thus, Dna K and bacterial phosphodiesterases are novel antibiotic targets, and inhibition of GRP78 is of therapeutic utility for cancer and also for bacterial and viral infections.

OSU-03012, is a derivative of the drug celecoxib (Celebrex), and lacks COX2 inhibitory activity (Zhu et al., 2004; Johnson et al., 2005). COX2 is over-expressed in several tumor types and drugs that inhibit COX2 i.e. Celecoxib have been shown to cause tumor cell specific increases in cell death, and that are also associated with a lower rate of growth (Koehne and Dubois, 2004; Cui et al., 2005; Kang et al., 2006; Klenke et al., 2006). Non-transformed cells such as primary hepatocytes are significantly less sensitive to the drug. 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 biological actions.

Compared to the parent drug celecoxib (Celebrex), 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 in our hands 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 cancer patients. Studies from the Phase I trial noted that 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 $\sim$1–2 μM. After 28 days of treatment the C max was $\sim$2–3 μM with the peak C max in some patients being $\sim$8 μM. Some patients were on this trial with stable disease for up to 9 months without any DLTs.

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

Abbreviations: CAR, cox sakie and adenovirus receptor; CD, cluster of differentiation; 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; TAD, tadalafil also called Cialis; VAR, vardenafil also called Levitra; PTEN, Phosphatase and tensin homolog; R, receptor; dn, dominant negative; COX, cyclooxygenase; P, phospho-; ca, constitutively active; WT, wild type; PERK, PKR like endoplasmic reticulum kinase; HSP, heat shock protein; GRP, glucose regulated protein.

present manuscript of ~1.0–8.0 μM 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 phosphatidyl-inositol 3 kinase signaling enhanced the toxicity of OSU-03012 (Yacoub et al., 2006; Park et al., 2008; Booth et al., 2012a; Booth et al., 2012b). However, our data has also strongly argued that OSU-03012 toxicity, and in addition its radiosensitizing and chemo-sensitizing effects, could not simplistically be attributed to suppression of AKT signaling (Park et al., 2008; Booth et al., 2012a; Booth et al., 2012b). Specifically, our prior studies have demonstrated 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 endoplasmic reticulum and plasma membrane localized HSP70 family chaperone GRP78/BiP/HSPA5, also called Dna K in prokaryotes, and a caspase-independent, cathepsin-dependent and autophagy-dependent form of tumor cell death (Yacoub et al., 2006 Park et al., 2008; Booth et al., 2012a; Booth et al., 2012b). 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 cancer 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 1α)/XBP1 (X-box binding protein 1) system and ATF6 (activating transcription factor 6). 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. The prokaryotic homologue of GRP78, Dna K, also plays an essential role in bacterial cell biology where it chaperones proteins such as Rec A which is essential for bacterial DNA replication and resistance where engulfed to the respiratory burst of macrophages (Roux, 1990; Earl et al., 1991; Anderson et al., 1992; Hogue and Nayak, 1992; Xu et al., 1997; Carleton and Brown, 1997; Xu et al., 1998; Bolt, 2001; Shen et al., 2002; Dimcheff et al., 2004; He, 2006; Reid et al., 2014; Moreno and Tiffany-Castiglioni, 2014; Spurgers et al., 2010; Bredeche et al., 2001). When high levels of unfolded protein are present in the ER, e.g. in a tumor cell or in a virally infected cell or in a rapidly dividing bacterial cell, GRP78 disassociates from PERK and IRE1 resulting in their activation, and GRP78 binds to the unfolded proteins in the ER as a chaperone (Chen et al., 2014; Luo and Lee, 2013; Lee, 2007, and references therein). Activation of PERK-eIF2α 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 by 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 eIF2α thereby preventing eIF2α 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; Booth et al., 2014b).

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 eIF2α dependent as the ability of OSU-03012 to cause high PERK phosphorylation was not reflected in the more modest phosphorylation increase of eIF2α (Park et al., 2008 Booth et al., 2012a). The induction of PERK activity by OSU-03012 was associated with reduced GRP78 and HSP90 expression and modestly increased HSP70 expression (Booth et al., 2014a; Booth et al., 2014b). 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 eIF2α phosphorylation (Booth et al., 2014a; Booth et al., 2014b).

IRE1 is the oldest and most conserved branch of the unfolded protein response (UPR)/ER stress response in metazoans (Chen and Brandizzi, 2013). 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 (Pavitt and Ron, 2012). 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 (Hollien and Weissman, 2006 Hollien et al., 2009). Thus low amounts of IRE1 activity could promote survival, with higher levels of activity promoting cell death. We recently noted that single agent OSU-03012 lethality was enhanced by knock down of IRE1α or of XBP-1 and that OSU-03012/sildenafil toxicity was also enhanced by knock down of IRE1 or XBP1 (Booth et al., 2014a).

We have published studies that demonstrated phosphodiesterase 5 (PDE5) inhibitors enhanced the toxicity of standard of care chemotherapies in bladder and pediatric CNS tumors (Roberts et al., 2014; Booth et al., 2014c). 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; Booth et al., 2014b). As noted previously GRP78 is essential for virus replication, including the hemorrhagic filoviridae virus, Ebola (Roux, 1990; Earl et al., 1991; Moreno and Tiffany-Castiglioni, 2014; Anderson et al., 1992; Hogue and Nayak, 1992; Xu et al., 1997; Carleton and Brown, 1997; Xu et al., 1998; Bolt, 2001; Shen et al., 2002; Dimcheff et al., 2004; He, 2006; Reid et al., 2014; Spurgers et al., 2010; Bredeche et al., 2001). The present studies were performed to further understand the biologic interactions between OSU-03012 and PDE5 inhibitors in the contexts of mammalian tumor cell biology, virus infectivity and replication, and bacterial cell growth and viability.

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. 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). Antibody reagents, other kinase inhibitors, caspase inhibitors cell culture reagents, and non-commercial recombinant adenoviruses have been previously described (Booth et al., 2014a Roberts et al., 2014; Booth et al., 2014b; Booth et al., 2014c; Booth et al., 2014d). BT549, BT474, HT1080, HEK293, MDCK, VERO E6 cells were purchased from the ATCC and were not further validated in the Dent lab. Previously characterized semi-established GBM5/GBM6/GBM12/GBM14 glioblastoma 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 CO$_2$) 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) CO$_2$) 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 CO$_2$). 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 EGF and 2 mM heparin. CD133+ glioma cells from this population were isolated by fluorescence-activated cell sorting analysis. Cells e.g. GBM12, grew as neurospheres and were characterized for multiple stem cell markers, including CD44, SOX2, CD133, CD15, CD36, Integrin B6 and MAP2 (Booth et al., 2014a; Booth et al., 2014b; Booth et al., 2014c). Neurosphere GBM cells had an approximate 10-fold greater tumorigenicity in vivo than parental wild type GBM cells (data not shown). For short-term cell killing assays and immunoblotting, cells were plated at a density of 3×10^3 per cm^2 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. *Nota bene*: DMSO has profound inhibitory effects on bacterial cell liquid culture growth and care must be taken to avoid DMSO anti-proliferative effects.

Bacterial strains

Neisseria gonorrhoeae strains were routinely maintained on GC medium base (Difco) agar with Kellogg's supplement I and 12 µM Fe (NO$_3$)$_3$ at 37°C in a 5% CO$_2$ atmosphere. Cell growth studies were performed in Chelex-treated defined medium (CDM) to which drugs or vehicle control was added (Ostberg et al., 2013). *Escherichia coli* bacteria were transformed with plasmids to confer ampicillin and kayamycin resistance and maintained on TB plates/in broth supplemented with 0.25 µg/ml penicillin and 0.25 µg/ml kayamycin. *MRSE* bacteria were cultured on sheep blood agar and after over-night colony formation 10 ml of TSA broth was inoculated with mild shaking at 37°C. Bacterial density (A 600) was measured/time = 0 and then media infused with OSU-03012 and/or sildenafil. Bacterial density/A600 was determined 4 h later.

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 (Booth et al., 2014a Booth et al., 2014b; Booth et al., 2014c; Booth et al., 2014d; Roberts et al., 2014). 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 refs (Booth et al., 2014a; Booth et al., 2014b; Booth et al., 2014c; Roberts et al., 2014; Booth et al., 2014d). Cells were infected with these adenoviruses at an approximate m.o.i. as indicated in the Figure/Legend (usually an moi 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) and Zeptometrix (Buffalo, NY) or kindly provided by colleagues (CMV-GFP, Dr. McVoy (Dept. Pediatrics, VCU)). Virus titers per ml were: Ad5-GFP (10^5); CMV-GFP (3×10^4); Cox sakie virus B4 (10^7); Influenza A (10^5); Influenza B (10^5); Measles (10^7); Mumps (10^7); RSVa (10^5); RSVb (10^5); Chikungunya (10^6). Infection of cells at the indicated doses of virus (N^{00}-N^{-7} orders of magnitude dilution).

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 (Booth et al., 2014a Booth et al., 2014b; Booth et al., 2014c; Roberts et al., 2014; Booth et al., 2014d). 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 doubled 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, Valencia, CA; 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 nM siRNA (scrambled or experimental) was diluted in serum-free media. Four micro liters 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 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 (50×10^3 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); 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-parafinized and H&E stained (special thanks to Dr. Hope Richards, VCU Dept. of Pathology). Images were taken at 10× magnification (special thanks to Dr. Steven Grant, VCU, Dept of Hematology/Oncology). Athymic nude mice (∼20 g) were injected with 1×10^7 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); OSU-03012 (10 mg/kg) + sildenafil (5 mg/kg) for 7 days.

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

Prolonged high concentration dosing of athymic mice with [OSU-03012 + sildenafil] did not cause any obvious frank damage to normal tissues, as we have observed previously for OSU-03012 alone (Fig. 1A, pictures). In contrast, treatment of animals carrying established breast cancer tumors with lower doses of [OSU-03012 + sildenafil] significantly reduced tumor volumes after 7 days of treatment and reduced the long-term viability of in vivo treated cells, as judged using ex vivo tumor cell colony formation assays (Fig. 1A, graphical). Treatment of GBM cells (wild type or stem-like derived cells) with OSU-03012 and sildenafil resulted in similar levels of killing, with stem cells over-expressing GRP78 tending to be more sensitive to the drug combination (Fig. 1B). Over-expression of GRP78 suppressed the lethality of [OSU-03012 + sildenafil] treatment whereas knock down of GRP78 enhanced OSU-03012 lethality (Fig. 1C).

In prior studies we have shown that OSU-03012 reduces the expression of GRP78 through protein destabilization/
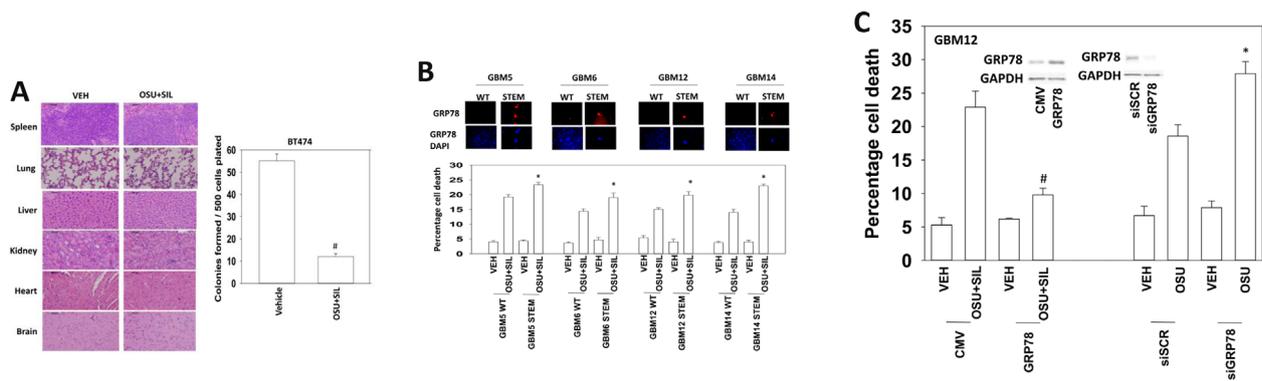

