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Autophagy and endocrine resistance in breast cancer

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Abstract

The American Cancer Society estimates that over 200,000 new breast cancer cases are diagnosed annually in the USA alone. Of these cases, the majority are invasive breast cancers and almost 70% are estrogen receptor-α positive. Therapies targeting the estrogen receptor-α are widely applied and include selective estrogen receptor modulators such as tamoxifen, a selective estrogen receptor downregulator such as Fulvestrant (Faslodex; FAS, ICI 182,780), or one of the third-generation aromatase inhibitors including letrozole or anastrozole. While these treatments reduce breast cancer mortality, many estrogen receptor-α-positive tumors eventually recur, highlighting the clinical significance of endocrine therapy resistance. The signaling leading to endocrine therapy resistance is poorly understood; however, preclinical studies have established an important role for autophagy in the acquired resistance phenotype. Autophagy is a cellular degradation process initiated in response to stress or nutrient deprivation, which attempts to restore metabolic homeostasis through the catabolic lysis of aggregated proteins, unfolded/misfolded proteins or damaged subcellular organelles. The duality of autophagy, which can be either pro-survival or pro-death, is well known. However, in the context of endocrine therapy resistance in breast cancer, the inhibition of autophagy can potentiate resensitization of previously antiestrogen resistant breast cancer cells. In this article, we discuss the complex and occasionally contradictory roles of autophagy in cancer and in resistance to endocrine therapies in breast cancer.

Keywords

3-methyladenine; antiestrogen resistance; aromatase inhibitor; autophagy; bafilomycin A1; breast cancer; endoplasmic reticulum stress; fulvestrant; hydroxychloroquine; tamoxifen; unfolded protein response

Endocrine therapy resistance in estrogen receptor-α-positive (ER+) breast cancer, whether acquired or *de novo*, remains an important clinical problem. While adjuvant endocrine therapy reduces breast cancer mortality, many ER+ tumors will eventually recur. Mechanisms of antiestrogen resistance are still poorly understood; however, preclinical studies suggest that several druggable targets offer the potential to restore endocrine therapy

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sensitivity, such as key components of prosurvival autophagy signaling. Autophagy, or 'self-eating', is a mechanism by which a cell digests its own subcellular organelles or unfolded/misfolded/aggregated proteins. Under normal conditions, this provides a quality-control mechanism, removing damaged organelles and proteins. In response to a stressor, this autophagic digestion recovers energy in an attempt to maintain/restore metabolic homeostasis. Targeting autophagy through chemical inhibitors, such as hydroxychloroquine (HCQ) or 3-methyladenine (3-MA), or by RNAi targeting of *beclin-1* can restore antiestrogen sensitivity in some resistant breast cancer cells. Several clinical trials have been initiated to investigate the role of autophagy in different cancer types, including metastatic breast cancer. One clinical trial of particular interest in this regard is the Preventing Invasive Breast Neoplasia with Chloroquine (PINC) study, which involves the inhibition of autophagy while concurrently treating with tamoxifen in patients with ductal carcinoma *in situ* of the breast.

The American Cancer Society (ACS) estimates that over 200,000 new cases of breast cancer are diagnosed in the USA each year [1]. Breast cancer remains the second highest killer of all cancers in women, second only to lung and bronchial cancer, with more than 40,000 reported deaths in women in the USA last year [1,2]. Over 1.15 million new cases of breast cancer are estimated to have been diagnosed worldwide last year, resulting in over 411,000 deaths in women. Breast cancer is the leading cause of cancer mortality in women worldwide [3]. Therapies targeting the ER are widely applied and include selective estrogen receptor modulators such as tamoxifen (TAM), a selective estrogen receptor downregulator such as Faslodex (FAS; fulvestrant [FAS], ICI 182,780), or one of the third-generation aromatase inhibitors (AIs) including letrozole or anastrozole. Antiestrogens are less toxic than cytotoxic chemotherapy and TAM has represented the 'gold standard' in first-line endocrine therapy for over 30 years [4]. More recently, AIs have begun to replace TAM as the first-line endocrine therapy of choice for ER+ postmenopausal breast cancer [5]. FAS, an antiestrogen drug lacking the agonist estrogenic affects of TAM, downregulates the ER through enhanced ubiquitin-mediated degradation of the receptor and has a different modality of action when compared with TAM [6,7]. In some patients, FAS is as effective as an AI [8]. While clinical studies demonstrate that adjuvant endocrine therapy reduces mortality, many ER+ breast tumors that initially respond to therapy develop acquired resistance [9–11]. For the most part, advanced ER+ breast cancer remains an incurable disease, highlighting the importance of understanding endocrine therapy resistance.

Two different types of antiestrogen resistance are generally described, *de novo* or intrinsic resistance and acquired resistance. A primary mechanism of *de novo* resistance to antiestrogen therapy is the lack of detectable ER expression [12,13]. Acquired resistance appears to occur through many different mechanisms, several of which involve changes in the ER including mutations, altered patterns of phosphorylation by growth factors and their downstream kinases, and altered expression of ER coregulators [13]. Much of our current understanding of antiestrogen resistance is based on studies focused on TAM resistance in experimental models of breast cancer. While these endocrine resistance studies have implicated many causative genes (reviewed in [13,14]), more recent studies associate autophagy and cell stress responses with endocrine resistance and thus open up a new area of research in this field (see recent reviews [15,16]).

