GX15-070 (Obatoclax) Induces Apoptosis and Inhibits Cathepsin D- and L–Mediated Autophagosomal Lysis in Antiestrogen-Resistant Breast Cancer Cells

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Abstract

In estrogen receptor–positive (ER+) breast cancer cells, BCL2 overexpression contributes to antiestrogen resistance. Direct targeting of the antiapoptotic BCL2 members with GX15-070 (obatoclax), a BH3-mimetic currently in clinical development, is an attractive strategy to overcome antiestrogen resistance in some breast cancers. Recently, GX15-070 has been shown to induce both apoptosis and autophagy, yet the underlying cell death mechanisms have yet to be elucidated. Here, we show that GX15-070 is more effective in reducing the cell density of antiestrogen-resistant breast cancer cells versus sensitive cells and that this increased sensitivity of resistant cells to GX15-070 correlates with an accumulation of autophagic vacuoles. Formation of autophagosomes in GX15-070-treated cells was verified by changes in expression of the lipidation of microtubule-associated protein-1 light chain-3 and both confocal and transmission electron microscopy. While GX15-070 treatment promotes autophagic vacuole and autolysosome formation, p62/SQSTM1, a marker for autophagic degradation, levels accumulate. Moreover, GX15-070 exposure leads to a reduction in cathepsin D (CTSD) and L (CTSL1) protein expression that would otherwise digest autolysosome cargo. Thus, GX15-070 has dual roles in promoting cell death: (i) directly inhibiting antiapoptotic BCL2 family members, thereby inducing apoptosis; and (ii) inhibiting downstream CTSD and CTSL1 protein expression to limit the ability of cells to use degraded material to fuel cellular metabolism and restore homeostasis. Our data highlight a new mechanism of GX15-070-induced cell death that could be used to design novel therapeutic interventions for antiestrogen resistant breast cancer. Mol Cancer Ther; 12(4); 448–59. ©2013 AACR.

Introduction

Approximately two thirds of newly diagnosed invasive breast tumors express the estrogen receptor-α (ER) protein (ER-positive; ER+; ref. 1) and most will be treated with an endocrine therapy such as an antiestrogen or aromatase inhibitor. Antiestrogens can inhibit ER function and/or expression, blocking the ER-regulated signaling that induces breast cancer cell survival and proliferation (2). Tamoxifen (TAM), a selective ER modulator (SERM), is the most frequently prescribed antiestrogen and is effective in increasing overall survival and reducing the incidence of ER+ disease in high-risk women (3). The selective ER downregulator (SERD) fulvestrant [Faslodex, ICI182780 (ICI)], does not exhibit the partial agonist activities of some SERMs and is often an effective treatment option following relapse on tamoxifen or an aromatase inhibitor (4, 5). Despite the widespread clinical efficacy of antiestrogens in the treatment of ER+ breast cancers, approximately half of these women will exhibit de novo or acquired resistance to endocrine therapies (6).

Breast cancer cells can acquire resistance to antiestrogens through changes in molecular signaling that affect cell proliferation and death. The B-cell lymphoma 2 (BCL2) gene family encodes central regulatory proteins with both antiapoptotic (BCL2, BCLW, BCL-xL, MCL1, and A1) and proapoptotic functions (BAX, BAK, and BH3-only proteins; ref. 7). BH3-only members interact with the core antiapoptotic BCL2 proteins to promote apoptosis by activating BAX and/or BAK, which leads to downstream cytochrome c release (8). BCL2 also interacts with beclin-1 (BECN1), a critical regulator of autophagy that facilitates autophagosome production (9). Before systemic therapy, BCL2 overexpression often correlates with ER+ and is usually a favorable prognostic indicator (10). However, BCL2 levels decrease in
tumors that respond to 3 months of tamoxifen therapy, whereas BCL2 expression is high in tumors that remain 3 months after tamoxifen (11, 12). Thus, targeting the antiapoptotic BCL2 family members may be a useful strategy to overcome antiestrogen resistance in some breast cancers.