Fig. 1. OSU-03012 and Sildenafil interact to kill tumor cells in vivo but do not damage normal tissues. (A). Athymic mice were treated with vehicle (Cremophore); OSU-03012 (50 mg/kg) + Sildenafil (10 mg/kg); for 14 days (*nota bene*: in ref 39 and below that anti-tumor effects were observed using half 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 and imaged under 10× magnification. Athymic nude mice were injected with 1×10^7 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); OSU-03012 (10 mg/kg) + sildenafil (5 mg/kg) for 7 days after which tumors were isolated, and plated as single cells to determine the ex vivo tumor colony formation ability of in vivo treated tumor cells (n = 3 +/− SEM) # $P < 0.05$ less than vehicle control treated. **(B)** GBM cells (wild type parental and stem cell media selected (Booth et al., 2014a; Booth et al., 2014b) were treated with vehicle or with OSU-03012 (1 μM) and sildenafil (2 μM) in combination. Cell viability was determined 24 h later via trypan blue exclusion assay (n = 3 +/− SEM) * $P < 0.05$ greater than corresponding value in wild type cells. **(C)** GBM cells were either transfected with an empty vector plasmid or a plasmid to express GRP78; or were transfected with a scrambled siRNA or an siRNA to knock down GRP78. Twenty four h after transfection cells were treated with vehicle, OSU-03012 (1 μM for GRP78 over-expression studies; 2 μM for siRNA studies), sildenafil (2 μM), or the drugs in combination as indicated. Cell viability was determined by trypan blue exclusion assay 24 h after the start of drug treatment (n = 3 +/− SEM). # $P < 0.05$ less than corresponding value in CMV transfected; *$P < 0.05$ greater than corresponding value in siSCR transfected.

lower half-life without significantly altering GRP78 promoter activity (Booth et al., 2014a). Furthermore, previous studies have also shown that over-expression of HSP90 does not significantly protect cells from OSU-03012 toxicity as a single agent and that over-expression of GRP78 has a significant though still partial (~75%) inhibitory effect on drug toxicity (Park et al., 2008 Booth et al., 2014a). Sildenafil and OSU-03012 interacted to increase PERK and eIF2α phosphorylation and reduce expression of GADD34, Nck1 and GRP78 (Fig. 2A). Similar data were obtained using the chemically dissimilar PDE5 inhibitor tadalafil (Cialis) (data not shown). 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 eIF2α 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 eIF2α and that this inhibitory effect on eIF2α is overcome by the addition of sildenafil which reduces, below basal levels, the expression of GADD34 and Nck1, and de facto thus also eIF2α –associated PP1 activity. To test this hypothesis further, cells were treated with OSU + SIL for 12 h and total eIF2α protein immuno-precipitated; in cells treated with OSU + SIL the amount of PP1 and PP1
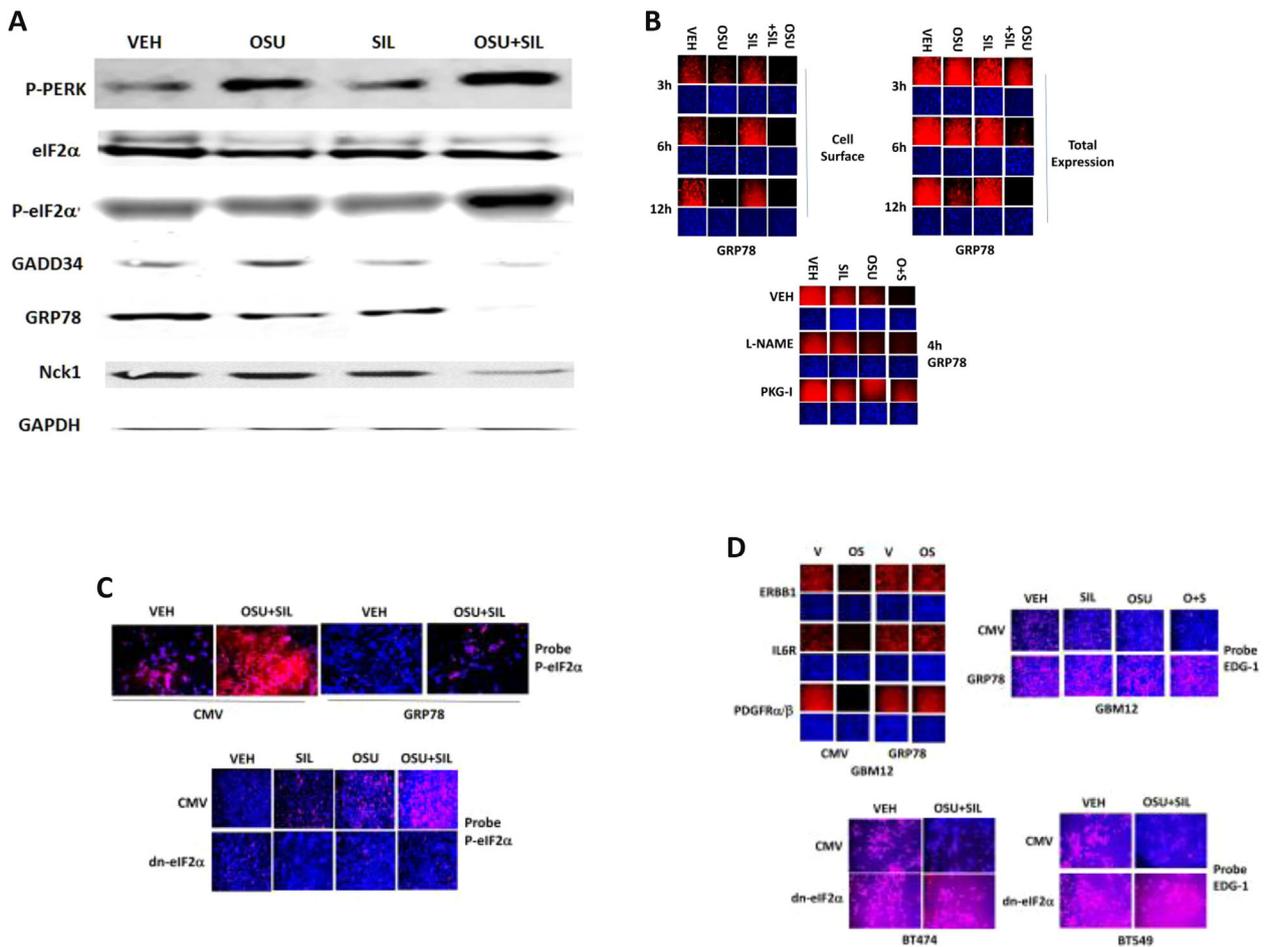

Fig. 2. Treatment of tumor cells with [OSU-03012 + sildenafil] rapidly reduces the expression of GRP78 and increases PERK and eIF2α activity. (A) GBM12 cells were treated with vehicle; OSU-03012 (1 μM); sildenafil (2 μM) or the drugs in combination and 3 h after treatment cells were isolated and subjected to SDS PAGE followed by western immunoblotting to detect the indicated total proteins and phospho-proteins present in the samples. (B) *Upper images*: GBM12 cells were treated with vehicle; OSU-03012 (1 μM); sildenafil (2 μM) or the drugs in combination and 3–12h after treatment cells were fixed with 2% (v/v) paraformaldehyde containing Triton X100 as indicated to permeabilized cells. Immuno-staining of GRP78 was performed using standard techniques and IF images detected using a Hermes Wiscan machine. *Lower images*: GBM12 cells were treated with vehicle; OSU-03012 (1 μM); sildenafil (2 μM) or the drugs in combination in the presence of absence of L-NAME (10 μM) or Guanosine 3′,5′-cyclic monophosphothioate, Rp-Isomer sodium salt (1 μM) and after 4 h treatment were fixed with 2% (v/v) paraformaldehyde containing Triton X100 to permeabilized cells. Immuno-staining of GRP78 on permeabilized cells was performed using standard techniques and IF images detected using a Hermes Wiscan machine. (C) *Upper*: GBM12 cells were transfected with empty vector plasmid or a plasmid to express GRP78. After 24 h cells were treated with vehicle or OSU-03012 (1 μM) and sildenafil (2 μM). Cells were fixed in situ after 2 h and immuno-fluorescence performed to determine the phosphorylation of eIF2α S51. *Lower*: GBM12 cells were transfected with empty vector plasmid or a plasmid to express dominant negative eIF2α S51A. Cells were fixed in situ after 2 h and immuno-fluorescence performed to determine the phosphorylation of eIF2α S51. (D) *Upper*: GBM12 cells were transfected with empty vector plasmid or a plasmid to express GRP78. After 24 h cells were treated with vehicle or OSU-03012 (1 μM) and sildenafil (2 μM). Cells were fixed in situ after 2 h and immuno-fluorescence performed to determine the expression of ERBB1, IL6R, PDGFR and EDG-1. *Lower*: BT474 and BT549 mammary carcinoma cells were transfected with empty vector plasmid or a plasmid to express dominant negative eIF2α S51A. After 24 h cells were treated with vehicle or OSU-03012 (1 μM) and sildenafil (2 μM). Cells were fixed in situ after 2 h and immuno-fluorescence performed to determine the expression of the S1P receptor 1 (also known as EDG-1).

activity co-precipitating with eIF2α was reduced (data not shown).

GRP78 is predominantly found within the endoplasmic reticulum where it plays a vital role in assisting correct protein folding and regulating multiple arms of the endoplasmic reticulum stress sensing machinery, but is also localized to the outer side of the plasma membrane. We noted that OSU-03012 as a single agent rapidly reduced the plasma membrane levels of GRP78, but had a longer time course for knock down of intracellular GRP78 pools (Fig. 2B). Similar data were obtained in multiple other GBM cell lines (data not shown). Combined exposure of cells to sildenafil and OSU-03012 very rapidly reduced the expression of GRP78 in all cellular locations. The OSU-03012 + sildenafil –induced decrease in GRP78 levels was weakly blocked by the nitric oxide synthase inhibitor L-NAME but was more substantially reduced by a cGMP dependent kinase inhibitor. Reduced GRP78 expression

facilitates PERK dimerization and PERK activation, and as judged by elevated eIF2α phosphorylation, and over-expression of GRP78 or expression of eIF2α S51A prevented the drug-induced increased phosphorylation of this factor (Fig. 2C). Treatment of cells with sildenafil and OSU-03012 rapidly decreased the expression of multiple growth factor receptors, an effect that was abolished by over-expression of GRP78 (Fig. 2D).

We next determined in HuH7 and HT1080 cells the impact of increasing concentrations of OSU-03012 with or without sildenafil on the expression of GRP78 and the chaperone proteins HSP70 and HSP90. OSU-03012 decreased expression of GRP78, HSP70 and HSP90 in HuH7 and HT1080 cells, effects that were magnified by co-exposure with sildenafil and occurred in a time-dependent fashion (Fig. 3A). Similar data for these and other chaperone proteins were found in GBM cells (Fig. 3B and C).
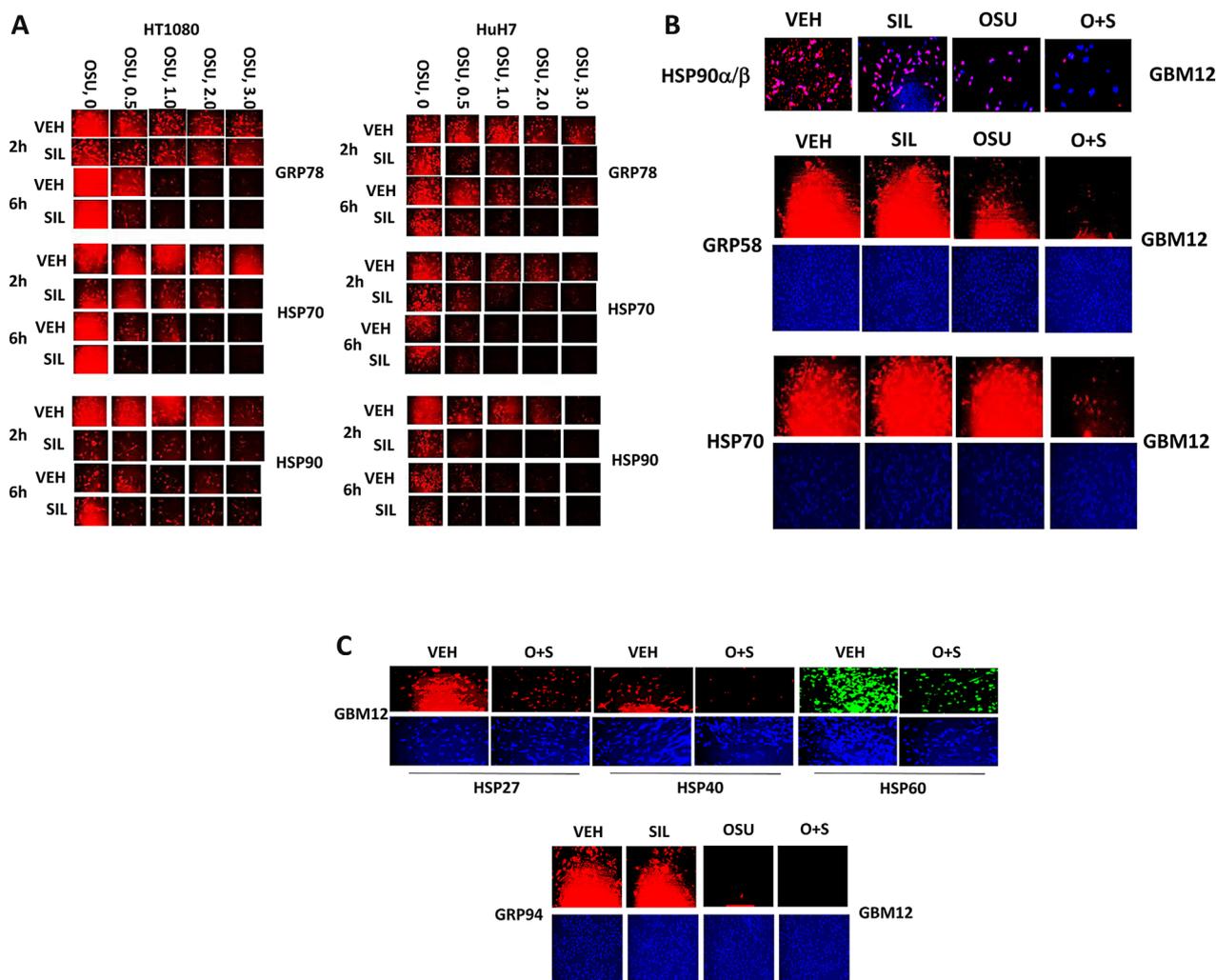

Fig. 3. The regulation of chaperone function and expression by OSU-03012 and Sildenafil. (A) HT1080 sarcoma and HuH7 hepatoma cells were treated with vehicle, sildenafil (2 μM), OSU-03012 (0.5–3.0 μM) as single agents or in the indicated combinations for 2 h or 6 h. Cells were then fixed with 2% (v/v) paraformaldehyde containing Triton X100 to permeabilize cells. Immuno-staining of total GRP78, HSP70 and HSP90 expression was performed using standard techniques and IF images detected using a Hermes Wiscan machine. (B) and (C) GBM12 cells were treated with vehicle, sildenafil (2 μM), OSU-03012 (1.0 μM) 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 GRP58, HSP70, HSP90, HSP27, HSP40, HSP60 and GRP94 expression was performed using standard techniques and IF images detected using a Hermes Wiscan machine.

