Sequential Cytoprotective Responses to Sigma1 Ligand–Induced Endoplasmic Reticulum Stress

Joel M. Schrock, Christina M. Spino, Charles G. Longen, Stacy M. Stabler, Jacqueline C. Marino, Gavril W. Pasternak, and Felix J. Kim

Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, Pennsylvania (C.M.S., C.G.L., F.J.K.); and Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, New York (J.M.S., S.M.S., J.C.M., G.W.P.)

Received June 7, 2013; accepted September 3, 2013

ABSTRACT

The Sigma1 receptor (Sigma1) is an endoplasmic reticulum (ER) integral membrane protein that is highly expressed in a number of cancer cell lines. Small molecule compounds targeting Sigma1 (Sigma1 ligands) inhibit cancer cell proliferation and induce apoptotic cell death in vitro and inhibit tumor growth in xenograft experiments. However, the cellular pathways activated by Sigma1 protein-ligand interaction are not well defined. Here, we find that treatment with some Sigma1 ligands induces ER stress and activates the unfolded protein response (UPR) in a dose- and time-responsive manner in a range of adenocarcinoma cell lines. Autophagy is engaged after extended treatment with Sigma1 ligands, which suggests that protracted UPR results in autophagy as a secondary response. Inhibition of UPR by RNAi-mediated knockdown of inositol-requiring enzyme 1α and activating transcription factor 4 abrogates autophagosome formation, as does knockdown of essential autophagy gene products Beclin1 and autophagy protein 5. Knockdown of Sigma1 also suppresses IPAG [1-(4-iodophenyl)-3-(2-adamantyl) guanidine] induced UPR marker and autophagosome levels, indicating that this response is indeed Sigma1-mediated. We find that UPR activation precedes autophagosome formation and autophagy precedes apoptosis in Sigma1 ligand-treated cells. These processes are reversible, and washout of IPAG before cell death results in a return of autophagosomes and UPR markers toward basal levels. However, inhibition of Sigma1 ligand–induced UPR or autophagy accelerates apoptotic cell death. Together, these data suggest that UPR and autophagy are engaged as primary and secondary cytoprotective responses, respectively, to Sigma1 ligand–induced disruption of cancer cell protein homeostasis.

Introduction

In eukaryotic cells, the endoplasmic reticulum (ER) is the primary site of synthesis, folding, and assembly of secreted and integral membrane proteins and their macromolecular complexes (Ron and Walter, 2007). Maintenance of ER protein homeostasis relies on the timely convergence of multiple pathways that detect homeostatic protein concentration thresholds and control the ebb-and-flow of ER proteins (Ron and Walter, 2007; Mu et al., 2008; Jonikas et al., 2009). Disruption of ER homeostasis activates stress response pathways, including the unfolded protein response (UPR) (Xu et al., 2005; Ron and Walter, 2007; Kim et al., 2008). The mammalian UPR comprises at least two phases: an initial alarm phase followed by a cytoprotective, adaptive phase in which UPR factors are upregulated to enhance the cellular capacity to process increased concentrations of unfolded protein (Marciniak and Ron, 2006; Ron and Walter, 2007; Kim et al., 2008).

It has been proposed that severe or prolonged ER stress can overwhelm the UPR and the cell may engage autophagy as a secondary survival response (Bernales et al., 2006, 2007; Ogata et al., 2006; Yorimitsu et al., 2006; Ron and Walter, 2007). Growing evidence suggests that ER stress, the unfolded protein response, and autophagy are likely integrated signaling pathways that modulate cell survival and growth (Levine and Klionsky, 2004; Hoyer-Hansen and Jaattela, 2007; Ron and Walter, 2007; He and Klionsky, 2009).

This work was supported in part by a Kimmel Cancer Center, Thomas Jefferson University, Drexel University Consortium Pilot Study Award; the National Institutes of Health National Institute on Drug Abuse [Grants R01 DA06241, R56 DA02815, and T32 DA07274]; an award from the Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research and the Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center; and a grant from the Translational and Integrative Medicine Fund of Memorial Sloan-Kettering Cancer Center.

dx.doi.org/10.1124/mol.113.087809.

[This article has supplemental material available at molpharm.aspetjournals.org.]

ABBREVIATIONS: 4-IBP, N-(2-benzylpiperidin-4-yl)-4-iodobenzamide; ATF4, activating transcription factor 4; ATG5, autophagy protein 5; Bafl, Baflofinycin A1; DMSO, dimethylsulfoxide; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; GFP, green fluorescent protein; IPAG, 1-(4-iodophenyl)-3-(2-adamantyl) guanidine; IRE1α, inositol-requiring enzyme 1α; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein 1 light chain 3; p38MAPK, mitogen-activated protein kinase p38; PARP, poly(ADP-ribose) polymerase; PERK, protein kinase-like endoplasmic reticulum kinase; PGRMC1, progesterone receptor membrane component 1; PRE-084, 2-(4-morpholinethyl) 1-phenylcyclohexanecarboxylate hydrochloride; siRNA, small interfering RNA; SKF10047, hydrochloride, [2S-(2a,6a,11R*)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride; UPR, unfolded protein response.
Autophagy describes a set of bulk cellular degradation pathways by which cells can maintain energy levels under conditions of metabolic stress as well as a mechanism by which large aggregates of misfolded proteins and damaged cellular components, including damaged organelles, are sequestered into membrane-bound vesicles called autophagosomes and subsequently targeted for lysosomal degradation (Levine and Klionsky, 2004; Levine and Kroemer, 2008; Mizushima et al., 2008). Thus, autophagy plays an important role in the maintenance of cellular homeostasis and disease prevention, and defective autophagy pathways have been implicated in pathologies including neurodegenerative disease and cancer (Levine and Kroemer, 2008; Mizushima et al., 2008; Mathew et al., 2009). Autophagy may serve a cytoprotective role in cancer cells that allows survival under the challenging metabolic conditions of the tumor cell environment (Degenhardt et al., 2006; Levine and Kroemer, 2008; Mizushima et al., 2008; Mathew et al., 2009). Furthermore, protein degradation mechanisms such as autophagy may serve to mitigate the higher intrinsic levels of proteotoxic stress in tumor cells (Solimini et al., 2007).