Autophagy

Autophagy (macroautophagy) is a conserved evolutionary process that can enable cells to maintain homeostasis in unfavorable environmental conditions. An autophagic 'self-eating' allows the cell to recover energy from damaged or unnecessary subcellular components. However, if the insult is too severe and autophagy persists at a high level, it becomes pro-

death; an autophagic cell death is often referred to as programmed cell death-2 (apoptosis is programmed cell death-1). Basal levels of autophagy help clear injured organelles or long-lived proteins; hypoxia, nutrient or growth factor deprivation, accumulation of misfolded or unfolded proteins in the endoplasmic reticulum or infection can each increase the extent of autophagy (reviewed in [17]).

The process of autophagy involves the segregation of cytoplasm and intracellular organelles in double membrane-bound structures called autophagosomes. Autophagosomes then fuse with lysosomes to form autolysosomes, facilitating the degradation of the sequestered cellular material by lysosomal hydrolases. Under starvation conditions, degraded organelles or proteins are recycled and converted into metabolic intermediates that can be used to fuel the cell. Under hypoxia, autophagy removes reactive oxygen species-generating mitochondria, thereby protecting the cell [18].

The autophagy-related family of proteins (Atg) comprise the distinct molecular machinery necessary for the induction and formation of autophagosomes, autophagosome-vesicle fusion, lysis and release of degraded molecules back into the cytosol (reviewed in [19]). Table 1 summarizes the primary autophagy-related genes and their effects on the autophagy pathway. The process of autophagy is best defined in yeast. Critical to the initiation of autophagy is the activation of Atg1 (mammalian homolog: Unc-51-like kinase [ULK]-1 and -2), which is negatively regulated by the serine/threonine protein kinase target of rapamycin (TOR) [20,21]. Under low-nutrient conditions where TOR is repressed, the kinase activity of Atg1 enables binding of Atg1 to Atg13 and Atg17 (mammalian homolog: focal adhesion kinase family-interacting protein of 200 kD; FIP200 and RB1CC1), thereby creating a scaffold for recruiting other Atg proteins [22]. Unlike yeast, mammalian cells can form stable ULK-Atg13-FIP200 complexes regardless of nutrient conditions.

In mammalian cells, autophagosome degradation is driven by p62/sequestosome-1 (SQSTM1), which binds directly to ubiquitinated proteins and microtubule-associated protein-1 (MAP1) light chain-3 (LC3), linking the ubiquitinated proteins to the autophagic machinery [23–25]. Formation of the autophagosome double membrane occurs *de novo* and not from either pre-existing organelles or by the annealing of single membranes, and requires the actions of vacuolar protein sorting 34 (Vps34), p150, Atg4 and beclin-1 (BECN1) [26–28]. Beclin-1 activity is regulated by B-cell lymphomia/leukemia-2 (Bcl-2) and is discussed more thoroughly later. LC3 determines autophagosome size and membrane curvature [19]; the Atg12/Atg5/Atg16 complex and the LC3-phosphatidylethanolamine (LC3-PE or LC3-II) complex participate in elongation of the autophagosome membrane. Atg9 is the only integral membrane protein identified in autophagosome formation, where it may function as a carrier of membrane materials. Atg9 is dependent on ULK1 and Atg13 for transportation from the trans-Golgi network to late endosomes [29–33]. The cysteine protease Atg4 cleaves pro-LC3 to expose a C-terminal glycine residue, enabling Atg12-Atg5 to conjugate LC3 to PE, via an amide bond; LC3-PE levels are often used as a measure of autophagy induction (reviewed in [34]). The early autophagosome fuses with a lysosome to form the late autolysosome. This fusion process is dependent upon the lysosomal membrane protein-2 (LAMP-2) and the small GTPase Rab7 [35,36]. After fusion of the lysosome, the resulting autolysosome degrades its protein/organelle load and inner membrane. In mammalian autophagy, degradation occurs through the actions of cathepsins B, D and L [17,37]. The resulting products of the catalytic degradation process are then transported to the cytosol and recycled. The process of autophagy described above is shown in Figure 1.

Bcl-2 & the regulation of autophagy in breast cancer

The Bcl-2 family contains two distinct functional groups, the anti-apoptotic group that includes Bcl-2 and Bcl-XL, and the proapoptotic group including the Bax and Bak proteins. Bcl-2 is an antiapoptotic protein that exhibits oncogenic potential through its ability to regulate the intrinsic apoptotic pathway. The molecular activity of Bcl-2 involves binding to mitochondrial Bax, thereby preventing Bax activation, mitochondrial outer-membrane permeabilization and apoptosis. Bcl-2 is overexpressed in over 60% of breast tumors; overexpression of Bcl-2 correlates with chemotherapeutic and radiation resistance [38,39]. Moreover, a recent clinicopathological investigation that measured both beclin-1 and Bcl-2 in breast cancer tissue indicated that beclin-1 is inversely correlated with Bcl-2 expression. Increased Bcl-2 expression is associated with the estrogen receptor, increased histological grade and distant metastases [40]. These data highlight the role of Bcl-2 in breast cancer and resistance.