Recently, a new series of small molecules that mimic BH3-only proteins have been generated, constituting a new class of potentially useful drugs. By mimicking BH3-only proteins (such as NOXA, PUMA, BID, BAD, and BIM), antiapoptotic BCL2 members can be sequestered, thus, allowing BAK and BAX to activate the intrinsic apoptotic pathway. Among the BH3-mimetics, GX15-070 (GX; obatoclax) is an indole bipyrrole compound that can inhibit all known prosurvival BCL2 family members (13, 14). GX15-070 is currently under investigation in phase II clinical trials for the treatment of leukemia, lymphoma, myelofibrosis, and mastocytosis (15, 16). Previous studies report that GX15-070 overcomes resistance to lapatinib, a tyrosine kinase inhibitor often used in HER2-amplified breast cancer (17). While GX15-070 can induce mitochondrial apoptosis (18), the precise molecular mechanisms of cell death by GX15-070 is unclear. Several reports suggest that GX15-070 may induce autophagy and other forms of death as an alternate mechanism to caspase-dependent apoptosis (17, 19, 20).

We examined the effects of GX15-070 and an antiestrogen using two separate models of antiestrogen resistance in breast cancer (21). Comparisons were made between MCF7 (ER+, estrogen-dependent, antiestrogen-sensitive) and MCF7/RR (ER+, estrogen-independent, tamoxifen-resistant; ICI182780-sensitive, derived from MCF7 cells selected against ICI182780; ref. 24) cells. In this report, we show that inhibiting antiapoptotic BCL2 family expression with GX15-070 induces cell death in antiestrogen-resistant breast cancer cell lines through the completion of apoptosis and a cathepsin-mediated inhibition of autophagy. These findings have important clinical implications and provide a mechanistic rationale for the use of GX15-070 in combination with an antiestrogen for the treatment of ER+ breast cancers.

**Materials and Methods**

**Cell culture and reagents**

MCF7 human breast cancer cells were provided by Dr. Marvin Rich (Karmanos Cancer Institute, Detroit, MI); MCF7/RR, LCC1, and LCC9 cells were established as previously described (21, 22, 24). MCF7 and MCF7/RR cells were cultured in improved Minimal Essential Media (IMEM; Invitrogen) with phenol red and supplemented with 5% FBS. LCC1 and LCC9 cells were routinely grown in phenol red–free IMEM supplemented with 5% charcoal-stripped calf serum. Cells were authenticated by DNA fingerprinting and tested regularly for *Mycoplasma* infection. GX15-070 was purchased from Selleck Chemicals; ICI182780 (Faslodex; fulvestrant) and Z-VAD-FMK from Tocris Bioscience; Bafilomycin A1 (BAF) from EMD Biosciences; 4-hydroxytamoxifen (tamoxifen), hydroxychloroquine (HCQ), BAPTA-AM, and 3-methyladenine (3-MA) were from Sigma-Aldrich. The cathepsin L (CTSL1; 1-naphthalenesulfonyl-Ile-Trp-aldehyde) and D (Ac-Leu-Val-Phe-aldehyde) inhibitors were from Enzo Life Sciences and Bachem, respectively.

**Cell proliferation**

Cells were seeded at a density of 5 × 10^3 per well in 96-well plates and, 24 hours later, treated with the indicated concentration of GX15-070 or vehicle control. Cells were incubated with drug for 48 hours or 6 days, with media containing either drug or vehicle being replaced every 3 days. Following treatment, cells were stained with a crystal violet staining solution as previously described (25). Sodium citrate buffer was used to extract the dye, and absorbance was measured at 550 nmol/L using a microplate reader (Bio-Rad). Cell density was calculated from the crystal violet assay.

**RNA interference**

ATG7 and BECN1 siRNA were from Cell Signaling and Origene, respectively. Cells were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions.

**Western blot analysis**

LCC1, LCC9, MCF7, and MCF7/RR cells were plated in 6-well dishes and the following day treated with 100 nmol/L GX ± 100 nmol/L ICI/TAM, 100 nmol/L ICI/TAM, or vehicle control. Lysates were harvested 48 hours later for protein analysis as previously described (3). Protein expression was measured by probing proteins with the following antibodies overnight at 4°C: ATG7, BECN1, CTSB, CTS1, LCC3B, PARP (Cell Signaling); p62 (BD Transduction Labs); BCL2 (Enzo Life Sciences); and CTSL1 (eBioscience). To confirm equal loading of the gels, membranes were reprobed for β-actin (Santa Cruz Biotechnology).