Several chemotherapeutic agents have been shown to induce autophagy (Rubinsztein et al., 2007). However, in many cases it remains unclear whether cell death occurs by autophagy, whether cell death is associated with autophagy, or whether autophagy is a survival response to cytotoxic chemotherapy (Levine and Klionsky, 2004; Hippert et al., 2006; Levine and Kroemer, 2008; Mathew et al., 2009). Emerging data suggest that autophagy participates in integrated responses to cellular stress that determine cell death versus survival. The proteins and pathways that regulate these integrated stress responses are just beginning to be clearly defined (Levine and Klionsky, 2004; Hoyer-Hansen and Jaattela, 2007; Ron and Walter, 2007; He and Klionsky, 2009).

Although first proposed as members of the opioid receptor family based upon their affinity for an opioid-related ligand (Martin et al., 1976), sigma receptors are now considered distinct binding sites unrelated to any classic receptors. The cloned sigma1 receptor (Sigma1) predicts a 26-kD integral membrane protein that is enriched in the ER (Hanner et al., 1996; Aydar et al., 2002; Hayashi and Su, 2007). Sigma1 has been proposed to function as a molecular chaperone at the ER-mitochondrion interface in certain cell types (Hayashi and Su, 2007). However, the physiologic role of Sigma1 in tumor cells remains unclear.

Sigma1 is highly expressed in various tumor cell lines, including breast and prostate adenocarcinoma (Vilner et al., 1995b; Spruce et al., 2004; Aydar et al., 2006). Some Sigma1 ligands may be effective antitumor agents (Vilner et al., 1995a; Berthois et al., 2003; Spruce et al., 2004; Piergentili et al. 2010). Some Sigma1 ligands inhibit cell proliferation, induce apoptotic cell death in vitro, and inhibit tumor growth in mouse xenograft experiments (Berthois et al., 2003; Spruce et al., 2004). However, the mechanisms of Sigma1-mediated actions remain largely unknown. In vitro, ligand treatment results in apoptotic cell death following extended treatment, with time and dose depending on the compound and cell line (Vilner et al., 1995a; Berthois et al., 2003; Spruce et al., 2004; Piergentili et al. 2010). We examined whether Sigma1 ligand treatment induces cascades of cytoprotective signaling in response to ligand-induced disruption of ER protein homeostasis.

**Materials and Methods**

**Chemicals.** Sigma1 ligands, IPAG [1-(4-iodophenyl)-3-(2-adamantyl)guanidine], haloperidol hydrochloride 4-[(4-[4-[4-(chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone hydrochloride], PRE-084 hydrochloride [2-(4-morpholinethyl) 1-phenylcyclohexanecarboxylate hydrochloride], (+)-SKF10047 hydrochloride [2S-[2α,6α,11β]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride], and (+)-pentazocine were obtained from Tocris Bioscience (Bristol, UK) or the U.S. National Institutes of Health National Institute on Drug Abuse. Baflomycin A1, inhibitor of the vacuolar type H+-ATPase (V-ATPase), and thapsigargin were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell Lines and Transfections.** The MDA-MB-468, T47D, MCF7 breast adenocarcinoma, PC3, prostate adenocarcinoma, Panc1 pancreatic adenocarcinoma, and HepG2 hepatocellular carcinoma cell lines were all acquired from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium with 4.5 g/l glucose, 5% fetal bovine serum, nonessential amino acids, and penicillin/streptomycin. Cells were seeded approximately 24 hours before the start of drug treatment in most assays. Human Beclin1, human Sigma1, human progesterone receptor membrane component 1 (PGRMC1), human inositol-requiring enzyme 1α (IRE1α), human activating transcription factor 4 (ATF4), and Control-A small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and human autophagy protein 5 (ATG5) siRNA was purchased from Cell Signaling Technologies (Beverly, MA). The siRNA transfections (100 nM per 35-mm well) were performed with Oligofectamine (Invitrogen/Life Technologies, Carlsbad, CA) or INTERFERin (PolyPlus Transfection SA, Illkirch, France) transfection reagent according to the manufacturer’s procedures. Cells were treated with the indicated drugs 72 hours after transfection for all RNA interference experiments except Sigma1, PGRMC1, and ATG5.

For Sigma1, PGRMC1, and ATG5 siRNA knockdown, 100 nM siRNA per approximately 100,000 cells were transfected with INTERFERin transfection reagent (PolyPlus Transfection SA). Then, 48 hours later, the cells were reseeded, allowed to attach and recover for 16–24 hours, and transfected again. Twenty-four hours after the second transfection of these siRNA, the cells were treated with IPAG or the indicated media, as indicated. Transfection of Control-A siRNA (Santa Cruz Biotechnology) was performed in parallel, using the conditions described earlier.

**Cell Death Assays.** We evaluated cell death by trypan blue exclusion assay, propidium iodide staining, as well as cleaved caspase 3 (Asp175) and cleaved poly(ADP-ribose) polymerase (PARP) (Asp214) immunoblot. Trypan blue exclusion and propidium iodide staining were used to quantify general cell death, and the presence of apoptotic cell death was confirmed by immunoblot analysis. Values were generated from at least six independent determinations, and statistical significance was determined as will be described.

**Immunoblot Analysis and Antibodies.** Cells were lysed and proteins extracted in a modified radioimmunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with 10% glycerol (v/v), Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and Halt phosphatase inhibitor cocktail (Pierce/Thermo Scientific, Rockford, IL). Approximately 10–20 µg of detergent soluble protein were resolved on precast NOVEX polyacrylamide Tris-glycine gels (Invitrogen). Immunoblots were performed in a 20 mM Tris-buffered 137 mM saline solution (pH 7.6) containing 0.1% Tween-20 [polyoxyethylene (20) sorbitan monolaurate] and 5% (w/v) blotting-grade nonfat dry milk (Bio-Rad Laboratories, Hercules, CA). All washes were performed in the same buffer without the blotting-grade nonfat dry milk. The Lumigen PS-3 enhanced chemiluminescence kit (GE Healthcare Bio-Sciences, Pittsburgh, PA) was used to reveal
immunoblotted proteins. The rabbit anti-Sigma1 antibody was generated in our laboratory as described elsewhere (Kim et al., 2012).