As noted in the Introduction section, the expression of GRP78 is essential for replication and productive virus release for many "*well known*" pathogenic viruses such as Ebola; Cytomegalovirus; Chikungunya, Measles; HIV; Influenza; Lassa; and Marburg (Roux, 1990; Earl et al., 1991; Moreno and Tiffany-Castiglioni, 2014; Anderson et al., 1992; Hogue and Nayak, 1992; Xu et al., 1997; Carleton and Brown, 1997; Xu et al., 1998; Bolt, 2001; Bredèche et al., 2001; Shen et al., 2002; Dimcheff et al., 2004; He, 2006; Spurgers et al., 2010; Reid et al., 2014). Some viruses such as Dengue fever virus are reported to actually infect cells through a cell surface expressed GRP78 protein (Quinones et al., 2008). Even the receptor tyrosine kinase ERBB1 has been argued to be an essential signaling co-factor for RSV infection. GRP78 also plays an essential role in the viability of unicellular parasites such as leishmania, malaria, and yeasts (Jensen et al., 2001; Cortes et al., 2003; Kimata and Kohno, 2011). Even in prokaryotes, three proteins, Dna K (GRP78), Dna J (HSP40) and GrpE (HSP27) are very similar and complex as functional homologues of mammalian proteins and play an essential role in bacterial growth and viability e.g. by regulating RecA expression (Noguchi et al., 2014). Phosphodiesterase enzymes, the clinical targets of multiple FDA approved PDE5 and PDE3 inhibitors in humans, are also expressed in bacteria, unicellular organisms and through evolution to mammals (Schmidt et al., 2005; Tuckerman et al., 2011; Kwan et al., 2014). Based on our data in the present manuscript using tumor cells, and the extensive literature on the vital role of GRP78 homologues as well as highly conserved variants of HSP27, HSP40, HSP60, GRP58, GRP94, HSP70 and HSP90 in the life cycles of viruses, bacteria and unicellular eukaryotes and mammals, we attempted to determine whether OSU-03012, with or without PDE5/PDE3 inhibitor co-exposure, could modify infectivity and life cycle completions of viruses and bacteria.

OSU-03012 + sildenafil treatment rapidly decreased expression of the cox sakie and adenovirus receptor (CAR) in a dose- and time-dependent fashion that was modestly reduced in a cell type dependent fashion by over-expression of GRP78 (Fig. 4A and B). Pre-treatment of cells with OSU-03012 + sildenafil significantly reduced the ability of a serotype 5 virus expressing GFP to infect cells and reduced the production of GFP when drug treatment occurred after virus infection (Fig. 4C–E). OSU-03012 + sildenafil treatment following virus infection also reduced the ability of a serotype 5 adenovirus to replicate as judged by infected cell killing. Pre-treatment of cells with OSU-03012 + sildenafil or OSU-03012 + sildenafil treatment following virus infection suppressed the ability of cox sakie B4 virus to kill infected cells 18 h after infection (Figure 4F). Over-expression of GRP78 enhanced the abilities of adenovirus and cox sakie virus to kill and abolished the protective effect of OSU-03012 + sildenafil treatment (Fig. 4G and H). Knock down of GRP78 also suppressed virus –induced killing (Fig. 4I and J).

Treatment of cells with OSU-03012 + sildenafil significantly lowered the expression of the virus receptors NPC1 and TIM1 (Ebola virus, Marburg virus, Hepatitis A) effects that were only modestly inhibited by over expression of GRP78 (Fig. 5A and B). Drug combination treatment also reduced expression of NTCP (Hepatitis B); CD81 (Hepatitis C); LAMP1 (Lassa fever); and ERBB1 (Rous sarcoma virus), effects that again were only partially reduced, and only in selected cell lines, by over-expression of GRP78 (Fig. 5C and D). Treatment of cells before or after virus infection with OSU-03012 and sildenafil suppressed infection and/or the killing of infected cells over a wide viral infectivity range by various BSL-3 and BSL-2 restricted human pathogenic viruses (Fig. 6A–F). Over-expression of GRP78 enhanced the lethality of Measles and Influenza B viruses (Fig. 6G and H). Unfortunately, Virginia Commonwealth University does not have a faculty member with whom we could collaborate for translational in vivo virus studies, and thus in vivo validation of our in vitro findings will have to be the subject of a future manuscript.

Bacteria express a homologue of GRP78, Dna K. Treatment of antibiotic sensitive wild type *E. coli* bacteria with OSU-03012 significantly reduced proliferation and enhanced penicillin/streptomycin toxicity (Fig. 7A). Treatment of laboratory generated ampicillin and kayamycin resistant *E. coli* bacteria with OSU-03012 significantly reduced proliferation (Fig. 7B). We know that OSU-03012 reduces the half-life of mammalian GRP78 and in the present studies we discovered that OSU-03012 could down-regulate DNA K expression (Fig. 7C). Bacteria also contain di-cyclic GMP phosphodiesterases that limit intracellular concentrations of cyclic nucleotides which when highly elevated are toxic (Tuckerman et al., 2011). Thus of particular note was that antibiotic resistant *E. coli* were also killed by the PDE5 inhibitors sildenafil (Viagra) or tadalafil (Cialis). In dose-response studies OSU-03012, sildenafil or tadalafil profoundly suppressed bacterial growth at very low clinically relevant concentrations (Fig. 7D). The decline in Dna K expression correlated with reduced expression of a Dna K chaperoned protein, Rec A. In the case of the chaperones GrpE (HSP27) and DNA J (HSP40), treatment with either sildenafil, tadalafil or OSU-03012 caused the appearance of a slower migrating species of the protein on SDS PAGE, that in mammalian cells would be linked to increased protein phosphorylation (Fig. 7C). These findings with OSU-03012 also correlated with significant morphological changes in the coliform bacteria, with surviving Gram-stained *E. coli* cells treated with OSU-03012 (2 μM) for 3 h appearing either elongated or "fat" in appearance (Fig. 7C).

Similar data to that obtained in *E. coli* with OSU-03012 were also found with the *Neisseria gonorrhoeae* strains FA19, FA1090, MS11 and 1291 (Fig. 8A, data not shown). OSU-03012 treatment did not appear to alter the morphology of these coccoid bacteria, though as before, we noted that OSU-03012 reduced expression of Dna K and Rec A (Fig. 8B). In FA19, OSU-03012 and PDE5 inhibitors could interact to cause a greater than additive amount of bacteria killing (Fig. 8C). Data in prior panels had shown that unlike the growth inhibitory antibiotic chloramphenicol, OSU-03012 exhibited true bacteriocidal effects on cells that had yet to enter log-phase growth. Finally, using F89 and HO41 drug resistant *N. gonorrhoeae* isolates we determined whether OSU-03012 exhibited any antibiotic effect and whether it could enhance/restore the bacteriocidal properties of approved standard of care antibiotics. Treatment of F89 and HO41 bacteria with OSU-03012 caused a dose-dependent reduction in bacterial growth (Fig. 9A). Treatment of F89 and HO41 bacteria with OSU-03012 variably enhanced the lethality of Ceftriaxone, Ciprofloxin, or Azithromycin over a 5 h time course (Figures 9B and 9 C). Methicillin-resistant Staphylococcus epidermidis (MRSE) bacteria were noted to be at least as sensitive to OSU-03012 as were *N. gonorrhoeae* (F89, HO41), possibly moreso i. e. 2 μM OSU-03012 was not growth inhibitory in F89 and HO41 but reduced MRSE growth by ∼50%, and co-exposure of bacteria to OSU-03012 and sildenafil modestly enhanced the cyto-toxic effect beyond that of OSU-03012 alone (Fig. 9D).