**Reverse transcription PCR**

RNA was extracted using TRIzol (Invitrogen). Two microgram RNA was used from each sample as a template for cDNA synthesis with the High Capacity RNA-to-cDNA Kit (Invitrogen). PCR amplification was conducted using the following primers (purchased from Integrated DNA Technologies): cathepsin B (CTSB): 5'-GCCGCC-GAGCTCATGTGGCAGCTGGCGCTCC-3' (forward) and 5'-ATTATCCCGGGTTAGACGTCCCTTCCCACTAC-TG-3' (reverse), cathepsin D (CTSD): 5'-GTGCTG-CCAGTCAGCCTCGTCAGCA-3' (forward) and 5'-CCTGC-TCAGGTAGAAGGAGAAGATG-3' (reverse), and CTSL1:
AAGGACTCATGACCTGCATCAA-3' (forward) and 5'-AAGGACTCATGACCTGCATCAA-3' (reverse).

Apoptosis and autophagosome formation assays

Cells were plated in 6-well tissue culture plates 1 day before treatment with 100 nmol/L GX ± 100 nmol/L ICI/TAM, 100 nmol/L ICI/TAM, or vehicle control. Forty-eight hours posttreatment, cells were harvested and stained as described in the Enzo Life Sciences Annexin V–FITC Apoptosis Detection Kit for flow cytometry. Accumulation of autophagic vesicles was measured using a modified monodansylcadaverine cytometry. Forty-eight hours posttreatment, cells were harvested before treatment with 100 nmol/L GX/C6

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Autophagosome maturation

LCC9 cells (1 × 10^5) were seeded onto 18 mm × 18 mm glass coverslips and 24 hours later were transfected with LC3 tagged with a GFP and/or a p62 cDNA tagged with a red fluorescent protein (RFP). An mRFP-GFP tandem fluorescent-tagged LC3 vector (Addgene) was used to assess whether GX15-070 inhibited autophagic flux (26). The following day, cells were treated with 500 nmol/L GX15-070, 100 nmol/L ICI182780, or 5 nmol/L BAF in CCS-IMEM. The pH of autolysosomes was measured in LCC9 cells using the LysoSensor Green dye (Invitrogen) as indicated by the supplier. Twenty-four hours posttreatment, cells were fixed, mounted on coverslips, and visualized as previously described (27).

Orthotopic xenografts in athymic nude mice

LCC1 or LCC9 cells were injected orthotopically into the mammary fat pads of 5-week-old ovariectomized athymic nude mice as previously described (28). Mice were sacrificed after 9 weeks; tumors were removed at necropsy, fixed in neutral buffered formalin, and processed using routine histologic methods.

Immunohistochemistry

Five-micrometer sections from LCC1 and LCC9 paraffin-embedded tissues were stained with mouse anti-BCL2 (Dako, 1:150) antibody as previously described (28). A computer-assisted counting technique with a grid filter to select cells was used to quantify the immunohistochemical staining of BCL2 (29).

Electron microscopy

Following 24 hours of treatment with 500 nmol/L GX15-070 or vehicle control, LCC9 cells were harvested and fixed in a glutaraldehyde/paraformaldehyde solution. Embedding sectioning and staining were carried out as previously described (30). After fixation, cells were double stained with uranyl acetate and lead citrate. Electron micrographs of ultrathin sections (90 nm) were viewed at a magnification of ×10,000/×15,000 with a JEOL JM1010 transmission electron microscope.