The anti–green fluorescent protein (GFP), anti–β-actin, anti-Beclin1, anti-ATF4, anti-PGRC1, and all horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnologies. The anti–microtubule-associated protein 1 light chain 3B (LC3B), anti-ATG5, anti–phospho-mitogen-activated protein kinase p38 (p38MAPK) (Thr180/Tyr182), anti–phospho-stress-activated protein kinase/e-Jun N-terminal kinase (JNK) (Thr185/Tyr185), anti–IRE1α, anti–protein kinase-like endoplasmic reticulum kinase (PERK), anti–phospho-eukaryotic initiation factor 2α (eIF2α) (Ser51), anti–GFP78/BiP, anti–cleaved caspase 3 (Asp175), and anti–cleaved PARP (Asp214) were all accessible from Cell Signaling Technologies.

**Microscopy and Quantification of Autophagosome Formation.** The human GFP-LC3 expression plasmid, pEGFP-LC3 (a gift from Drs. Grazia Ambrosini and Gary K. Schwartz, Memorial Sloan-Kettering Cancer Center), was stably transfected into MDA-MB-468 and selected with 0.5 mg/ml G418 sulfate. Stable populations were generated and compared with parental MDA-MB-468 for Sigma1 expression and autophagic and growth inhibitory response to Sigma1 ligands. GFP-LC3 translocation (puncta formation) was assessed by microscopy, and autophagic degradation (flux) was assessed by an immunoblot-based GFP-LC3 cleavage assay in MDA-MB-468 (GFP-LC3) stable cell populations. For microscopy-based experiments, cells were seeded onto Laboratory-Tek II glass chamber slides (Nalge Nunc International, Rochester, NY). After the indicated number of hours of drug treatment, cells were washed with room temperature Dulbecco’s modified phosphate-buffered saline solution containing calcium and magnesium, and were fixed and permeabilized with room temperature BD Cytofix-Cytoperm solution (BD Biosciences, San Jose, CA). Images of GFP-LC3 puncta were acquired with a Zeiss Axioscop2 Imaging widefield microscope using Axiosvision LE software (Carl Zeiss Microscopy GmbH, Jena, Germany). Puncta were counted using the spot quantification program in the Fluoro-Chem software package (Alpha Innotech/ProteinSimple, San Jose, CA) and confirmed in parallel by manual counting.

**Autophagic Flux Assays.** We evaluated autophagic flux (turnover of autolysosome cargo) using two methods previously described elsewhere (Bernales et al., 2006; Hosokawa et al., 2006; Mizushima and Yoshimori, 2007; Klonksy et al., 2008). Lipid-conjugated GFP-LC3 translocates to autophagosomes that conditionally fuse with lysosomes, which leads to autolysosomal degradation of LC3 and release of GFP in the case of active autophagic flux (Bernales et al., 2006; Hosokawa et al., 2006). In this GFP-LC3 degradation assay, cleaved GFP was detected by immunoblot (Bernales et al., 2006; Hosokawa et al., 2006). Autophagic flux was also verified by inhibiting autolysosomal degradation with the specific inhibitor of the vacuolar type H+-ATPase, Bafilomycin A1. In this assay, absence of cleaved and released GFP is an indicator that ligand-induced autophagic flux has been inhibited by Bafilomycin A1 (Mizushima and Yoshimori, 2007; Klonksy et al., 2008).

**Sigma1 Ligand-Binding Assay.** Radioactive [123I]IPAG was synthesized essentially as described elsewhere (Kimes et al., 1992) and subsequently purified and confirmed against cold standard by high-performance liquid chromatography. Protein concentrations for immunoblotting of the Sigma1 binding assay were determined by modified Lowry assay, high-performance liquid chromatography. Protein concentrations for saturation binding assays were performed in triplicate and quantified as the mean number of GFP-LC3 puncta per cell

were generated using nonlinear regression analysis (Prism; GraphPad Software, Inc., San Diego, CA).

**Statistical Analysis.** Statistical significance was determined by one-way analysis of variance followed by Bonferroni’s post-test using Prism software (GraphPad).

**Results**

**Some Sigma1 Ligands Induce Autophagosome Formation and Autophagic Flux.** MDA-MB-468 and T47D breast adenocarcinoma cells, which natively express Sigma1 (herein referred to as Sigma1 ligands): IPAG, haloperidol, PRE-084, and (+)SKF10047. In all our experiments, we noticed that some Sigma1 ligands, the putative antagonists, decreased cell size by approximately 16 to 24 hours after treatment (Kim et al., 2012) (and shown for 24-hour treatment of MDA-MB-468 in Supplemental Fig. 1). In view of evidence that autophagy plays a role in cell growth (Hosokawa et al., 2006; Scott et al., 2007), we evaluated the potential involvement of this bulk sequestration and degradation process in Sigma1 ligand–mediated decrease in cell size.

Initially, we tested for the activation of autophagy using an established immunoblot-based assay to detect LC3 lipidation (Mizushima and Yoshimori, 2007; Klonksy et al., 2008). In these experiments, treatment with Sigma1 putative antagonists (IPAG, haloperidol) but not agonists [PRE-084, (+)SKF10047] converted LC3 to LC3II, an indication of LC3 lipid conjugation and autophagosome formation (Fig. 1G).