Beclin-1 was originally identified as a Bcl-2-interacting protein [41], the Bcl-2 family being a group of proteins containing Bcl-2 homology domains. Beclin-1 binds to Bcl-2, Bcl-W, Bcl-X_L and Mcl-1, which results in the inhibition of autophagy [41–43]. Table 2 summarizes the Bcl-2 family members' effects on apoptosis and autophagy. In several cell types, binding of Bcl-2 to beclin-1 inhibits the binding and activation of Vps34, decreasing Vps34-mediated PI3K activation and subsequently inhibiting autophagy. In cases of nutrient starvation, or when cells are treated with Bcl-2 inhibitors that reduce Bcl-2 protein levels, Bcl-2 and beclin-1 dissociate and autophagy is stimulated [41–43]. Conversely, proapoptotic Bcl-2 family members, such as Bad, Bik, BNIP3L, Noxa, Puma and Bim_{EL}, may induce autophagy by competitively binding to Bcl-2 family members and disrupting the interaction between beclin-1 and Bcl-2; thereby freeing beclin-1 [44–46]. A recent study into the role of Mcl-1 in autophagy reported that, in response to glucose deprivation and hypoxia, Mcl-1 is rapidly degraded and autophagy becomes activated. Furthermore, Mcl-1 overexpression prevents LC3-positive punctate formation, indicating a key regulatory role of Mcl-1 in autophagy [47].

Several Bcl-2 inhibitors are currently undergoing clinical trials. While the use of Bcl-2 inhibitors are predominately focused on leukemias and lymphomas, a potential role for these inhibitors in breast cancer is now evident [48]. For example, preclinical studies investigating the role of Bcl-2 in MCF-7 breast cancer cells show that silencing Bcl-2 by siRNA increases autophagy and cell death, highlighting the possible use of Bcl-2 inhibitors as a therapeutic strategy in breast cancer [49]. Gossypol, a BH3 mimetic isolated from cotton seeds, induces beclin-1-dependent and -independent autophagy, resulting in cytoprotection and survival of MCF-7 breast cancer cells [50]. These studies likely reflect an important role for Bcl-2 family members in the regulation of autophagy in breast cancer.

Unfolded protein response & the regulation of autophagy in breast cancer

The unfolded protein response (UPR) pathway is activated in response to the accumulation of aggregated unfolded/mis-folded proteins within the endoplasmic reticulum (EnR) lumen. In response to this accumulation, the EnR protein chaperone glucose-regulated protein 78 (GRP78; BiP; HSPA5) is released from each of inositol requiring enzyme-1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like endoplasmic reticulum kinase, enabling their respective activation. IRE1 dimerizes and becomes autophosphorylated, resulting in its activation and ability to perform the unconventional (cytosolic) splicing of the X-box binding protein-1 (XBP1) mRNA. XBP1 splicing creates the transcriptionally-active XBP1-S form [16,51], which can confer estrogen independence and antiestrogen

resistance upon estrogen-dependent breast cancer cells [16,52] and is now known to be associated with a poor response to TAM [53].

Unfolded protein response stimulation promotes the activation of autophagy through different mechanisms. EnR stress results in phosphorylation of eIF2α by PKR-like endoplasmic reticulum kinase. Activated eIF2α increases ATF4 expression, which then increases the transcription of Atg12 and can thereby promote autophagy [54]. IRE1 activation also leads to the phosphorylation of c-Jun-terminal kinase, resulting in the phosphorylation of Bcl-2 at the T69, S70 and S87 residues in the unstructured loop of Bcl-2 [55]. Phosphorylation of Bcl-2 can cause dissociation of the Bcl-2/beclin-1 complex and thus may activate autophagy. Figure 2 illustrates the interaction between UPR signaling and autophagy. Calcium released from the EnR following stress also promotes autophagy by activating beclin-1 [17]. Therefore, the stimulation of UPR by mechanisms such as nutrient starvation, hypoxia or therapeutic drugs may result in the subsequent activation of a prosurvival autophagy.

Dichotomy of autophagy in cancers

In cancer, autophagy can serve as either a ‘tumor suppressor’ or as a ‘tumor promoter’. Allelic loss of vital autophagy components, such as beclin-1, is often found in breast, ovarian and prostate cancers [56–59]. Moreover, inhibition of beclin-1 or deletion of Atg5 in immortalized epithelial kidney cells or breast cancer cell lines is associated with increased proliferation and tumorigenicity [60,61]. Genetically altered heterozygous beclin-1 knockout mice exhibit an increased incidence of hepatocellular carcinoma, lung adenocarcinoma and mammary hyperplasia [62]. Increased susceptibility to hepatitis B-induced hepatocellular carcinoma, when compared with their wild-type counterparts [63,64], is also reported. Brain tumors often have reduced beclin-1 compared with the normal surrounding tissue and reduced beclin-1 inversely correlates with malignancy [65]. Vps34 expression is also dysregulated in cancers. Vps34 overexpression in colon cancer cell lines reduced tumorigenicity, while heterozygous deletion of the Vps34 gene *uvrag* is often observed in colon tumors [66]. Knockdown of Atg4, the protease that cleaves LC3, increases the severity of chemically-induced fibrosarcomas in mice [67]. These data suggest that disruption of the autophagic process is a key event in tumorgenesis.

While direct modulation of the components of autophagy is observed in different cancers, mutations indirectly affecting autophagy are also reported. *PI3K* mutations are found in over 20% of breast cancers and 30% of colorectal cancers. These mutations may indirectly influence autophagy through the stimulation of mTOR, which would prevent ULK activity and inhibit autophagy [62]. Another possible autophagy-regulating event, *p53* mutational inactivation, is observed in over 50% of all tumors [68]. Inactivating *p53* mutations, mutation in the *p53* activating kinases, overexpression of MDM2 that degrades p53 and loss of function of $p14^{ARF}$, are each documented in various cancers and these result in a loss/reduction in p53 activity (reviewed in [45]). Nuclear p53 can affect autophagy through transactivation of death-associated protein kinase-1 (DAPK-1) and the lysosomal protein damage-regulated autophagy modulator [69]. DAPK-1 is commonly dysregulated in human tumors and has both proapoptotic and proautophagy activities. For example, DAPK-1 phosphorylates myosin light chain, promoting membrane blebbing and apoptosis [70]. DAPK-1 is also implicated in autophagy through its ability to bind MAPB1, which interacts with LC3 to inhibit autophagy [70,71]. Transactivation of DRAM by p53 can activate autophagy and is necessary for the execution of a DNA damage-induced p53-mediated cell death. Furthermore, p53 inhibits mTOR and can thus activate autophagy.