Cathepsin activity

Cathepsin activity was determined using the commercial assay provided by Biovision according to the manufacturer’s protocol. Cells were seeded in 10 cm² dishes 24 hours before treatment with 100 nmol/L GX ± 100 nmol/L ICI, 100 nmol/L ICI182780, or vehicle control. Forty-eight hours posttreatment, cathepsin activity was measured using 10 mmol/L CTSB (Ac-RR-AFC) or L substrate (Ac-FR-AFC). A fluorometer (Tecan) was used to quantify the cleavage of synthetic substrate of CTSB and CTSL1. Cathepsin activity was expressed as relative fluorescence units (RFU) per microgram protein.

Statistical analysis

The statistical significance of differences between 2 groups was analyzed by 2-tailed Student t tests. For multiple group comparisons, Bonferroni multiple comparison test was applied following one-way ANOVA. Results were considered to be significantly different at P < 0.05. Statistical analysis was carried out using the Prism version 5.0 software.

Results

GX15-070 alone and in combination with an antiestrogen inhibits breast cancer cell density

Endogenous BCL2 protein expression was measured in MCF7, MCF7/RR, LCC1, and LCC9 breast cancer cells (Fig. 1A). Increased BCL2 expression was observed in estrogen-regulated MCF7 cells and estrogen-independent, antiestrogen-resistant LCC9 cells (Fig. 1A). We also measured total BCL2 protein by immunohistochemistry in LCC1 and LCC9 mammary tumor xenografts. Data from these studies revealed that LCC9 tumors had significantly higher BCL2 expression compared with LCC1 tumors (Supplementary Fig. S1A and S1B; P = 0.0005). Given that GX15-070 is a BH3 mimic that inhibits all known antiapoptotic BCL2 members (structure shown in Fig. 1B), we sought to determine the effect of GX15-070 on antiestrogen-sensitive and -resistant breast cancer cells. Increasing concentrations of GX15-070 (0, 0.1, 0.5, 1, and 10 µmol/L) inhibited both antiestrogen-sensitive (LCC1 and MCF7) and -resistant (LCC9 and MCF7/RR) cell density after 48 hours (Fig. 1C). When combined with the antiestrogen tamoxifen over the course of 6 days, GX15-070 had an additive effect on inhibiting the relative cell density of MCF7 and MCF7/RR cells (Fig. 1D), where-as no significant additive effect was observed in the LCC1 and LCC9 cells with GX+i-ICI (Fig. 1D). We also measured Annexin V–stained cells following GX15-070 exposure and observed that GX15-070 enhanced apoptosis consistent with its on-target effects (Supplementary Fig. S2A and S2B). These data suggested that GX15-070 enhanced apoptosis-mediated cell death in antiestrogen-sensitive and -resistant breast cancer cells.
GX15-070 induces BECN1-dependent autophagosome formation, but autophagy is not required for GX15-070 toxicity

While GX15-070 enhanced apoptosis, the pan-caspase inhibitor Z-VAD-FMK failed to abrogate killing by GX15-070 ± an antioestrogen (Supplementary Fig. S3). Furthermore, Z-VAD-FMK failed to reduce the number of apoptotic cells following GX15-070 exposure, suggesting that GX15-070-mediated apoptosis occurred via a caspase-independent mechanism (Supplementary Fig. S3). Because cell viability was not affected by a pan-caspase inhibitor and because others have linked autophagic cell death to GX15-070, we determined whether GX15-070 either alone or in combination with an antioestrogen could induce autophagosome formation in breast cancer cells. We measured the initial phase of autophagy in response to GX15-070 ± antioestrogen treatment by selectively labeling autophagosomes with a modified monodansylcadaverine. GX15-070 alone and in combination with an antioestrogen significantly increased the number of autophagic vacuoles in antioestrogen-sensitive and -resistant cell lines (Fig. 2A; *P* < 0.001).