Discussion

The present studies were initiated to determine the role of the ER chaperone protein GRP78/BiP/HSPA5 in regulating cell viability when cells are exposed to OSU-03012 alone, or when combined with phosphodiesterase 5 (PDE5) inhibitors. We discovered that sildenafil and other PDE5 inhibitors enhanced the ability of OSU-03012 to suppress GRP78 expression. This was associated with decreased expression of multiple growth
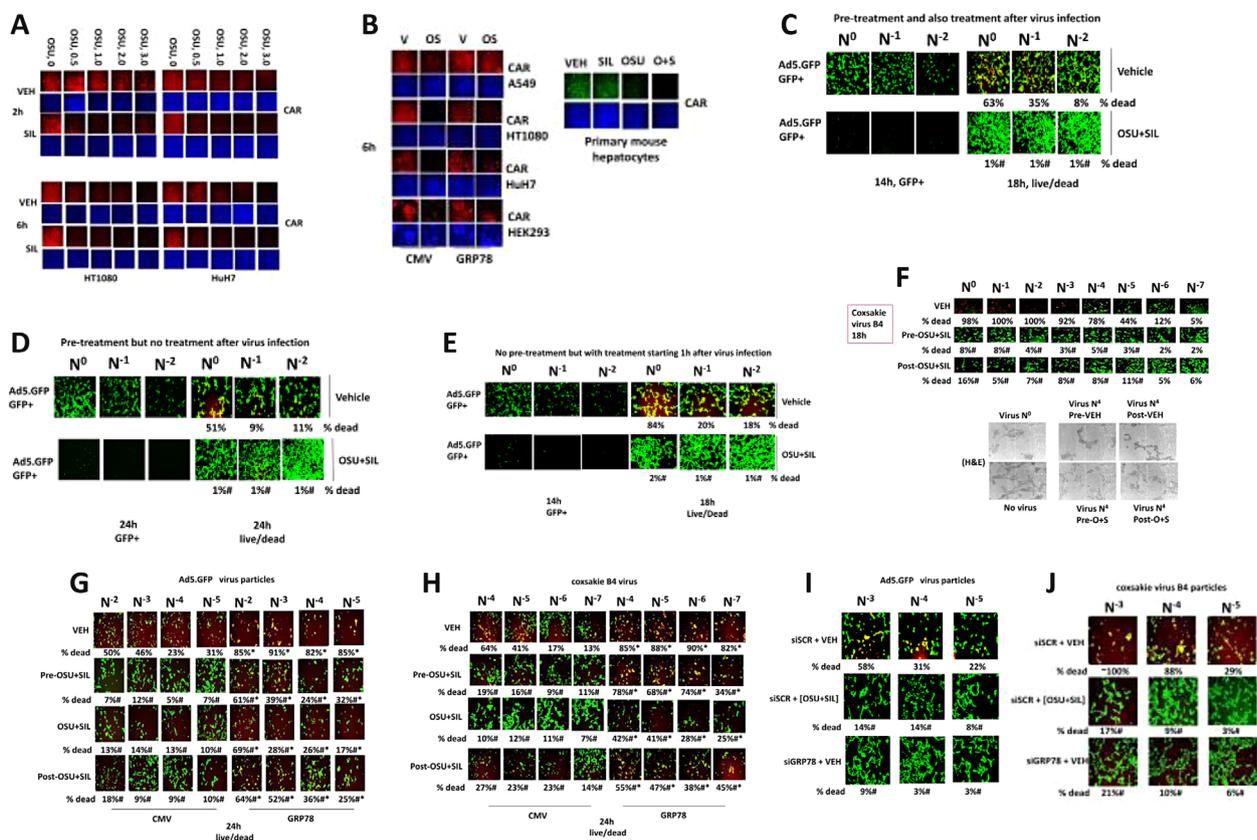
Fig. 4. The regulation of virus receptor expression and the virus reproductive cycle by OSU-03012 and Sildenafil. (A) HT1080 sarcoma and HuH7 hepatoma cells were treated with vehicle, sildenafil (2 μM), OSU-03012 (0.5–3.0 μM) as single agents or in the indicated combinations for 2 h or 6 h. Cells were then fixed with 2% (v/v) paraformaldehyde containing Triton X100 to permeabilize cells. Immuno-staining of the cox sakie and adenovirus virus receptor (CAR) expression was performed using standard techniques and IF images detected using a Hermes Wiscan machine. (B) A549 (NSCLC), HEK293 (kidney), HuH7 and HT1080 cells were transfected with empty vector plasmid or a plasmid to express GRP78. Primary mouse hepatocytes were not transfected. Twenty four h later cells were treated with vehicle control or with OSU-03012 (1 μM) and/or sildenafil (2 μM) for 6 h. Immuno-staining of the cox sakie and adenovirus virus receptor (CAR) expression was performed using standard techniques and IF images detected using a Hermes Wiscan machine. (C) HEK293 cells were pre-treated with vehicle or [OSU-03012 + Sildenafil] as above for 3 h. Cells (~10,000) were then infected at the indicated numbers of virus particles (total) with a recombinant serotype 5 adenovirus to express green fluorescent protein (GFP). The media was changed to media still containing drugs, as indicated, and the percentage of GFP+ cells determined after 14 h; and the percentage cell death was determined by live/dead assay determined after 18 h (n = 3 +/− SEM) # P < 0.05 less than corresponding value in vehicle treated cells. (D) HEK293 cells were pre-treated with vehicle or [OSU-03012 + Sildenafil] as above for 3 h. Cells (~10,000) were then infected at the indicated numbers of virus particles (total) with a recombinant adenovirus to express green fluorescent protein (GFP). The media was changed to drug free media and the percentage of GFP+ cells determined after 24 h; and the percentage cell death was determined by live/dead assay determined after 24 h (n = 3 +/− SEM) # P < 0.05 less than corresponding value in vehicle treated cells. (E) HEK293 cells (~10,000) were infected at the indicated numbers of virus particles (total) with a recombinant adenovirus to express green fluorescent protein (GFP). One h after infection the media was changed to contain vehicle or [OSU-03012 + Sildenafil] and the percentage of GFP+ cells determined after 14 h; and the percentage cell death was determined by live/dead assay determined after 18 h (n = 3 +/− SEM) # P < 0.05 less than corresponding value in vehicle treated cells. (F) HEK293 cells were continuously treated/pre-treated post-treated with vehicle or [OSU-03012 + Sildenafil] as above for 3 h. Cells (~10,000) were then infected at the indicated numbers of virus particles (total) with a cox sakie virus B4. The percentage cell death was determined by live/dead assay determined after 18 h (n = 3 +/− SEM) # P < 0.05 less than corresponding value in vehicle treated cells. (G) and (H) HEK293 cells were transfected with empty vector plasmid or a plasmid to express GRP78. After 24 h cells were infected with Ad5.GFP or with cox sakie virus B4 and treated before/during/after/always with vehicle or OSU-03012 (1 μM) and sildenafil (2 μM). The percentage cell death was determined by live/dead assay determined after 18 h where green cells = alive and red/yellow cells = dead (n = 3 +/− SEM). # P < 0.05 less than corresponding value in vehicle treated cells; * P < 0.05 greater than corresponding value in CMV transfected cells. (I) and (J) HEK293 cells were transfected with a scrambled siRNA (siSCR) or an siRNA to knock down GRP78 expression (siGRP78). Twenty four h after transfection cells are infected with increasing amounts of Ad5.GFP or cox sakie virus B4. The percentage cell death was determined by live/dead assay determined after 18 h where green cells = alive and red/yellow cells = dead (n = 3 +/− SEM) # P < 0.05 less than corresponding value in siSCR + vehicle treated cells.

factor and virus receptors, effects that were for some of these receptors, blocked by GRP78 over-expression. Our prior studies have linked OSU-03012–induced ER stress signaling by PERK as being causal in the toxicity of the drug (Park et al., 2008; Booth et al., 2014a; Booth et al., 2014b). The present studies demonstrated that the drug combination of OSU + SIL strongly activated both PERK and eIF2α that was associated with decreased GADD34 and Nck1 and GRP78 expression.

Reduced GADD34/Nck1 expression facilitates eIF2α signaling by reducing co-localization of the Ser/Thr phosphatase PP1 with eIF2α. Prolonged ER stress signaling results in tumor cell death (Fig. 10A).

Sildenafil was developed as an inhibitor of PDE5 with cardio-protective effects, and serendipitously became an approved therapeutic for erectile dysfunction (Zusman et al., 1999). PDE5 expression is not confined to the corpus cavernosum in
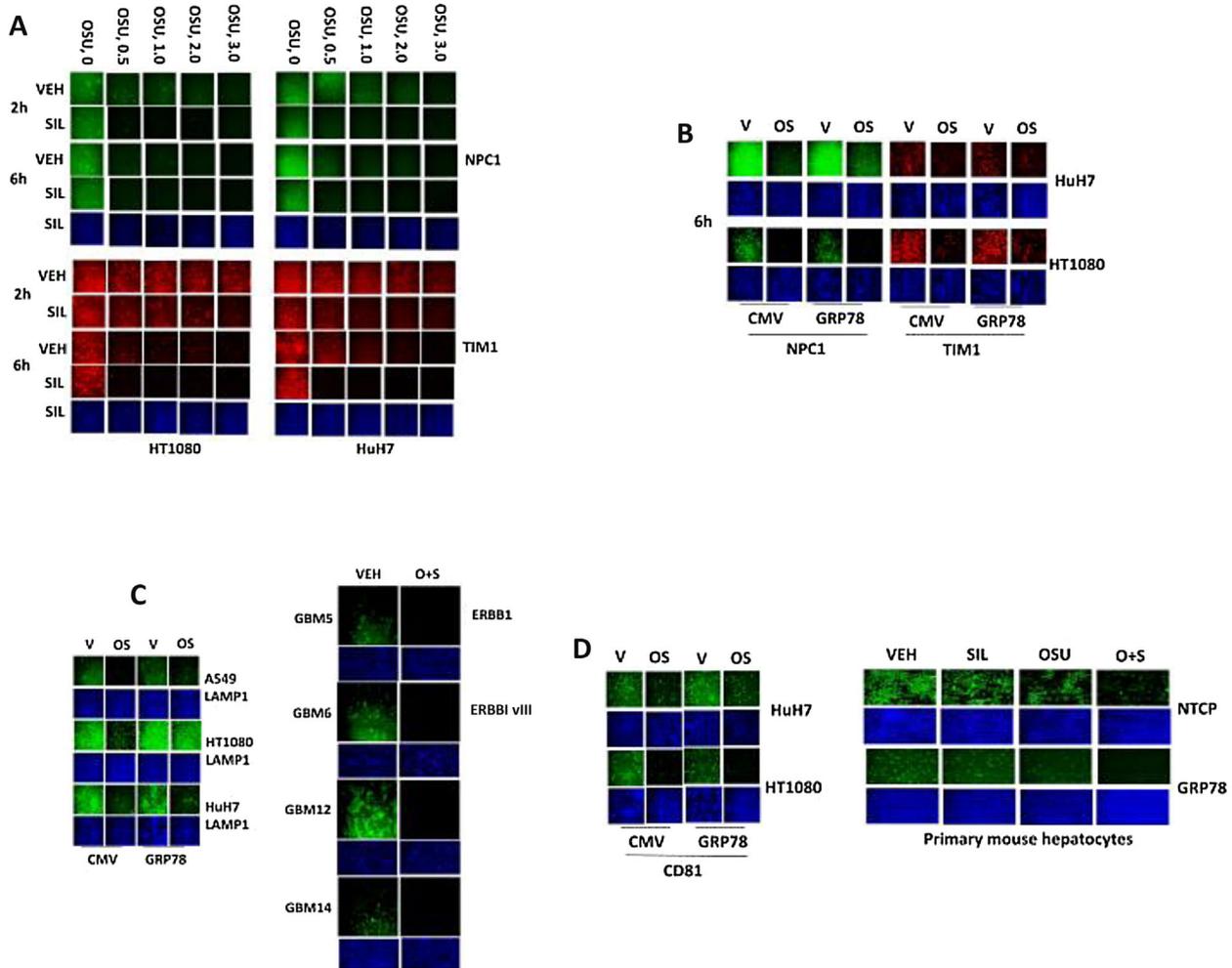
Fig. 5. OSU-03012 alone or in combination with sildenafil reduces expression of multiple virus receptor proteins. (A) HuH7 (hepatoma) and HT1080 (sarcoma) were treated with vehicle, OSU-03012 (0–3 μM), sildenafil (2 μM) or the drugs in combination. Two h and six h after drug exposure cells were fixed in place and permeabilized. Immuno-fluorescence was performed to determine the total cellular levels of TIM1 and NPC1 (Ebola/Marburg virus receptors). (B) HuH7 and HT1080 cells were transfected with empty vector plasmid or a plasmid to express GRP78. Twenty four h later cells were treated with vehicle control or with OSU-03012 (1 μM) and sildenafil (2 μM) for 6 h. Immuno-staining of NPC1 and TIM1 expression was performed using standard techniques and IF images detected using a Hermes Wiscan machine. (C) *Image set to the left:* A549, HT1080 and HuH7 cells transfected with empty vector plasmid or a plasmid to express GRP78. Twenty four h later cells were treated with vehicle control or with OSU-03012 (1 μM) and sildenafil (2 μM) for 6 h. Immuno-staining of LAMP1 expression was performed using standard techniques and IF images detected using a Hermes Wiscan machine. *Image set to the right:* GBM5/6/12/14 cells were treated with vehicle control or with OSU-03012 (1 μM) and sildenafil (2 μM) for 6 h. Immuno-staining of ERBB1 (full length) and ERBB1 vIII (GBM6) expression was performed using standard techniques and IF images detected using a Hermes Wiscan machine. (D) *Image set to the left:* HT1080 and HuH7 cells were transfected with empty vector plasmid or a plasmid to express GRP78. Twenty four h later cells were treated with vehicle control or with OSU-03012 (1 μM) and sildenafil (2 μM) for 6 h. Immuno-staining of CD81 expression was performed using standard techniques and IF images detected using a Hermes Wiscan machine. *Image set to the right:* Primary mouse hepatocytes were treated with vehicle control or with OSU-03012 (1 μM) and/or sildenafil (2 μM) for 6 h. Immuno-staining of NTCP expression (hepatitis B receptor) and of GRP78 was performed using standard techniques and IF images detected using a Hermes Wiscan machine.