We confirmed these results with a widely used microscopy-based assay to visualize and quantify the translocation of an amino-terminal GFP-LC3 into vesicular structures that appear as GFP-concentrated puncta characteristic of autophagosome formation (Klonksy et al., 2008). Because transient transfections can produce spurious GFP-LC3 aggregates (Kuma et al., 2007; Klonksy et al., 2008), we generated stable GFP-LC3 transfected populations of MDA-MB-468 (GFP-LC3). These cells were treated for 24 hours with 10 μM Sigma1 putative antagonists (IPAG, haloperidol) and 10 or 50 μM putative agonists [PRE-084, (+)SKF10047—data shown for 50 μM of each], and were compared with basal (nontreated) and dimethylsulfoxide (DMSO) treated controls (Fig. 1, A and B). Autophagosome formation in MDA-MB-468 (GFP-LC3) cells was quantified as the mean number of GFP-LC3 puncta per GFP-positive cell. In the microscopy-based assay, we found that among the four ligands tested, only IPAG and haloperidol significantly induced the formation of autophagosomes (Fig. 1). Treatment with 10 μM IPAG or haloperidol for 24 hours resulted in 32 ± 2 and 29 ± 4 puncta per cell, respectively (Fig. 1B). Basal and DMSO-treated cells produced fewer than eight puncta per cell (Fig. 1). The putative agonists PRE-084, (+)SKF10047, and (+)pentazocine produced fewer than eight puncta per cell, at drug concentrations up to 50 μM (data shown for PRE-084 and (+)SKF10047 in Fig. 1), and thus did not statistically significantly differ from basal or DMSO control in this assay.

To determine whether Sigma1 antagonists induce autolysosomal degradation of cargo proteins (autophagic flux) (Klonksy et al., 2008), we used an immunoblot-based assay to detect and quantify LC3 degradation (Bernales et al., 2006; Hosokawa et al., 2006; Mizushima and Yoshimori, 2007; Klonksy et al., 2008).
We evaluated autophagic flux in Sigma1 ligand–treated cells by detecting the conversion of GFP-LC3 to the lipid-conjugated form GFP-LC3II, and subsequent cleavage of GFP-LC3II. In the presence of active autophagic degradation (flux), LC3 is degraded in the autolysosome, whereas cytoplasmic GFP, which is relatively resistant to degradation, is cleaved and released from LC3 and can be detected as free GFP by immunoblot analysis (Bernales et al., 2006; Hosokawa et al., 2006).

IPAG and haloperidol increased GFP-LC3II levels and cleaved GFP (Fig. 1C). The lower band immediately below the prominent GFP-LC3 band is GFP-LC3II, the lipid-conjugated form of GFP-LC3 (Bernales et al., 2006; Hosokawa et al., 2006). In contrast to Sigma1 putative antagonist-induced GFP-LC3II conversion and cleavage, neither the putative agonist PRE-084 nor (+)SKF10047 increased levels of GFP-LC3II or cleaved GFP at concentrations up to 50 μM (Fig. 1C). However, in the presence of a small molecule inhibitor of autophagic degradation, vacuolar type H⁺-ATPase inhibitor Bafilomycin A1 (Baf A1), the combination of Baf A1 with IPAG decreased GFP-LC3 cleavage and release of cleaved GFP, suggesting suppression of autophagic flux (Fig. 1, D–F). Moreover, a supplemental increase in GFP-LC3 cleavage in IPAG-treated cells (Fig. 1, D–F).

Finally, using the immunoblot-based LC3II conversion assay, we found that IPAG induces autophagy in at least five other cancer cell lines, including two breast adenocarcinoma, one prostate adenocarcinoma, one pancreatic adenocarcinoma, and one hepatocellular carcinoma cell line (Fig. 1H).
**Sigma1 Ligand-Induced Autophagy Requires Essential Autophagy Genes.** Autophagy is a specific, highly regulated process that requires a series of essential autophagy gene products (Levine and Klionsky, 2004; He and Klionsky, 2009). To confirm that GFP-positive puncta formation and degradation were indeed products of autophagy, we evaluated the effects of RNAi mediated knockdown of Beclin1 and ATG5, both essential autophagy proteins (Levine and Klionsky, 2004). Knockdown of Beclin1 statistically significantly inhibited puncta formation, decreasing the mean number of puncta per cell from 28 ± 3 to 6 ± 1 in IPAG-treated cells, an 80% inhibition of IPAG-induced GFP-LC3 puncta formation (P < 0.001) (Fig. 2, A–C). Knockdown of ATG5 inhibited IPAG-induced puncta formation by approximately 60%, decreasing the mean number of IPAG-induced puncta per cell from 33 ± 2 in control siRNA transfected cells to 14 ± 1 in cells in which ATG5 was knocked-down (P < 0.001) (Fig. 2, D–F).

**Sigma1 Ligand Disrupts Endoplasmic Reticulum Protein Homeostasis and Activates Stress-Associated UPR.** We subsequently asked whether treatment with the Sigma1 putative antagonist IPAG immediately induces autophagy or whether it is activated downstream of other cellular events. Sigma1 is highly enriched in the endoplasmic reticulum and has been recently described to function as a molecular chaperone at the ER-mitochondrion interface (Hayashi and Su, 2007). Therefore, we asked whether Sigma1 ligand treatment could disrupt ER protein homeostasis and induce ER stress. ER stress is commonly associated with the formation of unfolded and/or misfolded proteins, and thus activates stress response pathways including the UPR (Schroder and Kaufman, 2005; Xu et al., 2005; Ron and Walter, 2007). The UPR comprises several signaling pathways that increase the protein folding and processing capacity of the ER. The three most extensively investigated sensors that initiate the UPR—IRE1α, PERK, and ATF6—transduce signals to a cascade of effectors (Schroder and Kaufman, 2005; Xu et al., 2005; Ni and Lee, 2007; Ron and Walter, 2007). We assayed for the IRE1α-JNK1/2 and PERK-eIF2α-ATF4 components of the UPR as well as the UPR-associated ER chaperone GRP78/BiP as indicators of activated UPR (Schroder and Kaufman, 2005; Xu et al., 2005; Ogata et al., 2006; Ron and Walter, 2007; Ni and Lee, 2007).

Stress-induced p38MAPK is a downstream target of the IRE1α-TRAF2-ASK1 (inositol-requiring enzyme 1/tumor necrosis factor-associated receptor 2/apoptosis signaling-regulated kinase 1) signaling complex that is activated in response to ER stress and subsequently phosphorylates and enhances apoptosis (Xu et al., 2005; Szegedi et al., 2006; Ron and Walter, 2007; Kim et al., 2008). In addition, a recent report also has demonstrated a role for p38MAPK in the control of macroautophagy (Webber and Tooze, 2010).