We next explored the effect of GX15-070 on autophagy regulation by measuring the formation of LC3-II, which
participates in elongation of the autophagosome membrane (31). In LCC9 cells, 48 hours of GX15-070 ± an antiestrogen significantly induced the formation of LC3-II (Fig. 2B; \( P \approx 0.0005 \)). When autophagy was blocked by RNA interference (RNAi)–targeting BECN1, a critical regulator of autophagy (31), LC3-II cleavage was significantly reduced (Fig. 2B; \( P \approx 0.0005 \)). In contrast, the increase in autophagosome formation observed after GX15-070 exposure was not suppressed by the addition of RNAi-targeting ATG7, a protein necessary for the formation of the preautophagosomal structure (ref. 31; Fig. 2B). To assess whether GX15-070-induced autophagosome formation was calcium-dependent, LCC9 cells were transfected with a GFP-tagged version of LC3, and the following day treated with 500 nmol/L GX15-070 and 10 μmol/L of the calcium chelator BAPTA-AM. Formation of LC3-GFP aggregates increased with GX15-070 and BAPTA + GX treatment, suggesting that GX15-070-mediated LC3 punctae formation was largely independent of calcium signaling (Fig. 2C).
To determine whether inhibition of autophagy could suppress GX15-070-mediated cell death, we used several autophagy inhibitors in combination with GX/C6 ICI. LCC1 and LCC9 cells were treated with 500 nmol/L GX/C6, 100 nmol/L ICI182780, or a combination of ICI182780, GX15-070, and one of the autophagy inhibitors, 3-MA (5 mmol/L; early-stage inhibitor), HCQ (10 μmol/L; late-stage inhibitor), or BAF (5 nmol/L; late-stage inhibitor). 3-MA slightly suppressed activity of the drug combination in LCC1 (P = 0.04) but not LCC9 cells (Fig. 2D). Because 3-MA inhibits autophagy by blocking autophagosome formation via inhibition of type III phosphoinositide 3-kinase (PI3K), these data suggested that GX15-070-induced autophagy was independent of PI3K. BAF, which results in the accumulation of autophagosomes, promoted drug combination lethality in LCC1 and LCC9 cells (Fig. 2D; P = 0.0006 and 0.003, respectively), whereas HCQ+GX+ICI reduced cell viability equally to GX+ICI (Fig. 2D). Despite BECN1-dependent LC3-II processing following GX15-070 exposure, GX15-070 reduced equally the cell density of LCC9 cells transfected with control or BECN1 siRNA (Supplementary Fig. S4).

GX15-070 induces autophagic vacuole and lysosome formation in antiestrogen resistant LCC9 breast cancer cells

To confirm our observation that GX15-070 induces autophagosome formation, we used electron microscopy to examine LCC9 cells treated with GX15-070. Untreated control cells seemed to have a normal cytoplasm with few autophagic vacuoles (Fig. 3; arrows). Twenty-four hours of GX15-070 exposure resulted in an accumulation of lysosomes (marked “L”) and autophagic vacuoles (Fig. 3B). Under higher magnification, autophagic vesicles were observed to have typical double-membrane boundaries containing electron dense material (Fig. 3B). Mitochondria (marked “M”) were also enlarged in the GX15-070–treated cells (Fig. 3B), suggesting that the cell was in the later stages of apoptosis (32). The electron micrographs revealed that GX15-070 increased lysosome and autophagic vacuole formation, leading us to examine proteins involved in autolysosome formation and degradation.

GX15-070 treatment results in the accumulation of LC3-II and p62 proteins

We next investigated the role of GX15-070 on later events of the autophagy process. In mammalian cells, p62/sequestosome-1 (SQSTM1) is implicated in autophagic cargo recognition and is lost in the final stages of autophagy during autolysosome degradation (31). Using Western blot analysis, we found an accumulation of p62 protein in antiestrogen-sensitive and -resistant breast cancer cells following treatment with GX15-070 (Fig. 4A). By immunofluorescence, we measured LC3 punctae and p62 protein expression in LCC9 cells treated with 100 nmol/L ICI182780, 500 nmol/L GX15-070, ICI+GX, or the late-stage autophagy inhibitor, 5 nmol/L BAF. 4’,6-Diamidino-2-phenylindole (DAPI) staining shows the location of the nuclei; when merged with LC3-GFP and red fluorescent p62 cDNA, the levels of LC3 punctae and p62 were elevated in GX15-070- and BAF-treated cells compared with both vehicle- and ICI182780-treated cells (Fig. 4B). Accumulation of LC3 punctae and p62 suggested that GX15-070 functioned as a downstream autophagy inhibitor.