the human penis and is expressed in the wider vasculature, myocardium and tumor cells (Shan et al., 2012 Karami-Tehrani 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; i.e. 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 ~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 NFκB 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
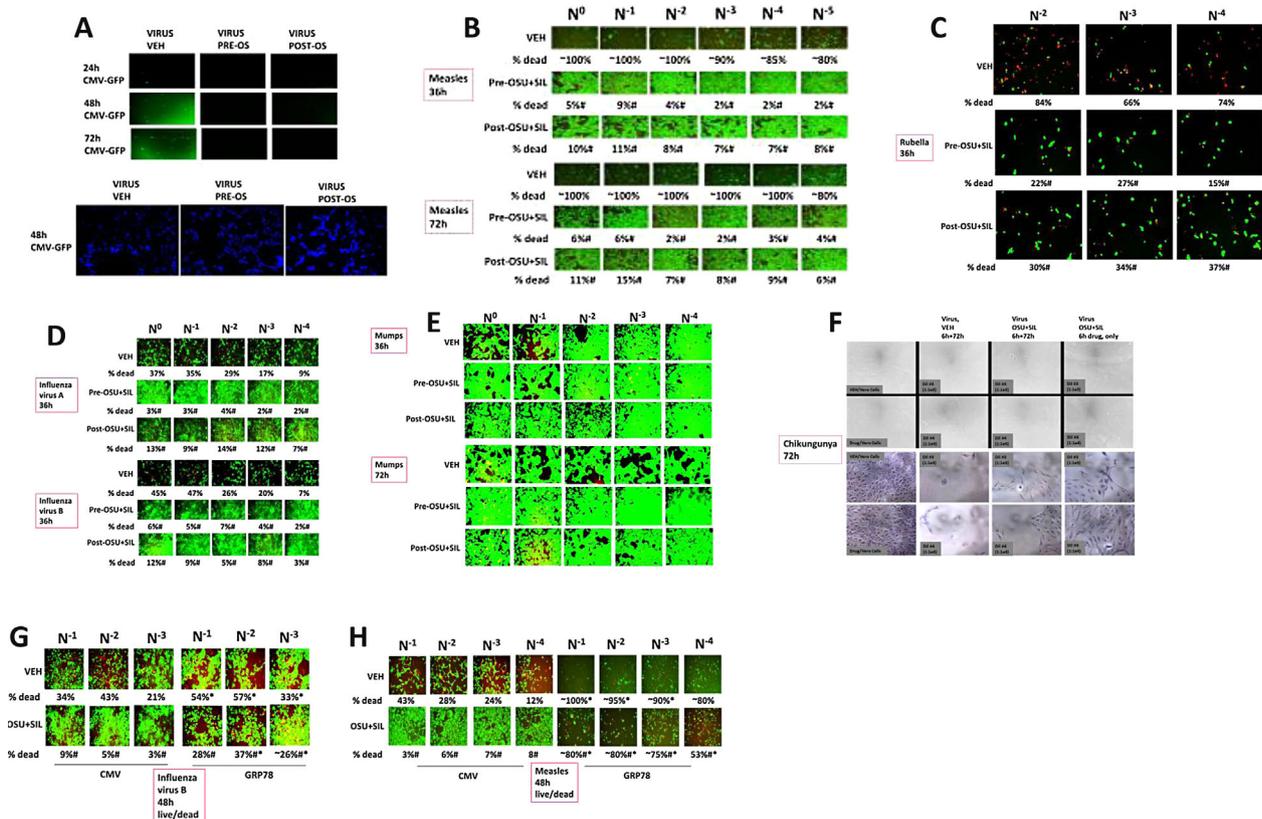
Fig. 6. OSU-03012 and PDE5 inhibitors modulate the ability of multiple viruses to infect and to replicate. (A) (i) MRC-5 fibroblasts were pre-treated with vehicle or [OSU-03012 + Sildenafil] for 3 h. Cells were then infected with Cytomegalovirus-GFP virus (multiplicity of infection, m.o.i. of 5). Cells were imaged 24–72h after infection to determine the number of GFP+ cells and to compare the relative intensity of GFP+ fluorescence. (ii) MRC-5 fibroblasts were infected with the indicated amounts of Cytomegalovirus-GFP virus (multiplicity of infection, m.o.i. of 5) and 1 h following infection, fibroblasts were treated with vehicle or [OSU-03012 + Sildenafil]. Cells were imaged 24–72h after infection to determine the number of GFP+ cells and to compare the relative intensity of GFP+ fluorescence. (B)-(H) Cells were infected with the indicated viruses at the approximate IC50 for virus –induced cell death based on Zeptometrix internal data/manufactuer's instructions. Portions of cells were: (i) infected with virus followed 1 h afterwards by treatment with vehicle or with OSU-03012 (1.0 μM) and sildenafil (2 μM). After 18h-96h as indicated in each panel cell viability/morphology was determined using a live/dead assay where green cell = alive; yellow/red cell = dead. (ii) Cells were pre-treated with vehicle or with OSU-03012 (1.0 μM) and sildenafil (2 μM), followed by viral infection, and subsequent incubation in the absence of drugs. Infected cells were cultured for 18h-96h and cell viability/morphology determined. For panels G and H, studies with GRP78 over-expression were performed in an identical fashion to those in Figure 4G and H (n = 3 +/− SEM) # P < 0.05 less than corresponding value in vehicle treated cells; * P < 0.05 greater than corresponding value in empty vector CMV transfected cells.

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., 2005; Zhu and Strada, 2007; Zhu et al., 2009). Our prior findings showed that OSU + SIL interacted to cause toxic JNK activation (Booth et al., 2012a; Booth et al., 2014a). 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.,

2005). 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 (Booth et al., 2014a).

Based on an unbiased search of the National Library of Medicine database it is self evident that GRP78 and evolutionary conserved homologues of GRP78 play essential roles in the biology and life cycles of viruses, bacteria, protosoal and yeast cells as well as higher eukaryotic cells, in particularly tumor cells that express high levels of many "activated" oncogenic signaling proteins (Roux, 1990 Earl et al., 1991; Anderson et al., 1992; Hogue and Nayak, 1992; Xu et al., 1997; Carleton and Brown, 1997; Xu et al., 1998; Bolt, 2001; Bredèche et al., 2001; Shen et al., 2002; Dimcheff et al., 2004; He, 2006; Spurgers et al., 2010; Moreno and Tiffany-Castiglioni, 2014; Reid et al., 2014). The bacterial GRP78 homologue,
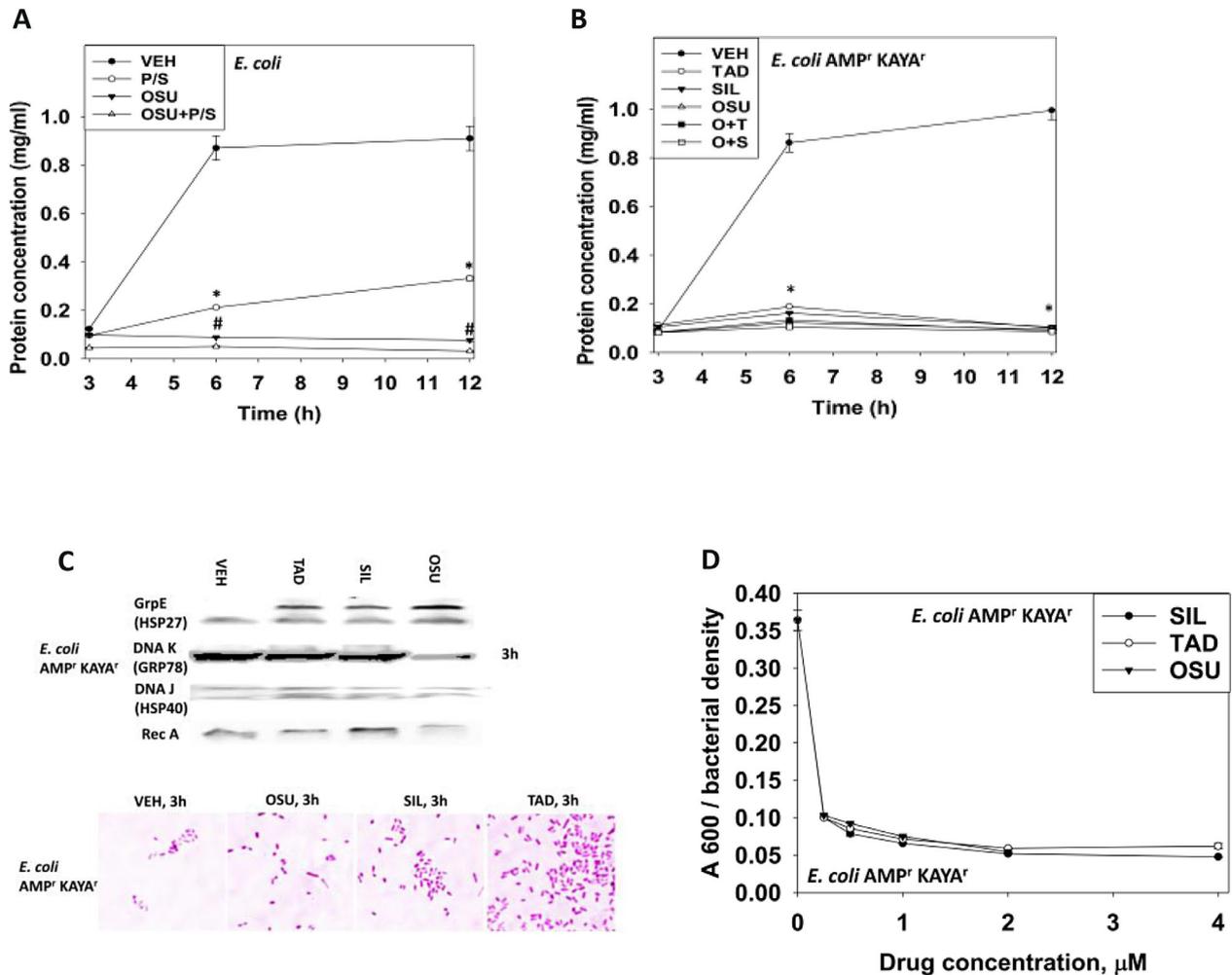
Fig. 7. OSU-03012 as an anti-bacterial agent in *E. coli*. (A) Antibiotic sensitive *E. coli* bacteria were treated with vehicle or with OSU-03012 (4 μM) for 3 h. Cell treatments were then split into two and as indicated bacteria were then treated with vehicle or with a laboratory Pen/Strep solution (0.05 v/v final concentration) (n = 3 +/− SEM) * *P* < 0.05 less than vehicle control; # *P* < 0.05 less than corresponding pen/strep treated value. (B) and (C) *E. coli* bacteria transformed to be resistant to ampicillin and kayamycin (AMPʳ KAYAʳ) were grown in AMP+ KAYA+ media and cells treated with OSU-03012 (2 μM); sildenafil (2 μM); tadalafil (2 μM), or in combination as indicated. Bacterial cell numbers were determined by assessing the protein concentration of the bacterial cell pellet 3 h, 6h and 12 h after drug exposure (n = 3 +/− SEM) * *P* < 0.05 less than corresponding vehicle value. The total protein content of each treatment condition was determined 3 h after addition of antibiotic. Portions of bacteria from each treatment condition were isolated and SDS PAGE and immuno-blotting performed to determine the expression of Dna J, Dna K, RecA and GrpE. *Lower images*: *E. coli* bacteria Gram stained and examined under a X100 oil immersion magnification. (D) *E. coli* bacteria transformed to be resistant to ampicillin and kayamycin (AMPʳ KAYAʳ) were grown in AMP+ KAYA+ media and cells treated with OSU-03012 (0.5–4.0 μM); sildenafil (0.25–2.0 μM); or tadalafil (0.25–2.0 μM). Bacterial cell numbers were determined by protein concentration in the bacterial cell pellet 6 h after drug exposure (n = 3 +/− SEM).

Dna K, is essential for bacterial growth and mRNA/protein stability, and we found that OSU-03012 down-regulated Dna K expression; the expression of chaperoned proteins e.g. Rec A, and rendered bacteria more sensitive to anti-bacterial drugs and in some cases, the phosphodiesterase inhibitor tadalafil. Thus Dna K represents a new target for antibiotic development. Furthermore, our data with sildenafil (Viagra) and tadalafil (Cialis) also demonstrates that bacterial phosphodiesterases may represent a new target for antibiotic development. Determining whether OSU-03012 combined with antibiotics and/or PDE inhibitors can restore drug sensitivities in addition to called "super-bugs," e.g. *MRSA/Klebsiela pneumonia*, is beyond the scope of the present study (Fig. 10B).