We evaluated all of the above-mentioned markers of ER stress after treatment with increasing doses of Sigma1 ligands to compare UPR with the dose-responsive activation of autophagy, described earlier. We found that Sigma1 putative agonists activate the UPR in a dose-responsive manner (data shown for IPAG in Fig. 3). In contrast, Sigma1 putative agonists did not activate any of these markers (data not shown). Interestingly, the unfolded protein response to IPAG-induced endoplasmic reticulum stress (Fig. 3) occurs at lower doses than the autophagic response (Fig. 2). Indeed, treatment with 1 µM IPAG, a dose that does not produce autophagosomes, resulted in a salient and significant activation of at least seven markers of UPR (Fig. 3). Whereas the mean EC_{50} of LC3 lipid conjugation (i.e., LC3II induction) is 7 µM, the EC_{50} values for induction of ATF4, IRE1α, GRP78/BiP, and phosphorylation of eIF2α (Ser51), JNK (Thr183/Tyr185), and p38MAPK (Thr180/Tyr182) are 0.5, 0.9, 1.4, 2.3, 1.6, 1.7, and 0.5 μM, respectively. These mean values were generated from two independent determinations. Phosphorylated PERK, indicated by decreased electrophoretic mobility compared with nonphosphorylated PERK, could be detected after treatment with 1 µM IPAG (Fig. 3). Together, these data demonstrate that Sigma1 antagonist induction of UPR occurs at 3- to 14-fold lower concentrations than required for autophagosome formation (Fig. 3).

Next, we asked whether autophagy occurs before or after UPR. Cells were treated with 10 µM IPAG for 1, 6, 12, and 24 hours (Fig. 4). Of the seven ER stress and UPR markers evaluated in this experiment, salient induction of five was
detected by 1 hour of treatment, and three were clearly induced between 1 to 6 hours (Fig. 4, A–D). In contrast, significant formation of autophagosomes, measured by GFP-LC3 puncta and LC3II immunoblot, was detected between 6 to 12 hours (Fig. 4E). Clearly, UPR is induced before the detectable formation of autophagosomes.

Despite the activation of stress markers and increase in JNK phosphorylation, we observed no change in steady-state BCL2 protein levels up to 24 hours of treatment with 10 μM IPAG (Fig. 4F). However, when treatment was extended to 48 hours, we observed a marked decrease BCL2 (data not shown), consistent with the appearance of apoptotic cell death at this treatment time point (Fig. 7).

Finally, we found that IPAG induces UPR in a range of cancer cell lines, including at least two other breast adenocarcinoma (T47D and MCF-7), one prostate adenocarcinoma (PC3), one pancreatic adenocarcinoma (Panc1), and one hepatocellular carcinoma cell line (HepG2) (Fig. 4G).

**Sigma1 Ligand-Induced UPR and Autophagy Are Suppressed by Sigma1 RNAi.** To confirm that the Sigma1 ligand–induced UPR and autophagy are indeed Sigma1 mediated, we used siRNA to knockdown Sigma1 in MDA-MB-468(GFP-LC3) cells, and we evaluated IPAG-induced autophagy (Fig. 5). Significant Sigma1 knockdown was detectable >90 hours after transfection of Sigma1 selective siRNA, suggesting a stable, long protein half-life, consistent with previous reports (Hayashi and Su, 2007). We found that approximately 60% knockdown of Sigma1 resulted in a corresponding decrease of

---

**Fig. 3.** Dose-responsive induction of UPR by Sigma1 ligand. MDA-MB-468 cells were treated for 24 hours with increasing doses of IPAG (1 to 20 μM). (A) Induction of BiP protein levels. (B) Phosphorylation of p38MAPK (Thr180/Tyr182). (C) Induction of IRE1α protein levels and phosphorylation of JNK (Thr183/Tyr185). (D) Phosphorylation of PERK (phospho-PERK, P-PERK), induction of ATF4 protein levels, and phosphorylation of eIF2α (Ser51). (E) Induction of LC3II protein levels. (F) Immunoblot revealing Sigma1 protein levels. (G) Quantification of autophagosomes and UPR marker induction after 24-hour treatment with 1 μM IPAG. Data were generated from at least three independent determinations and are presented as the mean fold induction over DMSO-treated control. Error bars represent S.E.M. *P < 0.05; ***P < 0.001. BiP, GRP78/BiP.

**Fig. 4.** Time-course of Sigma1 ligand–induced UPR and autophagy. Time-course of Sigma1 antagonist–induced ER stress was evaluated by immunoblot analysis of UPR markers. Cells were treated for indicated times with 10 μM IPAG. (A) Induction of IRE1α protein levels and phosphorylation of JNK (Thr183/Tyr185). (B) Phosphorylation of PERK (phospho-PERK, P-PERK), induction of ATF4 protein levels, and phosphorylation of eIF2α (Ser51). (C) Phosphorylation of p38MAPK (Thr180/Tyr182). (D) Induction of GRP78/BiP (BiP) protein levels. (E) Time-action histogram of autophagosome formation in MDA-MB-468 (GFP-LC3). Data are representative of at least 10 fields and 300 cells for each drug concentration. P < 0.001 for 24-hour IPAG treatment compared with 0 (basal), 1, and 6 hours; P < 0.05 for 24-hour compared with 12-hour IPAG treatment. (F) Immunoblot revealing BCL2 protein levels. (G) Treatment of the following cell lines for 24 hours with IPAG (10 μM) or PRE-084 (50 μM) and immunoblot of detergent soluble whole-cell lysates with BiP antibody: breast adenocarcinoma (MCF-7, T47D), prostate adenocarcinoma (PC3), hepatocellular carcinoma (HepG2), and pancreatic adenocarcinoma (Panc1).
[125I]IPAG binding in a radioligand binding saturation assay, decreasing maximal IPAG binding (B_max) from 1837 fmol/mg to 748 fmol/mg, demonstrating the selectivity of the siRNA knockdown and of IPAG (Fig. 5A). In our functional assays we were able to knockdown Sigma1 to approximately 20% of basal levels (Fig. 5, B–D). Consistent with Sigma1-mediated effects of IPAG, we found that approximately 80% knockdown of Sigma1 resulted in suppression of IPAG-induced UPR, evaluated by induction GRP78/BiP, IRE1α, and ATF4 protein levels (Fig. 5B).