GX15-070 blocks autophagic degradation through attenuation of cathepsin activity

In the final stages of autophagy, mature autolysosomes are subjected to proteolytic degradation, leading to a reduced level of autophagic contents and substrates such as p62 (33). Therefore, we focused on the lysosomal hydrolases CTSB, CTSD, and CTSL1, as potential targets of GX15-070. Forty-eight hours of GX15-070 exposure resulted in a 5-fold inhibition of CTSD and CTSL1 protein...
expression in LCC1, LCC9, and MCF7 cells (Fig. 5A). While GX15-070 modestly reduced CTSB activity in LCC1 cells, ICI182780 alone stimulated expression of the 3 cathepsins in antiestrogen-sensitive cells (Fig. 5A). We further examined the mechanism of GX15-070 by measuring cathepsin mRNA expression in LCC1 and LCC9 cells. CTSB, CTSD, and CTSL1 mRNA levels remained unchanged after GX-ICI exposure (Supplementary Fig. S5).

We next assessed whether cathepsin activity was attenuated by GX15-070 by measuring activity of B and L in LCC1 and LCC9 cells exposed to 100 nmol/L GX-ICI, 100 nmol/L ICI182780, or vehicle control. GX-ICI significantly inhibited CTSL1 activity in LCC1 and LCC9 cells after 48 hours (Fig. 5B; P = 0.0001 and 0.0001, respectively). While GX15-070 slightly reduced CTSB activity in LCC1 cells (P = 0.004), CTSB activity was unaffected by GX15-070 in LCC9 cells (Fig. 5C). Without a specific substrate, activity for CTSD could not be determined. To further elucidate how GX15-070 affects autophagosome maturation, we transfected LCC9 with an mRFP-GFP tandem-tagged LC3. Typically, GFP-LC3 is degraded by hydrolases following autophagosome–lysosome fusion (26); however, GX15-070 induced an accumulation of mRFP-GFP tandem-tagged LC3. Typically, GFP-LC3 is degraded by hydrolases following autophagosome–lysosome fusion (26); however, GX15-070 induced an accumulation of mRFP and GFP, suggesting that a reduction in cathepsin protein expression inhibited the degradation of GFP (Supplementary Fig. S5). Acidic pH is required for cathepsin activity, which is indicative of inhibition of autophagic flux (Fig. 6B). CTSL1 and CTSD inhibition also increased autophagosome formation in both LCC1 and LCC9 cells (Fig. 6C; P = 0.0001). Taken together, these data provide strong evidence that GX15-070 inhibits autophagic degradation through a CTSL1- and CTSD-dependent mechanism, which causes cancer cells to lose their ability to recycle subcellular components through autophagy and restore metabolic homeostasis.

**Inhibition of cathepsin L and D results in breast cancer cell death by blocking autophagosomal degradation**

We next sought to determine whether known inhibitors of CTSL1 and CTSD would also reduce breast cancer cell growth through the inhibition of autophagosomal lysis. Relative cell proliferation of LCC1 and LCC9 cells was inhibited by 20 μmol/L of the CTSL1 and 50 μmol/L of the CTSD inhibitor alone and in combination with 100 nmol/L ICI182780 after 48 and 24 hours, respectively (Fig. 6A; P = 0.0001). Cell death was accompanied by an accumulation of p62 in antiestrogen-sensitive and -resistant cells (Fig. 6B). CTSL1 and CTSD inhibition also increased autophagosome formation in both LCC1 and LCC9 cells (Fig. 6C; P = 0.0001). Taken together, these data provide strong evidence that GX15-070 inhibits autophagic degradation through a CTSL1- and CTSD-dependent mechanism, which causes cancer cells to lose their ability to recycle subcellular components through autophagy and restore metabolic homeostasis.