Low levels of GRP78 expression are essential for regulating the ER stress response of normal non-transformed cells and for folding of the relatively low levels of protein production in non-transformed cells. In tumor cells with very high protein expression levels or in the reproduction cycle of viruses in higher eukaryotes, the GRP78 protein, together with other chaperone proteins including HSP27, HSP40, HSP60, HSP90, GRP94 and HSP70, is essential for the ATP-dependent correct folding of highly expressed host and viral proteins, including the virus capsid proteins. (Roux, 1990 Earl et al., 1991; Anderson et al., 1992; Hogue and Nayak, 1992; Xu et al., 1997; Carleton and Brown, 1997; Xu et al., 1998; Bolt, 2001; Bredèche et al., 2001; Shen et al., 2002; Dimcheff et al., 2004; He, 2006; Spurgers et al., 2010; Moreno and Tiffany-Castiglioni, 2014;
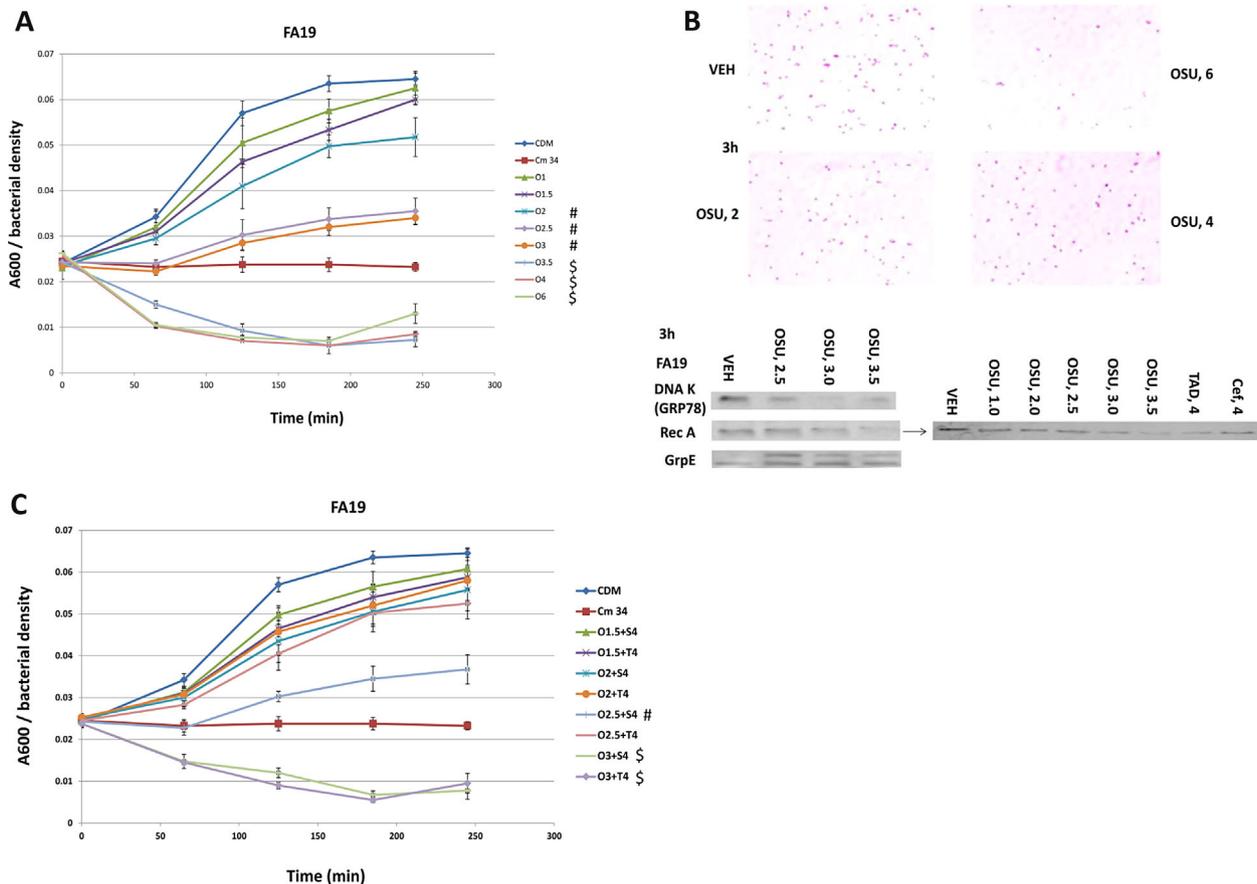
Fig. 8. OSU-03012 kills *Neisseria gonorrhoeae* bacteria. (A) *Neisseria gonorrhoeae* (FA19) were grown in liquid culture and treated with vehicle or with OSU-03012 (0–8 μM). The optical density of each treatment was determined 0–4h after treatment as indicated in each graphical presentation (n = 3 +/− SEM) # P < 0.05 less than CDM value; $ P < 0.05 less than corresponding time = 0 density value. (B) From bacteria isolated during Panel A: *Upper images*: the morphology of the coccoid bacteia did not alter following OSU-03012 exposure. *Lower western blot*: OSU-03012 reduces expression of Dna K and Rec A but not GrpE in FA19 bacteria. (C) *Neisseria gonorrhoeae* (FA19) were grown in liquid culture and bacteria were treated with vehicle or with OSU-03012 (1.5–3.0 μM) as indicated in the presence or absence of sildenafil (4 μM) or tadalafil (4 μM). The optical density of each treatment and the bacterial protein content was determined after 0–240 min (n = 3 +/− SEM) # P < 0.05 less than value in CDM treated cells; $ P < 0.05 less than time = 0 value.

Reid et al., 2014). Because viruses synthesize large amounts of protein, they induce a rapid intense ER stress response in infected cells including stimulation of additional GRP78 expression that is essential for the correct viral protein folding and the overall transient cell survival purposes of the virus. Some viral proteins are known to suppress the intense PERK-eIF2α signaling response to viral protein production via association of PP1 with eIF2α, to ensure that "just the right amount" of ER stress is signaled in a virus infected cell. Thus an infected cell with a high protein load only signals a diminuted stress response. i.e. promoting a survival ER stress response rather than a ER stress death response that would usually be generated by such an intense unfolded protein response (Rathore et al., 2013). Hence: OSU-03012 disrupts the "Goldilocks" virus modulation of ER stress.

OSU-03012, unlike the parent compound celecoxib, destabilizes the GRP78 protein causing protein half-life to be profoundly reduced, and OSU-03012 when combined with sildenafil/tadalafil rapidly further reduces the decline in total GRP78 levels by >> 90% in all tumor cell lines tested (Booth et al., 2012a). Treatment of adenovirus/cox sakie

virus virally infected cells with OSU-03012 and sildenafil decreased the release of active virus particles as judged by decreased cell lysis/cell death caused by virus reproduction. Furthermore, OSU-03012 and sildenafil treatment reduced expression of multiple membrane virus receptors essential for host infection by adenovirus/cox sakie virus; as well as by Ebola; Marburg, Chikungunya, Lassa fever; Hepatitis A; Hepatitis B; and Hepatitis C viruses. A vast number of human viruses rely on GRP78 expression and those of other chaperones for their life cycle, e.g. Ebola and Influenza, and for the further induction of GRP78 to facilitate production of functional native conformation viral proteins and, obviously, new virus particles. Hence collectively our data strongly argue that the combination of OSU-03012 and sildenafil or other PDE5 inhibitors e.g. tadalafil, could potentially be developed into a potent anti-viral therapy for viral diseases ranging from Ebola to Influenza to the common cold (Fig. 10C).

Based on our data it can also be hypothesized that virus infected cells treated with OSU + SIL may still be largely capable of replicating their genetic material and generating mis-
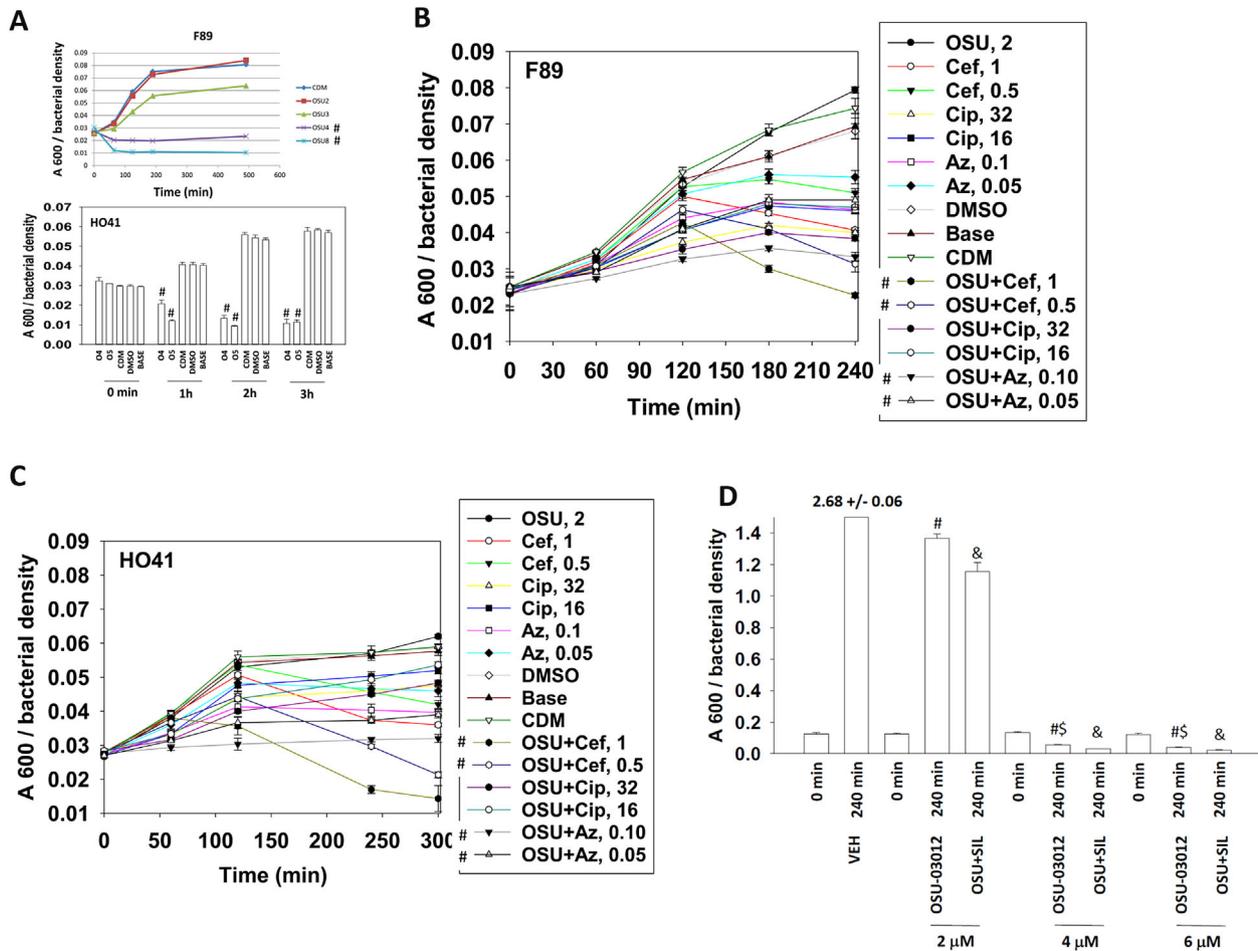
Fig. 9. OSU-03012 kills complete antibiotic drug resistant *Neisseria gonorrhoeae* and interacts with standard of care drugs to re-sensitize resistant bacteria to antibiotics. (A) Clinical isolate complete antibiotic drug resistant *Neisseria gonorrhoeae* (F89, HO41) were grown in liquid culture and bacteria were treated with vehicle or OSU-03012 (0–8 μM) as indicated. The optical density of each treatment was determined (n = 3 +/− SEM). # $P < 0.05$ less than CDM value; \$ $P < 0.05$ less than bacterial density value at time = 0. (B) and (C) Clinical isolate complete antibiotic drug resistant *Neisseria gonorrhoeae* strains (F89, HO41) were grown in liquid culture and bacteria were treated with vehicle or OSU-03012 (2 μM) in the presence or absence of Ceftriaxone (0.5–1.0 μg/ml), Azithromycin (0.05–0.10 μg/ml), Ciprofloxacin (16–32 μg/ml) as indicated. The optical density of each treatment was determined (n = 3 +/− SEM). # $P < 0.05$ less than corresponding bacterial density value in cells treated only with antibiotic alone. (D) Clinical isolate complete antibiotic drug resistant Methicillin-resistant Staphylococcus epidermidis (MRSE, RP62A) was grown in liquid culture and bacteria were treated with vehicle or OSU-03012 (0, 2, 4, 6 μM) as indicated in the presence or absence of sildenafil (2 μM), as indicated. The optical density of each treatment was determined at time = 0 and at time = 240 min (n = 3 in triplicate +/− SEM). # $P < 0.05$ less than corresponding value at 4 h in vehicle treated cells; \$ $P < 0.05$ less than bacterial density value at time = 0; & $P < 0.05$ less than corresponding OSU-03012 value alone treatment.

folded non-functional viral proteins. This has already been noted (Mirazimi and Svensson, 2000 Goodwin et al., 2011; Yu et al., 2011). Though the production of active virions will be profoundly reduced by OSU-03012, such infected cells will eventually die, likely through autophagic or necroptotic mechanisms, releasing cell and virus debris into the host. Viral genetic material and capsid proteins are highly immunogenic and have the potential to stimulate a vaccination protective effect in the host. Indeed, that we found drug combination treatment reduced GRP78 expression in primary hepatocytes with causing their cell death (see also: Chiu et al., 2009a; Chiu et al., 2009b). Thus OSU + SIL treatment may both incapacitate virus and bacterial reproduction and simultaneously stimulate host defenses and anti-virus/anti-bacterial host resistance.