While the transcriptional activation of *p53* promotes autophagy, cytosolic p53 can inhibit autophagy [72]. Preclinical studies show an increase in autophagy with an increased formation of LC3-containing autophagic vacuoles when p53 is knocked down, knocked out or otherwise inhibited [34,73]. Moreover, inhibition of nuclear transporters, resulting in the accumulation of p53 in the nucleus, prevents the inhibitory actions of p53 on autophagy. While the influence of p53 on autophagy is evident, the overall effect of p53 on the regulation of autophagy remains controversial. Function of the upstream activator of p53, $p14^{ARF}$, is also lost in many cancers and the mitochondrial form of $p14^{ARF}$ is a potent stimulator of autophagy [74].

Defects in autophagy may also promote tumorgenesis. Impaired autophagy hinders the ability of a cell to survive stressful environmental conditions and can result in increased cell death [59,60]. While this may seem anti-tumorigenic at first, chronic cell death leads to a prolonged inflammatory response that can be oncogenic. Cancer-related inflammation is often considered the seventh hallmark of cancer [75]. For example, chronic cell death in the liver stimulates inflammation, increases organ damage and raises the risk of developing hepatocellular carcinoma. Necrotic cell death leads to the release of cellular debris, activating various cell-surface receptors on neighboring cells that can stimulate survival pathways and enhance cell growth [75,76]. Moreover, autophagy also limits genotoxic damage by reducing the formation of reactive oxygen species (ROS) and clearing damaged mitochondria [75,77]. When autophagy is impaired, damaged mitochondria remain in the cell, increasing ROS production and the associated protein, organelle and DNA damage. Oxidative damage from the accumulation of ROS may promote tumorgenesis, thereby supporting inadequate autophagy as a contributor to oncogenesis (reviewed in [76]).

While autophagy is implicated as a tumor supressor in early tumorgenesis, a growing body of evidence implicates autophagy as a tumor promoter in late stage cancers. The ability of the autophagic process to provide cellular resilience to stressors such as glucose deprivation and hypoxia, two common stresses experienced by tumors, enables long-term cell survival [45,57,60,78]. Autophagy allows cancer cells to ‘eat themselves’, progressively reducing in size, to conserve and provide nutrients for survival functions. Since some of these cancer cells retain the ability of self-renewal, they can return to their original size and proliferate given proper conditions. Thus, some cancer cells with intact autophagy may exhibit cellular dormancy (reviewed in [59]).

In summary, the oncogenic activity of autophagy stimulation may be stage dependent. Autophagy appears to a tumor suppressor in early tumorigenesis, perhaps because the cells cannot easily adapt to the increased elimination of subcellular organelles as an energy source. During progression, those cells that survive will likely have been able to adapt their physiology such that they can survive with the increased basal level of autophagy, which may now be providing sufficient energy for survival without exceeding a threshold where cell death becomes inevitable.

Autophagy in breast cancer

The role of autophagy in breast cancer, like other types of cancer, is an area of active investigation. Clinicopathological investigation of breast cancer tissue indicated three discernable LC3-positive patterns; diffuse cytoplasmic, cytoplasmic/juxtanuclear and dense round 5 μm ‘stone-like’ structures [79]. Diffuse cytoplasmic or cytoplasmic/juxtanuclear LC3 staining correlates directly to with estrogen and progesterone receptor expression in breast tissue. Moreover, the ‘stone-like’ LC3 stained phenotype is associated with high-grade tumors and a less favorable outcome, suggesting the more autophagic a tumor, the more aggressive the tumor [79].

Evidence also supporting the oncogenic activity of autophagy was obtained through 3D morphogenic assays investigating the role of beclin-1 on mammary acini formation. Immortalized mouse mammary epithelial cells with homozygous beclin-1 produce an atypical solid acini structure, owing to the autophagy-competent central acini epithelial cells having an increased capacity to survive anoikis and the hypoxic environment of the central lumen [61]. Conversely, heterozygous deletion of beclin-1 in Immortalized mouse mammary epithelial cells results in normal mammary lumen formation. The defect in autophagy resulting from beclin-1 deletion leads to necrosis of the central epithelial cells, allowing normal mammary lumen formation [78]. Morphogenesis assays capture the physiological context found in normal tissues to model adhesion signaling in acini formation. The ability of cells to form atypical solid acini parallels breast cancer's invasion and metastatic potential *in vivo*; therefore, these data indicate a role of beclin-1 and autophagy in mammary acini development and in cancer progression. A role for beclin-1 in breast tumorigenesis is apparent in the significant variability of beclin-1 expression across different molecular subtypes; higher expression levels of beclin-1 are seen in the HER2-negative luminal-A or luminal-B breast cancers [80,81]. In combination with the mammary acini study, these data indicate that the role of beclin-1 in breast cancer may be more complex than that of just a tumor suppressor, consistent with data from other cancers.