We subsequently evaluated autophagosome formation (GFP-LC3 puncta). Knockdown of Sigma1 alone did not induce the formation of autophagosomes in the absence of Sigma1 ligand: 6 ± 2 puncta per cell compared with 7 ± 3 puncta per cell in control siRNA-transfected cells (Fig. 5, C and D). Treatment with 10 μM IPAG resulted in 28 ± 4 puncta per cell in control siRNA-transfected cells and a significant inhibition to 10 ± 2 puncta per cell in Sigma1-knockdown cells (Fig. 5, C and D). Together, these data demonstrate that Sigma1 is required for both IPAG-induced UPR and autophagy.

Inhibition of UPR Suppresses Sigma1 Ligand–Induced Autophagy. The results of our dose-response and time-action experiments suggest that ER stress-induced UPR is engaged upstream of autophagy; however, they do not demonstrate that ER stress is required to activate autophagy. Therefore, we subsequently tested the effects of inhibiting the ER stress response to Sigma1 antagonist treatment. To confirm that UPR precedes and is required for Sigma1 antagonist-induced autophagy, we inhibited UPR by siRNA-mediated knockdown of IRE1α or ATF4. In these experiments, after transfection of siRNA, MDA-MB-468 cells were treated for 24 hours with 10 μM IPAG (Fig. 6). Knockdown of IRE1α resulted in decreased autophagosome formation (Fig. 6, A, C, and D). The number of GFP-positive puncta per cell decreased from 24 ± 2 and 33 ± 2, respectively, when treated with IPAG or haloperidol alone to 9 ± 2 and 13 ± 2, respectively, when IRE1α was knocked down (Fig. 6, C and D). When siRNA was used to knockdown ATF4, IPAG or haloperidol treatment produced 5 ± 1 and 15 ± 2 GFP-positive puncta per cell, respectively (data

**Fig. 5.** Sigma1 ligand–mediated UPR and autophagy are Sigma1 dependent. MDA-MB-468 cells were treated for 24 hours with 10 μM IPAG, 90 hours after two transfections with either control or Sigma1 siRNA. (A) [125I]IPAG radioligand binding saturation curves in control siRNA transfected (solid line, closed squares) and Sigma1 knockdown (dashed line, open circles) cell membranes. Data are presented as mean ± S.D. and are representative of three determinations. Immunoblot confirming siRNA-mediated knockdown of Sigma1 is shown above the graph. (B) Immunoblot confirming siRNA-mediated knockdown of Sigma1 post-transfection and treatment in MDA-MB-468 cells and detection of Sigma1 and UPR markers GRP78/BiP (BiP), IRE1α, and ATF4. Immunoblot is representative of three determinations. (C and D) GFP-LC3 puncta formation in MDA-MB-468(GFP-LC3) cells treated for 24 hours with 10 μM IPAG after siRNA-mediated knockdown of Sigma1. Immunoblot of Sigma1 knockdown and representative fluorescent micrograph images are shown in C and GFP-LC3 puncta quantified in D.

**Fig. 6.** Inhibition of Sigma1 ligand–mediated UPR inhibits autophagy. (A and B) Immunoblot of IRE1α siRNA knockdown in A and ATF4 siRNA knockdown in B. 72 hours after transfection, MDA-MB-468(GFP-LC3) cells were treated for 24 hours with a combination of IPAG (10 μM). Knockdown of IRE1α or ATF4 abrogates IPAG-mediated induction of GFP-LC3 cleavage. (C and D) siRNA mediated knockdown of IRE1α and ATF4 abrogate GFP-LC3 puncta formation. Representative images in C and quantified in D.
shown for IPAG in Fig. 6, B–D). Together, these data suggest that Sigma1 ligand–induced autophagy occurs via UPR activation.

**Inhibition of Sigma1 Ligand–Induced UPR and Autophagy Accelerate Apoptotic Cell Death.** These results suggest that UPR and autophagy may function as primary and secondary survival responses, respectively, to Sigma1 ligand–induced ER stress. Previous studies with tunicamycin and thapsigargin demonstrated that ER stress can lead to autophagy as a survival response (Ogata et al., 2006; Yorimitsu et al., 2006). After 24 hours of treatment with IPAG and haloperidol, ligand-induced MDA-MB-468 cell death was not statistically significantly different than untreated (basal) or DMSO-treated controls, with 10% ± 3% and 7% ± 2% dead cells per well, respectively, compared with 9% ± 2% dead cells in DMSO-treated control samples (data for IPAG shown in Fig. 7). DMSO-treated control cell death was not significantly different than untreated or basal cell death rates. However, after 48 hours of continuous treatment, a significant percentage of IPAG treated cells begin to undergo apoptotic cell death, with an average of 30% ± 2% of the cells dying under these conditions (Fig. 7, A and B). By 72 hours of treatment with IPAG, >75% of cells died.

Consistent with this pattern, whereas control siRNA transfected cells survived 24 hours of IPAG and haloperidol treatment, with 9% ± 4% and 7% ± 2% dead cells per well, respectively, inhibition of UPR by IRE1α knockdown potentiated IPAG and haloperidol induced cell death with 47% ± 8% and 61% ± 8% dead cells, respectively, after 24 hours of Sigma antagonist treatment (Fig. 7, C and D). Knockdown of ATF4 also potentiated IPAG- and haloperidol-induced apoptosis, with 30% ± 9% and 53% ± 11% dead cells per well, respectively, whereas ATF4 knockdown alone did not significantly alter cell-death rates, with 7% ± 2% dead cells per well (data not shown). Thus, inhibition of UPR by siRNA knockdown of IRE1α or ATF4 abrogates autophagosome formation (Fig. 6) and potentiates Sigma1 antagonist-mediated apoptotic cell death (Fig. 7; data shown for IRE1α).