**Discussion**

Increased expression of BCL2 and/or BCLW plays a role in antiestrogen resistance by allowing cells to evade...
apoptosis (35). Using multiple cell lines and different endocrine therapies, we show that GX15-070, a small-molecule pan-inhibitor of antiapoptotic BCL2 family members, potentiates cell death in both antiestrogen-sensitive and -resistant human breast cancer cells. While reports describe apoptosis and autophagy as the major forms of death induced by GX15-070 (14, 18, 19), the downstream mediators of GX15-070-induced autophagy have yet to be elucidated. We show that GX15-070's anticancer efficacy is due to the blockade of antiapoptotic BCL2 family members and an increase in autophagy initiation without the complete digestion of autolysosomes; perhaps one form of apparent autophagic cell death. Furthermore, GX15-070 inhibits the protein expression of CTSD and CTSL1 that would ultimately limit cells from effectively recycling cargo that could be used to fuel cell metabolism and restore metabolic homeostasis. Using multiple cell lines affected differently by estrogen, we were able to establish a role for GX15-070 in antiestrogen-resistant breast cancer. BCL2 and CTSD are both estrogen-regulated genes (36, 37), so assays were conducted with or without estrogen to determine whether

Figure 5. GX15-070 inhibits autophagic degradation through attenuation of cathepsin activity. A, LCC1, LCC9, MCF7, and MCF7/RR cells were treated with the indicated amount of GX ± ICI/TAM, ICI/TAM, or vehicle control for 48 hours and expression of the indicated proteins were detected by immunoblot; β-actin served as the loading control; n = 3. B and C, LCC1 and LCC9 cells were assayed for CTSL1 and CTSB activity as described in Materials and Methods. Data represent the mean fluorescence unit relative to the vehicle control for 3 or more independent experiments; *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus control/vehicle treatment. D, LCC9 cells were incubated with CellLight LysoTracker Red (50 particles per cell) ± 500 nmol/L GX15-070 for 24 hours 15 minutes before fixation; cells were incubated with a LysoSensor Green dye. Merged (yellow) images are indicative of an acidic pH.
the effects of GX15-070 were mediated by ER signaling. We hypothesized that GX15-070 would inhibit the proliferation of cells that express high levels of antiapoptotic BCL2. While BCL2 members are known to regulate apoptosis (35), several antiapoptotic BCL2 members also affect autophagy through their interaction with BECN1 (9). In the antiestrogen-resistant LCC9 cells, we observed a strong induction of apoptosis and autophagy compared with their LCC1 parental cells following GX15-070 treatment alone. We noted a similar induction of apoptosis and autophagy in MCF7 cells compared with their tamoxifen-resistant MCF7/RR-derived cells. Thus, ER+ breast tumors that have high BCL2 expression seem to be good candidates for GX15-070 ± antiestrogen treatment.

GX15-070 seems to induce apoptosis as measured by Annexin V localization to the outer leaflet of the plasma membrane (Supplementary Fig. S3). To determine whether GX15-070-mediated cell death was caspase-dependent, we treated cells with GX15-070 in combination with the pan-caspase inhibitor Z-VAD-FMK. While Z-VAD-FMK inhibited PARP cleavage, it did not influence the effects of GX15-070 on cell density and Annexin V translocation (Supplementary Fig. S3). Thus, we hypothesized that GX15-070 killed breast cancer cells by a caspase-independent cell death mechanism. Z-VAD-FMK can potentiate necrotic cell death in certain cell types (38), suggesting that the drug combination killing could have resulted from off-target effects of Z-VAD-FMK. Further studies...
involving the effects of Z-VAD-FMK + GX on metabol-ic regulators and necroptosis are in progress. Recent studies have associated increased autophagy with endocrine resistance and imply that autophagy provides one mean for cells to delay an apoptotic cell death (35, 39–41). However, when autophagy persists at high levels, it is often associated with cell death (31). The BH3 mimetic GX15-070 can induce both apoptosis and autophagy (18, 19). Therefore, we expected to see both apoptosis and autophagy implicated in GX15-070-induced lethality in antiestrogen-sensitive and -resistant breast cancer cells. Indeed, GX15-070 promoted autophagic vacuole and lysosome formation in LCC9 cells (Fig. 3B). However, this was accompanied by an accumulation of p62 (Fig. 4A), which suggests an impaired ability to degrade contents of autop-hagic vesicles. We also observed swollen mitochondria in GX15-070–treated cells (Fig. 3B). Previous reports characterize mitochondria swelling with release of cytochrome c in the later stages of apoptosis (32).