In triple-negative breast cancer (ER negative, PR negative, HER2 negative), the effect of autophagy is just beginning to be elucidated. Recent studies report increased autophagic properties in the mitochondria of the metastatic triple-negative MDA-MB-231 when compared with the less metastatic MDA-MB-468 and noncancerous MCF7–10A cells [82]. However, investigation into the effect of phytochemical therapy and PPARγ ligands in triple-negative breast cancers shows an increase in cell death caused by autophagic activation and necrosis, suggesting there may be a threshold limitation between prosurvival and prodeath autophagy [83,84]. Various studies of the therapeutic response of experimental chemotherapies in breast cancer have implicated a prodeath role for autophagy. A lipid-modified estrogen derivative, developed to treat breast cancer independent of ER status, was shown to induce apoptosis and autophagy in the triple-negative MDA-MB-231 breast cancer cells [85]. Furthermore, this estrogenic compound interferes with mTOR activity, thereby inducing autophagy and promoting cell death. These observations suggest a possible therapeutic strategy for inhibiting triple-negative or ER-negative breast cancer growth through the stimulation of pro-death autophagy [85].

Autophagy in drug resistance

Preclinical studies using chemical inhibitors of autophagy (described in Table 3) or siRNA to knockdown vital autophagy genes demonstrate the role of autophagy in stress and chemotherapeutic sensitization of cancer cells. Most chemical inhibitors of autophagy lack specificity and often have off-target effects. Preclinical studies using these chemicals may benefit from siRNA knockdown of autophagy genes before concluding that the observed effects are due solely to autophagy inhibition. Inhibition of autophagy in glioblastoma, lung cancer, cervical cancer, prostate cancer, leukemia and breast cancer cells resensitized the cells to various therapeutic agents [86]. For example, upregulation of autophagy can protect cancer cells against various therapies including temozolomide, resveratrol, vitamin D3, anthocyanins, radiotherapy and TAM [16,76,86–89]. Treatment with temozolomide in malignant glioma cells stimulates autophagy without activating apoptosis and is associated with resistance to DNA-alkylating agents in some brain cancers [90]. These data suggest an important role of autophagy in promoting cancer therapeutic drug resistance.

Breast cancer studies have also revealed a role of autophagy in resistance. Autophagy protects MCF-7 breast cancer cells against epirubicin-mediated cell death, and inhibition of

autophagy through beclin-1 siRNA restored epirubicin effectiveness [91]. In addition, when treated with camptothecin or etoposide (DNA-damaging therapeutic drugs) autophagy can delay the onset of apoptotic cell death in breast cancer cells, an effect reversed by knockdown of the autophagy-dependent genes *Atg7* and *beclin-1* [44]. Furthermore, treatment of MCF-7 breast cancer cells with bortezomib, a proteasome inhibitor, results in a potent stimulation of autophagy and UPR. The authors speculate that the observed activation of UPR and autophagy is prosurvival, and therefore may explain the poor response to bortezomib in breast cancer patients [92]. HER2-targeted therapies, such as the monoclonal antibody herceptin and EGF receptor tyrosine kinase inhibitors, such as lapatinib, are sensitive to autophagy-mediated resistance [93,94]. Trastuzumab (herceptin) induces LC3-positive punctate formation in SKBr3 cells (HER2-amplified breast cancer cell line). Inhibition of autophagy by 3-MA and LY294002 increases cell death in response to trastuzumab, suggesting autophagy as a cytoprotective response [94]. Moreover, inhibition of autophagy restores EGF receptor-mediated cell death in lapatinib-resistant BT-474 cells (HER2 amplified breast cancer cell line) [93]. These data suggest that targeting autophagy can be sufficient to restore chemotherapeutic drug sensitivity and promote breast cancer cell death.

Studies have also investigated the outcome of autophagy stimulation in antiestrogen therapy. Bursch *et al.* treated MCF-7 estrogen-dependent ER+ breast cancer cells with the antiestrogens TAM and ICI, and found that dying cells showed increased cytosolic autophagosome formation [95]. These authors concluded that autophagy, stimulated by antiestrogens in MCF-7 cells, resulted in active cell death. However, more recently Samaddar *et al.* suggest that this conclusion more likely reflects cells' failed attempts at survival. Samaddar *et al.* demonstrated that in the surviving MCF-7 cellular population (~70%) after antiestrogen treatment, there was an increase in autophagosome formation. This group also hypothesized that whether autophagy promotes survival or cell death may be dependent on the number of autophagosomes in each cell, resulting in a threshold limit. Inhibiting autophagosome formation via 3-methyladenine (3-MA) or beclin-1 siRNA significantly enhanced antiestrogen-induced cell death in MCF-7 cells, further suggesting a prosurvival role of autophagy in anti-estrogen therapy [96]. Qadir *et al.* used siRNA with Atg5, beclin-1 and Atg7 to inhibit autophagy in MCF-7, T47D and TAM-resistant MCF7-HER2 cell lines, and reported that concurrent knockdown of autophagy and treatment with TAM resulted in increased mitochondrial-mediated apoptotic cell death and overall reduced cell viability [97]. Moreover, we have shown that inhibition of autophagy through beclin-1 shRNA or 3-MA treatment in the ICI resistant, TAM cross-resistant MCF7/LCC9 breast cancer cells partially restored antiestrogen therapy effectiveness [89].