In conclusion, treatment of cells with OSU + SIL rapidly reduces the expression of the key chaperone protein GRP78,

as well as reducing the functions of multiple other cell-survival chaperones. Loss of GRP78 expression reduces bacterial cell viability, prevents virus reproduction, and may facilitate a potential host vaccination effect. As enhanced GRP78 chaperone function, and those of other chaperones, has also been implicated in the biology of other debilitating human diseases, e.g. most notably Alzheimer's disease; as brain-permeant PDE5 inhibitors are considered to be potentially useful agents in Altzheimer's disease treatment; as OSU-03012 also crosses the blood-brain barrier; the possibilities of also developing an effective OSU + PDE5 inhibitor therapy regimen for this debilitating disease together with those previously mentioned in this manuscript could have profound health implications in many areas of human health (Zhang et al., 2013 Devan et al., 2014; Yang et al., 2014; García-Barroso et al., 2013).
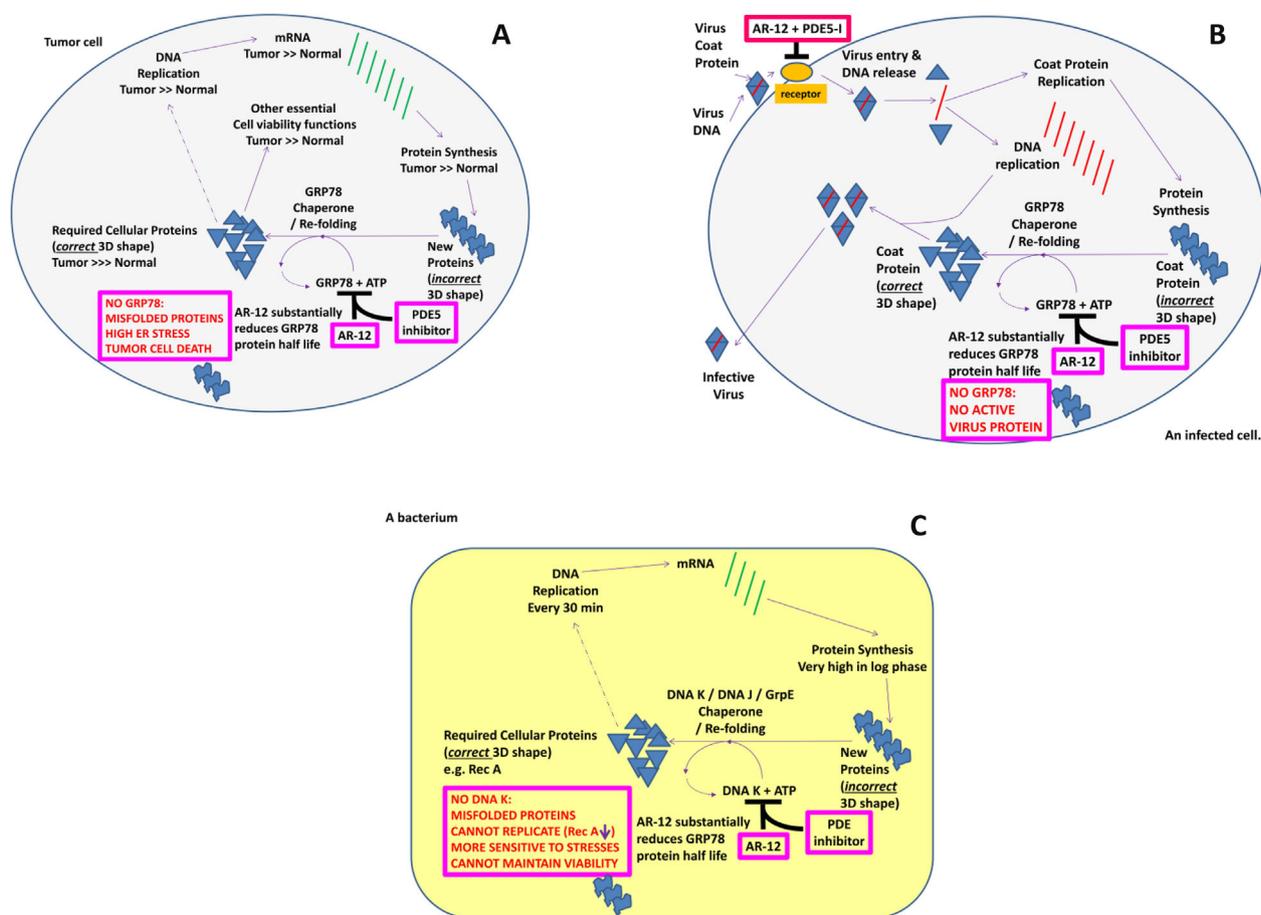
Fig. 10. Possible molecular mechanisms by which OSU-03012 (also called AR-12) and PDE inhibitors could: *Panel A*: kill tumor cells; *Panel B*: prevent production of new infective virions; *Panel C*: prevent bacterial growth. The ability of OSU-03012 (AR-12) alone, or enhanced by a PDE5 inhibitor, to reduce GRP78 expression and the function of multiple other chaperones will prevent the correct folding of essential tumor cell/virus/bacterial proteins resulting in dead tumor cells/inactive virus materials/dead bacteria. The combination of OSU-03012 and a PDE5 inhibitor will also reduce the expression of plasma membrane receptors whose expression is essential for bacterial and viral infection. Thus treatment of cells with OSU-03012 and a PDE5 inhibitor will both reduce bacterial/virus infection and virus production/bacterial growth simultaneously. The loss of GRP78 and other chaperones in tumor cells will result in much lower expression levels of key oncogenic kinases resulting in less tumor growth and more tumor cell death. Collectively our data strongly argue that it is a strong possibility that our drug combination will have profound anti-tumor/anti-viral/anti-bacterial capabilities.

Literature Cited

Anderson K, Stott EJ, Wertz GW. 1992. Intracellular processing of the human respiratory syncytial virus fusion glycoprotein: Amino acid substitutions affecting folding, transport and cleavage. J Gen Virol 73:1177–1188.

Black KL, Yin D, Ong JM, Hu J, Konda BM, Wang X, Ko MK, Bayan JA, Sacapano MR, Espinoza A, Irvin DK, Shu Y. 2008. PDE5 inhibitors enhance tumor permeability and efficacy of chemotherapy in a rat brain tumor model. Brain Res 1230: 290–302.

Bolt G. 2001. The measles virus (MV) glycoproteins interact with cellular chaperones in the endoplasmic reticulum and MV infection upregulates chaperone expression. Arch Virol 146:2055–2068.

Booth L, Cazanave SC, Hamed HA, Yacoub A, Ogretmen B, Chen CS, Grant S, Dent P. 2012a. OSU-03012 suppresses GRP78/BiP expression that causes PERK-dependent increases in tumor cell killing. Cancer Biol Ther 13:224–236.

Booth L, Cruickshanks N, Ridder T, Chen CS, Grant S, Dent P. 2012b. OSU-03012 interacts with lapatinib to kill brain cancer cells. Cancer Biol Ther 13:1501–1511.

Booth L, Roberts JL, Cruickshanks N, Grant S, Poklepovic A, Dent P. 2014a. Regulation of OSU-03012 toxicity by ER stress proteins and ER stress-inducing drugs. Mol Cancer Ther 13:2384–2398.

Booth L, Roberts JL, Cruickshanks N, Tavallai S, Webb T, Samuel P, Conley A, Binion B, Young HF, Poklepovic A, Spiegel S, Dent P. 2014b. PDE5 inhibitors enhance Celecoxib killing in multiple tumor types. J Cell Physiol Oct 9 DOI: 10.1002/jcp.24843. [Epub ahead of print].

Booth L, Roberts JL, Cruickshanks N, Conley A, Durrant DE, Das A, Fisher PB, Kukreja RC, Grant S, Poklepovic A, Dent P. 2014c. Phosphodiesterase 5 inhibitors enhance chemotherapy killing in gastrointestinal/genitourinary cancer cells. Mol Pharmacol 85:408–419.

Booth L, Roberts JL, Conley A, Cruickshanks N, Ridder T, Grant S, Poklepovic A, Dent P. 2014d. HDAC inhibitors enhance the lethality of low dose salinomycin in parental and stem-like GBM cells. Cancer Biol Ther 15:305–316.

Bredèche MF, Ehrlich SD, Michel B. 2001. Viability of rep recA mutants depends on their capacity to cope with spontaneous oxidative damage and on the DnaK chaperone protein. J Bacteriol 183:2165–2171.

Burugu S, Daher A, Meurs EF, Gatignol A. 2014. HIV-1 translation and its regulation by cellular factors PKR and PACT. Virus Res Jul 23. pii S0168–S1702.

Carleton M, Brown DT. 1997. The formation of intramolecular disulfide bridges is required for induction of the Sindbis virus mutant ts23 phenotype. J Virol 71:7696–7703.

Chen WT, Zhu G, Pfaffenbach K, Kanel G, Stiles B, Lee AS. 2014. GRP78 as a regulator of liver steatosis and cancer progression mediated by loss of the tumor suppressor PTEN. Oncogene 33:4997–5005.

Chen Y, Brandizzi F. 2013. IRE1: ER stress sensor and cell fate executor. Trends Cell Biol 23:547–555.

Choi CY, Park KR, Lee JH, Jeon YJ, Liu KH, Oh S, Kim DE, Yea SS. 2007. Isoeugenol suppression of inducible nitric oxide synthase expression is mediated by down-regulation of NF-kappaB, ERK1/2, and p38 kinase. Eur J Pharmacol 576:151–159.

Chiu HC, Soni S, Kulp SK, Curry H, Wang D, Gunn JS, Schlesinger LS, Chen CS. 2009a. Eradication of intracellular Francisella tularensis in THP-1 human macrophages with a novel autophagy inducing agent. J Biomed Sci 16:110.

Chiu HC, Kulp SK, Soni S, Wang D, Gunn JS, Schlesinger LS, Chen CS. 2009b. Eradication of intracellular Salmonella enterica serovar Typhimurium with a small-molecule, host cell-directed agent. Antimicrob Agents Chemother 53:5236–5244.

Cortes GT, Winograd E, Wiser MF. 2003. Characterization of proteins localized to a subcellular compartment associated with an alternate secretory pathway of the malaria parasite. Mol Biochem Parasitol 129:127–135.

Cui W, Yu CH, Hu KQ. 2005. In vitro and in vivo effects and mechanisms of celecoxib-induced growth inhibition of human hepatocellular carcinoma cells. Clin Cancer Res 11:8213–8221.

Dabo S, Meurs EF. 2012. DsRNA-dependent protein kinase PKR and its role in stress, signaling and HCV infection. Viruses 4:2598–2635.

Das A, Xi L, Kukreja RC. 2008. Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. J Biol Chem 283:29572–29585.

Deguchi A, Thompson WJ, Weinstein IB. 2004. Activation of protein kinase G is sufficient to induce apoptosis and inhibit cell migration in colon cancer cells. Cancer Res 64: 3966–3973.

Devan BD, Pistell PJ, Duffy KB, Kelley-Bell B, Spangler EL, Ingram DK. 2014. Phosphodiesterase inhibition facilitates cognitive restoration in rodent models of age-related memory decline. NeuroRehabilitation 34:101–111.

Dimcheff DE, Faasse MA, McAtee FJ, Portis JL. 2004. Endoplasmic reticulum (ER) stress induced by a neurovirulent mouse retrovirus is associated with prolonged BiP binding and retention of a viral protein in the ER. J Biol Chem 279:33782–33790.

Donnelly N, Gorman AM, Gupta S, Samali A. 2013. The eIF2α kinases: their structures and functions. Cell Mol Life Sci. 70:3493–3511.

Earl PL, Moss B, Doms RW. 1991. Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. J Virol. 65:2047–2055.

Friebe A, Koesling D. 2009. The function of NO-sensitive guanylyl cyclase: what we can learn from genetic mouse models. Nitric Oxide. 21:149–156.

García-Barroso C, Ricobaraza A, Pascual-Lucas M, Unceta N, Rico AJ, Goicolea MA, Sallés J, Lanciego JL, Oyarzabal J, Franco R, Cuadrado-Tejedor M, García-Osta A. 2013. Tadalafil crosses the blood-brain barrier and reverses cognitive dysfunction in a mouse model of AD. Neuropharmacology. 64:114–123.

Giannini C, Sarkaria JN, Saito A, Uhm JH, Galanis E, Carlson BL, Schroeder MA, James CD. 2005. Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. Neuro Oncol 7: 164–176.

Goodwin EC, Lipovsky A, Inoue T, Magaldi TG, Edwards AP, Van Goor KE, Paton AW, Paton JC, Atwood WJ, Tsai B, DiMaio D. 2011. BiP and multiple DNAJ molecular chaperones in the endoplasmic reticulum are required for efficient simian virus 40 infection. MBio 2 e00101–e00111.

Gorbatyuk MS, Gorbatyuk OS. 2013. The Molecular Chaperone GRP78/BiP as a Therapeutic Target for Neurodegenerative Disorders: A Mini Review. J Genet Syndr Gene Ther 4: pii 128.

Ha KS, Kim KM, Kwon YG, Bai SK, Nam WD, Yoo YM, Kim PK, Chung HT, Billiar TR, Kim YM. 2003. Nitric oxide prevents 6-hydroxydopamine-induced apoptosis in PC12 cells through cGMP-dependent PI3 kinase/Akt activation. FASEB J 17:1036–1047.

He B. 2006. Viruses, endoplasmic reticulum stress, and interferon responses. Cell Death Differ 13:393–403.

Hayden MA, Lange PA, Nakayama DK. 2001. Nitric oxide and cyclic guanosine monophosphate stimulate apoptosis via activation of the Fas-FasL pathway. J Surg Res 101:183–189.

Hogue BG, Nayak DP. 1992. Synthesis and processing of the influenza virus neuraminidase, a type II transmembrane glycoprotein. Virology 188:510–517.