GX15-070–induced LC3 processing has been suggested to depend on ATG7 (18). However, we show that LC3-II formation following GX15-070 exposure is dependent on BECN1 and independent of ATG7 (Fig. 2B). BECN1 contains a BH3 domain and its ability to initiate autophagy is inhibited by antiapoptotic BCL2 family members (9). It is possible that inhibition of antiapoptotic BCL2 members by GX15-070 releases free BECN1, allowing for BECN1-dependent autophagosome formation. However, GX15-070 reduces cell density in the presence of BECN1 siRNA (Supplementary Fig. S4), suggesting that GX15-070 toxicity promoted cell death through an unknown, PI3K–, BECN1–, and ATG7–independent mechanism. These different cell death mechanisms show the plasticity of breast cancer cell signaling to regulate cell fate in response to endocrine-based stress.

To determine the precise role of GX15-070 in autophagy maturation, we systematically measured the downstream events of autophagy. Previous reports suggest that defective autophagic degradation, reflected by the accumulation of undigested autophagosomes and p62 protein, may contribute to cell death induced by a combination of GX15-070 and lapatinib (42). However, the mechanism by which GX15-070 inhibits autophagy is unclear. In the present study, we showed that degradation of the autophagy substrate, p62, and clearance of autolysosomes is blunted in GX15-070–treated cells. This is further supported by data illustrating that GX15-070 prevents the degradation of GFP and mRFP–tagged LC3, suggesting GX15-070 inhibits autophagic flux (Supplementary Fig. S6). This led us to focus on studying CTSB and CTSL1, which are known to degrade autolysosome contents (43), and CTSD that is tightly regulated by estrogen (37) and often overexpressed in ER+ breast cancers (44).

Our data show that expression of CTSD, CTSL1, and to a lesser extent CTSB, protein was suppressed in GX15-070–treated cells (Fig. 5A). Importantly, CTSB, CTSD, and CTSL1 have each been implicated in tumor invasion, and metastasis (37, 45). Consistent with the protein expression data, we also reported reduced proteolytic activity of CTSB and CTSL1 (Fig. 5B). Despite decreases in protein expression, we did not observe any changes in CTSB, CTSD, or CTSL1 mRNA expression following GX15-070 exposure (Supplementary Fig. S5). Increased secretion of CTSD and CTSL1 could contribute to reduced intracellular levels; however, we measured cytosolic cathepsin expression following GX15-070 treatment and found no difference in cathepsin protein expression when compared with vehicle-treated cells (Unpublished Data). Thus, it is possible that GX15-070 modulated the posttranslational modification of cathepsins, which, in turn, attributed to the increase in LC3-II and p62 protein levels. To mimic the effects of GX15-070, we suppressed CTSD and CTSL1 with a chemical inhibitor and treated cells with ICI182780. As with GX15-070 treatment, we detected an accumulation of autophagosomes in cells with depressed CTSL1 and CTSD activity (Fig. 6C). This observation is consistent with the ability of CTSL1 to degrade lysosomal membrane components (43). Because CTSD and CTSL1 are implicated in breast cancer progression, inhibition of cathepsin activity using GX15-070 or other strategies may be clinically beneficial.

In summary, our results show that inhibition of antiapoptotic BCL2 expression by GX15-070 effectively reduces breast cancer cell growth alone and additively with an antiestrogen. Mechanistically, GX15-070 induces apoptosis by freeing up BH3-only BCL2 members and increasing autophagic vacuole formation in breast cancer cells. Despite the increase in autop-hagosome formation with GX15-070 exposure, the decision for the cell to undergo cell death is driven by GX15-070’s ability to impair cathepsin protein expression, which leads to the accumulation of autophagosomes and autolysosomes but interrupts completion of degra-dation of autophagic vacuoles. This study provides strong data supporting the potential use of GX15-070 and an endocrine therapy for the treatment of ER+ breast cancer cells with detectable BCL2, CTSD, and CTSL1 expression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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