Increased responsiveness of resistant breast cancer cells to anti-estrogen therapy requires concurrent inhibition of both Bcl-2 and beclin-1. Dual inhibition of Bcl-2 by the chemical inhibitor, YC137 and *beclin-1* knockdown increases apoptosis and decreases cell survival in response to antiestrogen therapy [89]. These data illustrate that inhibition of autophagy pathway, coupled with Bcl-2 inactivation, is more detrimental to antiestrogen resistant breast cancer cell survival than the individual inhibition of either pathway alone. Thus, dual targeting of synergistic molecular pathways may be beneficial to resensitizing antiestrogen-resistant breast cancers. Investigating the role of estrogen signaling in breast cancer cells by *beclin-1* has shown that overexpression of *beclin-1* results in decreased growth in response in estrogen, with a decrease in estrogen-regulated genes including *c-myc*, *c-fos* and *egr 1*. These decreases in estrogenic growth by *beclin-1* overexpression may appear anti-tumorigenic but *beclin-1* overexpression in breast cancer cells also leads to a loss of sensitivity to the antiestrogens raloxifene and TAM, further implying a role for autophagy in promoting antiestrogen resistance [98]. Recent preclinical studies into possible drug combinations to overcome autophagy-mediated TAM resistance suggest a possible

therapeutic benefit of combining histone deacetylase inhibitors or proteasomal inhibitors with antiestrogens [99,100]. Treatment of antiestrogen-resistant T47D and MCF7 ER+ breast cancer cells with bortezomib produces a potent induction of cell death and an inhibition of autophagy as measured by p62 and LC3 accumulation, suggesting the benefit of targeting autophagy in antiestrogen-resistant breast cancer [99]. Taken together, these data clearly support a role for autophagy in the promotion of antiestrogen resistance in breast cancer.

Since the weight of preclinical data indicates that inhibiting autophagy resensitizes some resistant cancer to specific therapies, it is not surprising that clinical trials targeting autophagy have recently been initiated (Table 4). Since it has been used extensively for the treatment of malaria, safety data for the use of HCQ in humans is already accessible. Clinical trials have been initiated using HCQ in combination with gefitinib in lung cancer, with docetaxel in prostate cancer, with temozolomide in glioblastoma multiforme, with ixabepilone in metastatic breast cancer, and with bortezomib in multiple myeloma. Of particular interest in ER+ breast cancer is a study in ductal carcinoma *in situ*, in which patients will receive TAM, chloroquine or a combination of both for 3 months before surgical removal of the tumor. It will be of great interest to see whether inhibition of autophagy in combination with TAM treatment reduces the growth and invasiveness of these breast tumors. The results of the clinical trials listed in Table 4 should hold promising answers to some of the questions pertaining to the role of autophagy in cancer. Moreover, several groups have recently investigated possible small molecular regulators of autophagy through LC3-GFP imaged-based high-throughput screening [101,102]. It is interesting to note that several of these autophagy modulators, either autophagy inducers or autophagy inhibitors, are already US FDA approved for the treatment of various diseases including cardiovascular disorders, schizophrenia and irritable bowel syndrome.

Expert commentary

Targeting autophagy, particularly when it is acting in a survival mode, has significant potential to lead to the development of novel agents and therapeutic regimens. Existing data already suggest that this could be beneficial in combination with both cytotoxic chemotherapy and with endocrine therapy in some cancers. While it is difficult to predict the outcomes of early trials using ‘first-generation’ inhibitors such as HCQ, the field is ripe for the development of more specific inhibitors or combinations of new inhibitors. Outcomes from the early trials indicated in Table 4 should begin to offer powerful new insights into these exciting opportunities.

Longer term success in targeting autophagy may require the development of a greater understanding of the signaling that both regulates and executes autophagy. While the basic machinery for its execution is defined in normal systems such as yeast, whether this provides an adequate definition of how autophagy signaling is present or altered in different human cancers remains unclear. Perhaps the greatest opportunity will lie in the identification of cancer-specific modifications in the regulatory signaling, rather than in the execution machinery. Such knowledge may best be obtained by the development of useful computational and/or mathematical models of the signaling-control mechanisms [15].

Five-year view

Greater detail on the control signaling of autophagy will likely emerge and provide new insights into how the extent and duration of prosurvival autophagy are regulated to allow cancer cells to survive for prolonged periods in the presence of natural (nutrient deprivation or immunologic suppression of growth leading to dormancy) or imposed (therapeutic intervention leading to resistance) stress. Data from clinical trials will show some evidence

for the activity of autophagy inhibitors, although the full value of this will await a better understanding of the redundancy in the signals controlling autophagy and the development of combination regimens that address this redundancy. Considering the complexity of this mechanism, systems biology-based approaches will generate the most useful insights, and initial computational and/or mathematical models of autophagy regulation and execution will emerge. While the true clinical potential will likely take longer than 5 years to realize, in part owing to the time needed for clinical follow-up and adequate outcome measures, interest and excitement in this field seems certain to rise substantially within the next 5 years.

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Key issues

- With over 70% of all breast cancer cases being estrogen receptor-α-positive, endocrine therapy remains the primary treatment for these breast cancer patients.
- Many breast tumors that initially respond to antiestrogen treatments eventually develop acquired resistance; preventing and overcoming antiestrogen resistance remain important clinical goals.
- Autophagy, the processes of 'self-eating', can enable cell survival in adverse environmental conditions, including nutrient deprivation and hypoxia.
- Several cancer therapies induce autophagy, such as radiation, temozolomide, cytotoxic drugs, antiestrogens and aromatase inhibitors.
- Inhibitors of autophagy restore antiestrogen sensitivity in endocrine-resistant breast cancer cells growing *in vitro*.
- Clinical trials involving autophagy inhibitors in combination with endocrine or cytotoxic therapies are now being initiated to study the role of autophagy in the survival and progression of cancers.