To determine whether Sigma1 ligand–induced autophagy functions as a cell death or survival pathway, or whether autophagy is simply associated with Sigma1 ligand–induced cell death, we evaluated the effects of inhibiting autophagy by siRNA mediated Beclin1 or ATG5 knockdown. Whereas treatment with 10 μM IPAG for 24 hours does not induce significant cell death (5% ± 3%), with no evidence of apoptosis, inhibiting autophagosome formation by siRNA knockdown of Beclin1 or ATG5 results in cell death at 24 hours of IPAG treatment (Fig. 7; Supplemental Fig. 2, respectively).

**Sigma1 Ligand–Induced UPR and Autophagy Are Reversible.** Interestingly, removal of Sigma1 ligand before activation of cell death pathways results in a rapid disappearance of autophagosomes (Fig. 8, A–C). Thus, Sigma1 ligand–induced autophagy is rapidly reversible if it is removed before progression beyond an irreversible cytoprotective threshold. Whether our observations reflect a sequestration and subsequent recycling after removal of drug or degradation of autophagosome
cargo remains unclear. The absence of cell death during this treatment period was confirmed by trypan blue exclusion assay (Fig. 8E).

These data suggest that constant treatment, and presumably constant ligand-receptor binding, is required for antagonist-induced stress response and autophagy. Upon removal (“washout”) of IPAG from the cell culture medium, UPR markers returned to basal levels at various time frames: within 1–6 hours for PERK, phospho-eIF2α, and phospho-p38MAPK; 6–24 hours for ATF4; and 24–48 hours for IRE1α and GRP78/BiP (Fig. 8D). Thus, UPR is engaged before autophagy and remains active for several hours after autophagy subsides, suggesting that UPR may act to maintain ER homeostasis at lower intensity of stress.

The activation of these signaling pathways, and likely others, suggests an integrated response to Sigma1 ligand–induced ER stress. Furthermore, the reversibility of autophagy and UPR, subsequent to washout of IPAG, supports the notion that these are cytoprotective responses.

**Discussion**

A growing number of reports suggest that autophagy may be part of an integrated response to cellular stress (Levine and Klionsky, 2004; Hoyer-Hansen and Jaattela, 2007; Ron and Walter, 2007; Kim et al., 2008; He and Klionsky, 2009). Our findings suggest a role for Sigma1 in this process. Here, we demonstrate that high-affinity Sigma1 ligands induce ER stress and subsequent UPR (Figs. 3 and 4). Severe or prolonged ligand-induced ER stress appears to overwhelm the cytoprotective, adaptive capacity of the UPR, and the cell subsequently engages autophagy. RNAi knockdown of Sigma1 suppresses IPAG-induced UPR and autophagy (Fig. 5), suggesting that Sigma1 is the principal mediator of these ligand-induced ER stress responses.

Treatment with two Sigma1 putative antagonists (IPAG, haloperidol) resulted in the activation of UPR and autophagosome formation whereas putative agonists [PRE-084, (+)SKF10047] did not induce either. Our results are consistent with published data demonstrating that putative antagonists mediate cell death whereas putative agonists do not elicit a detectable cytotoxic response (Spruce et al., 2004). One possible explanation for the absence of putative agonist effect may be the predominance of Sigma1 in a constitutive agonist conformation in cancer cells. Alternatively, because IPAG- and haloperidol-associated autophagosome formation is not blocked by PRE-084 and (+)SKF10047 (Kim FJ, Schrock JM, Stabler SM, Pasternak GW, unpublished observations), it is possible that putative antagonists and agonists bind to distinct Sigma1 populations within the cell, or it is possible that they bind to distinct regions of Sigma1 and thereby elicit different effects even when bound to the same protein.

Interestingly, it appears that binding affinity does not necessarily predict drug actions: ligands with similar affinities elicit distinct responses (Fig. 1). Among the ligands used here, we find that IPAG binds Sigma1 with high affinity, \( K_d 3 \text{nM} \)
PRE-084 and (±)SKF10047 bind Sigma1 with affinities (K_i values) of 2 and 40 nM, respectively (Narayanan et al., 2011). However, only IPAG and haloperidol elicit UPR and autophagy.

Although the pharmacologic properties of these prototypic compounds have been described in considerable detail (Spruce et al., 2004; Hayashi and Su, 2008; Su et al., 2010), the molecular mechanisms of Sigma1 ligand actions are largely unknown, and intracellular signaling pathways activated in response to ligand treatment are not well defined. Furthermore, although PRE-084 and (±)SKF10047 do not alter cell proliferation or survival, some putative agonists such as 4-IBP [N-(N-benzylpiperidin-4-yl)-4-iodobenzamide] have been reported to have cytostatic properties as well as to sensitize cancer cells to proapoptotic and proautophagic drugs (Megalizzi et al., 2007, 2009). Because the functional domains that mediate Sigma1 actions have not been clearly identified, it remains difficult to use biochemical approaches to determine how putative agonist and antagonist binding specifically modulate Sigma1 functions. Indeed, the agonist and antagonist designations of many Sigma1 compounds are based primarily on data from rodent behavior assays (Su et al., 2010). Thus, the difference between putative agonist and putative agonist responses remains unresolved at the molecular and cellular level.

It is noteworthy that Sigma1 does not have the properties of a traditional receptor, and thus designation of Sigma1 selective compounds as classically defined receptor antagonist or agonist may be inaccurate. We propose that structure-activity relationship studies to identify chemotypes with Sigma1-mediated ER stress response-inducing activities, regardless of antagonist or agonist designation, should be particularly informative in understanding the utility of Sigma1 ligands as cancer therapeutics.