Hollien J, Weissman JS. 2006. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. Science 313:104–107.

Hollien J, Lin JH, Li H, Stevens N, Walter P, Weissman JS. 2009. Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J Cell Biol 186:323–331.

Jensen AT, Curtis J, Montgomery J, Handman E, Theander TG. 2001. Molecular and immunological characterisation of the glucose regulated protein 78 of Leishmania donovani(1). Biochim Biophys Acta 1549:73–87.

Johnson AJ, Smith LL, Zhu J, Heerema NA, Jefferson S, Mone A, et al. 2005. A novel celecoxib derivative, OSU-03012, induces cytotoxicity in primary CLL cells and transformed B-cell lymphoma cell line via a caspase-and Bcl-2-independent mechanism. Blood 105:2504–2509.

Kang SG, Kim JS, Park K, Kim JS, Groves MD, Nam DH. 2006. Combination celecoxib and temozolomide in C6 rat glioma orthotopic model. Oncol Rep 15:7–13.

Karami-Tehrani F, Moeinifard M, Aghaei M, Atri M. 2012. Evaluation of PDE5 and PDE9 Expression in Benign and Malignant Breast Tumors. Arch Med Res 43:470–475.

Kashfi K, Rigas B. 2005. Is COX-2 a 'collateral' target in cancer prevention?. Biochem Soc Trans 33:724–727.

Kato M, Blanton R, Wang GR, Judson TJ, Abe Y, Myoishi M, Karas RH, Mendelsohn ME. 2012. Direct Binding and Regulation of RhoA by Cyclic GMP-dependent Protein Kinase Iα. J Biol Chem 287:41342–41351.

Kimata Y, Kohno K. 2011. Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells. Curr Opin Cell Biol 23:135–142.

Klenke FM, Gebhard MM, Ewerbeck V, Abdollahi A, Huber PE, Sckell A. 2006. The selective Cox-2 inhibitor celecoxib suppresses angiogenesis and growth of secondary bone tumors: an intravital microscopy study in mice. BMC Cancer 12:6–9.

Koehne CH, Dubois RN. 2004. COX-2 inhibition and colorectal cancer. Semin Oncol 31:12–21.

Komalavilas P, Shah PK, Jo H, Lincoln TM. 1999. Activation of mitogen-activated protein kinase pathways by cyclic GMP and cyclic GMP-dependent protein kinase in contractile vascular smooth muscle cells. J Biol Chem 274:34301–34309.

Kulp SK, Yang YT, Hung CC, Chen KF, Lai JP, Tseng PH, Fowble JW, Ward PJ, Chen CS. 2004. 3-Phosphoinositide-dependent protein kinase-1/Akt signaling represents a major cyclooxygenase-2-independent target for celecoxib in prostate cancer cells. Cancer Res 64:1444–1451.

Kwan BW, Osbourne DO, Hu Y, Benedik MJ, Wood TK. 2014. Phosphodiesterase DosP increases persistence by reducing cAMP which reduces the signal indole. Biotechnol Bioeng Sep 12 .[Epub ahead of print].

Lee AS. 2007. GRP78 induction in cancer: therapeutic and prognostic implications. Cancer Res 67:3496–3499.

Luo B, Lee AS. 2013. The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies. Oncogene 32:805–818.

Maurel M, Chevet E, Tavernier J, Gerlo S. 2014. Getting RIDD of RNA: IRE1 in cell fate regulation. Trends Biochem Sci 39:245–254.

Mirazimi A, Svensson L. 2000. ATP is required for correct folding and disulfide bond formation in rotavirus VP7. J Virol 74:8048–8052.

Moreno JA, Tiffany-Castiglioni E. 2014. The Chaperone Grp78 in protein folding disorders of the nervous system. Neurochem Res Aug 9. [Epub ahead of print].

Narayanan BA, Narayanan NK, Pittman B, Reddy BS. 2006. Adenocarcina of the mouse prostate growth inhibition by celecoxib: Downregulation of transcription factors involved in COX-2 inhibition. Prostate 66:257–265.

Ni M, Lee AS. 2007. ER chaperones in mammalian development and human diseases. FEBS Lett 581:3641–3651.

Noguchi A, Ikeda A, Mezaki M, Fukumori Y, Kanemori M. 2014. DnaJ-promoted binding of DnaK to multiple sites on σ32 in the presence of ATP. J Bacteriol 196:1694–1703.

Ostberg KL, DeRocco AJ, Mistry SD, Dickinson MK, Cornelissen CN. 2013. Conserved regions of gonococcal TbpB are critical for surface exposure and transferrin iron utilization. Infect Immun 81:3442–3450.

Panner A, Murray JC, Berger MS, Pieper RO. 2007. Heat shock protein 90alpha recruits FLIPS to the death-inducing signaling complex and contributes to TRAIL resistance in human glioma. Cancer Res 67:9482–9489.

Park MA, Yacoub A, Rahmani M, Zhang G, Hart L, Hagan MP, et al. 2008. OSU-03012 stimulates PKR-like endoplasmic reticulum-dependent increases in 70-kDa heat shock protein expression, attenuating its lethal actions in transformed cells. Mol Pharmacol 73:1168–1184.

Patel MI, Subbaramaiah K, Du B, Chang M, Newman RA, Cordon-Cardo C, Thaler HT, Dannenberg AJ. 2005. Celecoxib inhibits prostate cancer growth: Evidence of a cyclooxygenase-2-independent mechanism. Clin Cancer Res 11:1999–2007.

Pavitt GD, Ron D. 2012. New insights into translational regulation in the endoplasmic reticulum unfolded protein response. Cold Spring Harb Perspect Biol 4.pii: a 012278.

Potter LR. 2011. Guanylyl cyclase structure, function and regulation. Cell Signal 23:1921–1926.

Quinones QJ, de Ridder GG, Pizzo SV. 2008. GRP78: A chaperone with diverse roles beyond the endoplasmic reticulum. Histol Histopathol 23:1409–1416.

Rao R, Fiskus W, Ganguly S, Kambhampati S, Bhalla KN. 2012. HDAC inhibitors and chaperone function. Adv Cancer Res. 116:239–262.

Rathore APS, Ng ML, Vasudevan SG. 2013. Differential unfolded protein response during Chikungunya and Sindbis virus infection: CHIKV nsP4 suppresses eIF2α phosphorylation. Virology Journal 10:36.

Reid SP, Shurtleff AC, Costantino JA, Tritsch SR, Retterer C, Spurgers KB, Bavari S. 2014. HSPA5 is an essential host factor for Ebola virus infection. Antiviral Res 109:171–174.

Roberts JL, Booth L, Conley A, Cruickshanks N, Malkin M, Kukreja RC, Grant S, Poklepovic A, Dent P. 2014. PDE5 inhibitors enhance the lethality of standard of care chemotherapy in pediatric CNS tumor cells. Cancer Biol Ther 15:758–767.

Roller C, Maddalo D. 2013. The molecular chaperone GRP78/BiP in the development of chemoresistance: Mechanism and possible treatment. Front Pharmacol. 11; 4:10.

Roux L. 1990. Selective and transient association of Sendai virus HN glycoprotein with BiP. Virology 175:161–166.

Sano R, Reed JC. 2013. ER stress-induced cell death mechanisms. Biochim Biophys Acta 1833:3460–3470.

Sarfati M, Mateo V, Baudet S, Rubio M, Fernandez C, Davi F, Binet JL, Delic J, Merle-Beral H. 2003. Sildenafil and vardenafil, types 5 and 6 phosphodiesterase inhibitors, induce caspase-dependent apoptosis of B-chronic lymphocytic leukemia cells. Blood 101:265–269.

Schmidt AJ, Ryjenkov DA, Gomelsky M. 2005. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: Enzymatically active and inactive EAL domains. J Bacteriol 187:4774–4781.

Shan X, Quaile MP, Monk JK, French B, Cappola TP, Margulies KB. 2012. Differential expression of PDE5 in failing and nonfailing human myocardium. Circ Heart Fail 5:79–86.

Shen Y, Meunier L, Hendershot LM. 2002. Identification and characterization of a novel endoplasmic reticulum (ER) DnaJ homologue, which stimulates ATPase activity of BiP in vitro and is induced by ER stress. J Biol Chem. 277:15947–15956.

Spurgers KB, Alefantis T, Peyser BD, Ruthel GT, Bergeron AA, Costantino JA, Enterlein S, Kota KP, Boltz RC, Aman MJ, Delvecchio VG, Bavari S. 2010. Identification of essential filovirion-associated host factors by serial proteomic analysis and RNAi screen. Mol Cell Proteomics 9:2690–2703.

Thompson WJ, Piazza GA, Li H, Liu L, Fetter J, Zhu B, Sperl G, Ahnen D, Pamukcu R. 2000. Exisulind induction of apoptosis involves guanosine 3',5'-cyclic monophosphate phosphodiesterase inhibition, protein kinase G activation, and attenuated beta-catenin. Cancer Res 60:3338–3342.

Tong L, Heim RA, Wu S. 2011. Nitric oxide: a regulator of eukaryotic initiation factor 2 kinases. Free Radic Biol Med 50:1717–1725.

Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA. 2011. Cyclic di-GMP activation of polynucleotide phosphorylase signal-dependent RNA processing. J Mol Biol 407:633–639.

Vélez Acevedo RN, Ronpirin C, Kandler JL, Shafer WM, Cornelissen CN. 2014. Identification of regulatory elements that control expression of the tbpBA operon in Neisseria gonorrhoeae. J Bacteriol 196:2762–2774.

Xu A, Bellamy AR, Taylor JA. 1998. BiP (GRP78) and endoplasmin (GRP94) are induced following rotavirus infection and bind transiently to an endoplasmic reticulum-localized virion component. J Virol 72:9865–9872.

Xu Z, Jensen G, Yen TS. 1997. Activation of hepatitis B virus S promoter by the viral large surface protein via induction of stress in the endoplasmic reticulum. J Virol 71: 7387–7392.

Yacoub A, Park MA, Hanna D, Hong Y, Mitchell C, Pandya AP, et al. 2006. OSU-03012 promotes caspase-independent but PERK-, cathepsin B-, BID-, and AIF-dependent killing of transformed cells. Mol Pharmacol 70:589–603.

Yang Y, Chen S, Zhang J, Li C, Sun Y, Zhang L, Zheng X. 2014. Stimulation of autophagy prevents amyloid-β peptide-induced neuritic degeneration in PC12 cells. J Alzheimers Dis 40:929–939.

Yu H, Li S, Yang C, Wei M, Song C, Zheng Z, Gu Y, Du H, Zhang J, Xia N. 2011. Homology model and potential virus-capsid binding site of a putative HEV receptor Grp78. J Mol Model 17:987–995.

Zhang X, Yan G, Ji J, Wu J, Sun X, Shen J, Jiang H, Wang H. 2012. PDE5 inhibitor promotes melanin synthesis through the PKG pathway in B16 melanoma cells. J Cell Biochem 113:2738–2743.

Zhang J, Guo J, Zhao X, Chen Z, Wang G, Liu A, Wang Q, Zhou W, Xu Y, Wang C. 2013. Phosphodiesterase-5 inhibitor sildenafil prevents neuroinflammation, lowers beta-amyloid levels and improves cognitive performance in APP/PS1 transgenic mice. Behav Brain Res 250:230–237.

Zhang S, Sun Y, Chen H, Dai Y, Zhan Y, Yu S, Qiu X, Tan L, Song C, Ding C. 2014. Activation of the PKR/eIF2α signaling cascade inhibits replication of Newcastle disease virus. Virol J 11:62.

Zhu B, Vemavarapu L, Thompson WJ, Strada SJ. 2005. Suppression of cyclic GMP-specific phosphodiesterase 5 promotes apoptosis and inhibits growth in HT29 cells. J Cell Biochem 94:336–350.

Zhu B, Strada SJ. 2007. The novel functions of cGMP-specific phosphodiesterase 5 and its inhibitors in carcinoma cells and pulmonary/cardiovascular vessels. Curr Top Med Chem 7:437–454.

Zhu B, Strada S, Stevens T. 2005a. Cyclic GMP-specific phosphodiesterase 5 regulates growth and apoptosis in pulmonary endothelial cells. Am J Physiol Lung Cell Mol Physiol 289:L196–L206.

Zhu B, Zhang L, Alexeyev M, Alvarez DF, Strada SJ, Stevens T. 2009. Type 5 phosphodiesterase expression is a critical determinant of the endothelial cell angiogenic phenotype. Am J Physiol Lung Cell Mol Physiol 296:L220–L228.

Zhu J, Huang JW, Tseng PH, Yang YT, Fowble JW, Shiau CW, et al. 2004. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. Cancer Res 64:4309–4318.

Zusman RM, Morales A, Glasser DB, Osterloh IH. 1999. Overall cardiovascular profile of sildenafil citrate. Am J Cardiol 83:35C–44C.