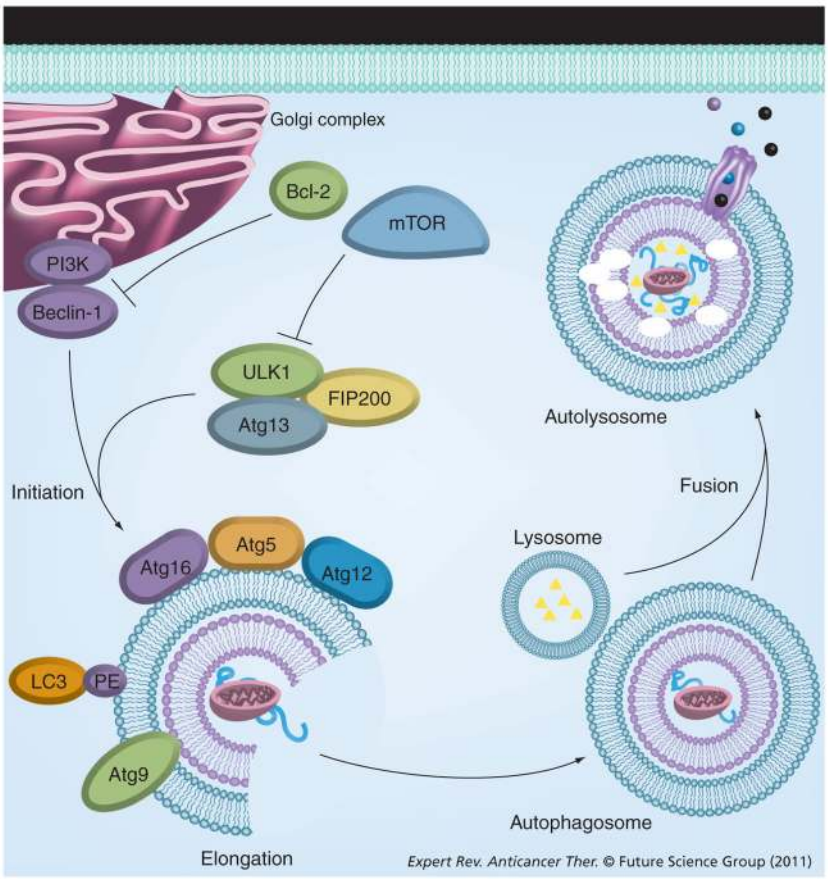

Figure 1. Cellular pathway of autophagy

The PI3K complex mediates the initiation of the phagophore membrane, enveloping labeled cytosolic proteins organelles and fat. mTOR and Bcl-2 can inhibit the initiation of autophagy. The Atg12-Atg5-Atg16 complex, LC3 and the transmembrane Atg9 are recruited to the phagophore and are necessary for elongation of the double membrane. Lysosomes fuse with the autophagosome, creating the autolysosome. The resulting products of the catalytic degradation process are transported to the cytosol and recycled.

LC: Light chain-3.

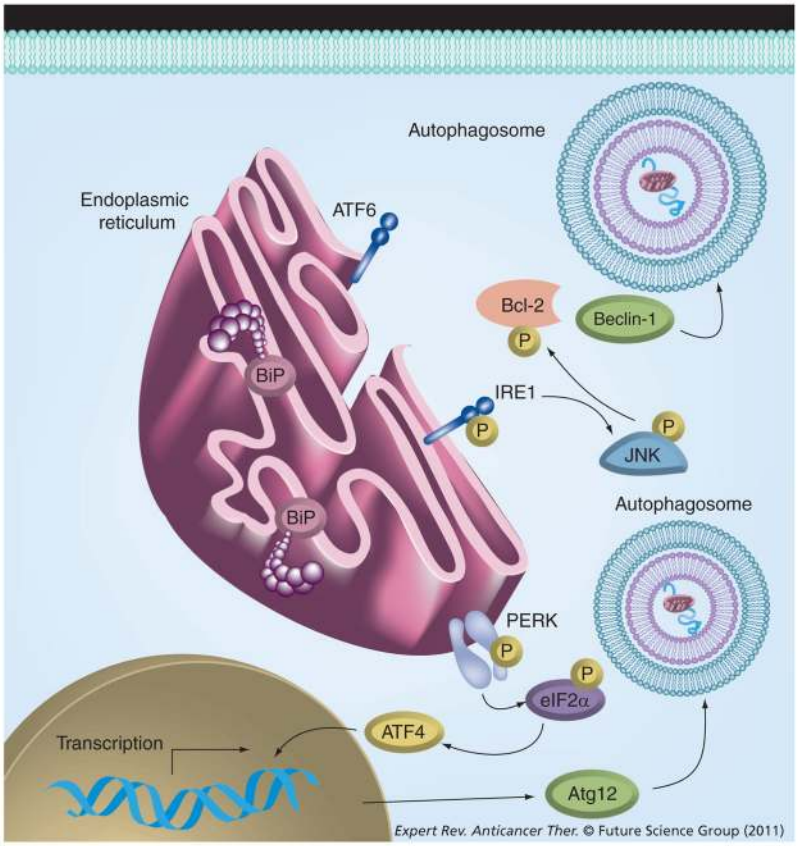

Figure 2. Effect of the unfolded protein response on autophagy

The unfolded protein response can activate autophagy through two distinct mechanisms. PERK activation leads to phosphorylation of eIF2α, resulting in increased ATF4 transcription. ATF4 promotes the transcription of Atg12, resulting in increased autophagy. Another mechanism of unfolded protein response-modulating autophagy is through activation of IRE1. IRE1 activates JNK, leading to the subsequent phosphorylation of Bcl-2. Bcl-2 phosphorylation prevents its binding to beclin-1, thereby promoting autophagy. ATF: Activating transcription factor; IRE1: Inositol requiring enzyme-1; PERK: PKR-like endoplasmic reticulum kinase.