Of the two proposed sigma receptor subtypes, the sigma1 receptor (Sigma1) has been cloned and widely characterized, and the sigma2 receptor subtype has remained a pharmacologically defined entity until recently. Xu et al. (2011) have identified the PGRMC1 protein complex as the putative sigma2 receptor. A growing body of evidence demonstrates the antineoplastic activities of compounds with affinity for sigma2 receptor as well as Sigma1, and many ligands bind both Sigma1 and the sigma2 receptor with varying affinities for each subtype (Crawford and Bowen, 2002; Spruce et al., 2004; Su et al., 2010; Narayanan et al., 2011). However, here we find that knockdown of PGRMC1 with siRNA does not alter IPAG induction of GRP78/BiP (marker of UPR) and has no effect or a modest effect on LC3II levels (marker of autophagosome formation), in further support of Sigma1 as the principal mediator of these responses in our model (Supplemental Fig. 3). Therefore, Sigma1 and sigma2 receptor ligand–mediated proliferation arrest and cell death of transformed cells may engage distinct cellular pathways.

Whether Sigma1 ligand–induced ER stress is a direct or indirect effect is also unclear. Either the proteins associated with Sigma1 are directly altered by ligand binding, causing deleterious protein folding in other processing (directly implicating Sigma1 as a chaperone or as a molecular scaffold), or Sigma1 ligand binding may indirectly cause protein-folding defects leading to the subsequent activation of ER stress response pathways. For example, altered ER calcium levels can lead to protein misfolding and subsequent UPR (Schröder and Kaufman, 2005; Xu et al., 2005; Hoyer-Hansen and Jaattela, 2007; Ron and Walter, 2007). Treatment with some Sigma1 ligands has been shown to modulate cytosolic calcium levels (Brent et al., 1996; Hayashi and Su, 2007). Recent evidence (Brinson et al., 2011) demonstrates that IPAG-induced intracellular calcium release occurs at concentrations that clearly exceed the UPR and autophagy activating concentrations presented herein (Figs. 1, 3, and 4). Therefore, it remains to be determined whether Sigma1 ligand–induced ER stress and UPR are caused by ER calcium release, or whether ER calcium release occurs as a result of Sigma1 ligand binding–induced protein-folding defects.

Regarding the rapid kinetics of Sigma antagonist actions upon washout of drugs, we propose at least two possibilities: 1) Sigma1 ligand–induced stress requires continuous ligand–receptor binding to continuously inhibit basal chaperone–protein associations; or 2) continuous Sigma1 ligand binding maintains chaperone–protein associations that sustain ER stress response. No evidence of either has been reported; however, the rapid reversibility of Sigma1 ligand–induced autophagy and the rapid disappearance of autophagosomes upon removal of drugs suggests that continuous treatment, and presumably constant intracellular IPAG binding, is necessary to sustain disruption of ER protein homeostasis. The availability of sufficient free compound within the cell to act on intracellular targets may also explain why concentrations of IPAG significantly higher than its binding affinity (K_i) are required for the cellular responses observed here and in the literature. This notion, applied to other compounds, is reviewed in detail elsewhere (Trainor, 2007).

Cytoprotective UPR and autophagy reach maximal levels in a dose- and time-responsive manner (Figs. 1, 3, and 4). Apoptotic cell death occurs when these levels are reached and sustained, presumably beyond the cellular capacity to suppress proteotoxic stress. Consistent with the notion that autophagy is a survival response to Sigma1 ligand–induced ER stress, siRNA-mediated knockdown of Beclin1 or ATG5 inhibits IPAG-induced autophagosome formation and facilitates apoptotic cell death (Figs. 2 and 7; Supplemental Fig. 2). Both Beclin1 and ATG5 siRNA-mediated knockdown suppressed Sigma1 ligand–induced GFP-positive puncta, demonstrating that components canonically associated with autophagosome formation are involved in Sigma1-mediated autophagic sequestration. However, whether Sigma1 ligand–induced autophagy corresponds with macroautophagy or a novel ER–associated sequestration and bulk degradation mechanism remains to be determined. The link between UPR and autophagy has been demonstrated using thapsigargin–induced ER stress (Ogata et al., 2006; Yorimitsu et al., 2006). However, we find that siRNA knockdown of Sigma1 does not inhibit thapsigargin–induced UPR and autophagy (Supplemental Fig. 4). This suggests that ER stress-mediated autophagosome formation may vary according to the effector used to disrupt ER homeostasis.

Interestingly, the dose-response and time-action curves reveal that at lower drug concentrations, IPAG activates the UPR but does not induce autophagy (Figs. 3 and 4). Furthermore, at these doses it does not induce cell death. This suggests that below threshold stress levels the UPR may be sufficient to preserve cell survival, and that lethal cytotoxicity occurs when Sigma1 ligand treatment exceeds these thresholds. As Sigma1
is enriched in the ER and its putative antagonists activate UPR and autophagy, our data suggest that Sigma1 functions as a regulatory component of ER protein homeostasis or proteostasis. Thus, these Sigma1 ligands are reminiscent of so-called proteostasis regulators that modulate ER protein folding capacity by coordinating the transcription and translation of chaperones that facilitate proper protein folding and transport in the ER (Mu et al., 2008).

Together, these findings point to the potential utility of novel chemotherapeutic drug combinations that include Sigma1 ligands in combination therapies designed to modulate protein synthesis, processing, assembly, and degradation, without necessarily inducing cell death. These data suggest that Sigma1 ligand doses, treatment timing, and combinations can be controlled to increase the therapeutic utility of these small molecules beyond their use as cytotoxic agents. We believe that rationally designed Sigma1 ligand–based therapies will require a better understanding of the molecular mechanisms that govern Sigma1 regulation of cellular stress and homeostasis.

Acknowledgments
The authors thank Dr. Francis Weiss-Garcia (MSKCC Monoclonal Antibody Core Facility) for assistance in generating the Sigma1 antibody, and Dr. Paul Campbell for critical reading of the manuscript. The authors also thank members of the Kim laboratory for technical assistance.

Authorship Contributions
Participated in research design: Kim, Schrock, Stabler, Spino, Longen, Marino.

Performed data analysis: Kim, Schrock.

Wrote or contributed to the writing of the manuscript: Kim, Schrock, Pasternak.

References

Vilner BJ, John CS, and Bowen WD (1995b) Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell lines. Cancer Res 55:408–413.

Address correspondence to: Dr. Felix J. Kim, Department of Pharmacology and Physiology, Drexel University College of Medicine, 245 North 15th St., Philadelphia, PA 19102-1192. E-mail: fkim@drexelmed.edu