Table 1

Selected autophagy-related genes.

Autophagy gene	HUGO gene symbol	Effect on autophagy
Atg3	*ATG3*	E2-like enzyme facilitates lipidation of LC3
Atg4A, *-B*, *-C*, *-D*	*ATG4A*, *ATG4B*, *ATG4C* and *ATG4D*	Cleaves pro-LC3 to form LC3
Atg5	*ATG5*	Forms a complex with Atg12-Atg16, resulting in lipidation of LC3
Atg7	*ATG7*	E1-like enzyme activates Atg12
Atg9A, *-B*	*ATG9A* and *-B*	Phagophore membrane expansion
Atg10	*ATG10*	E2-like enzyme facilitates the formation of Atg5-Atg12-Atg16 complex
Atg12	*ATG12*	Forms complex with Atg5-Atg16, resulting in lipidation of LC3
Atg13	*ATG13*	Part of the initiation complex with ULK1, Atg101 and FIP200
Atg16L1, *-L2*	*ATG16L1* and *-2*	Forms a complex with Atg5-Atg12, resulting in lipidation of LC3
Beclin-1	*BECN1*	Part of the initiation complex with Vps34
Atg101	*C12orf44*	Part of the initiation complex with ULK1, FIP200 and Atg13
Cathepsin B	*CTSB*	Lysosome enzyme
LAMP1, *-2*, *-3*	*LAMP1*, *-2* and *-3*	Lysosome autophagosome fusion
LC3 (A, B or C)	*MAP1LC3A*, *-B* and *-C*	Phagophore membrane curvature and expansion
MTOR	*MTOR*	Inhibits ULK1
PIK3C3 (Vps34)	*PIK3C3*	Part of the initiation complex with beclin-1
FIP200	*RB1CC1*	Part of the initiation complex with ULK1, Atg101 and Atg13
p62	*SQSTM1*	Cargo recognition
Rab7	*RAB7A*	Lysosome autophagosome fusion
ULK1	*ULK1*	Part of the initiation complex with Atg101, Atg13 and FIP200

HUGO: Human Genome Organisation; LAMP: Lysosomal membrane protein; LC: Light chain; ULK: Unc-51-like kinase.

Table 2

Role of Bcl-2 family members on apoptosis and autophagy.

Bcl-2 family member	HUGO gene symbol	Effect on apoptosis	Effect on autophagy
Bcl-2	*BCL2*	Antiapoptotic	Inhibits autophagy by binding to beclin-1
Bcl-w	*BCL2L2*	Antiapoptotic	Inhibits autophagy by binding to beclin-1
Bcl-X_L	*BCL2L1*	Antiapoptotic	Inhibits autophagy by binding to beclin-1
Mcl-1	*MCL1*	Antiapoptotic	Inhibits autophagy (to a lesser extent than Bcl-2, Bcl-w and Bcl-X_L) by binding to beclin-1
Bad	*BAD*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
t-Bid	*BID*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Bim_{EL}	*BCL2L11*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Noxa	*PMAIP1*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Puma	*BBC3*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
BNIP3L	*BNIP3L*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Bik	*BIK*	Proapoptotic	Promotes autophagy by competitively binding to Bcl-2, Bcl-w and Bcl-X_L
Bax	*BAX*	Proapoptotic	No effect
Bak	*BAK1*	Proapoptotic	No effect

HUGO: Human Genome Organisation.

Table 3

Commonly used inhibitors of autophagy.

Compound	Target and effect
Hydroxychloroquine or chloroquine	Lysosomal pH, prevents autophagosome–lysosome fusion
3-methyladedine	Class III PI3K inhibition, prevents autophagosom formation
Wormatin	Class III PI3K inhibition, prevents autophagosome formation
LY294002	Class III PI3K inhibition, prevents autophagosome formation
Bafilomycin A1	Vacuolar ATPase inhibition, prevents autophagosome–lysosome fusion

Table 4

Selected ongoing clinical trials inhibiting autophagy in cancer.

Cancer type	Treatment	Study phase	ClinicalTrials.gov identifier [201]
Glioblastoma multiforme	Hydroxychloroquine, radiation, temozolomide	I/II	NCT00486603
Multiple myeloma	Hydroxychloroquine, bortezomib	I/II	NCT00568880
Advanced non-small-cell lung cancer	Hydroxychloroquine, carboplatin, paclitaxel, bevacizumab	I/II	NCT00728845
Breast (metastatic)	Hydroxychloroquine, ixabepilone	I/II	NCT00765765
Colorectal (metastatic)	Hydroxychloroquine, capecitabine, oxaliplatin, bevacizumab	II	NCT01006369
Prostate	Hydroxychloroquine	II	NCT00726596
Renal cell carcinoma	Hydroxychloroquine, surgery	I	NCT01144169
Breast (DCIS)	Chloroquine, tamoxifen	I/II	NCT01023477
Prostate (metastatic)	Hydroxychloroquine, docetaxel	I/II	NCT00786682
Lung	Hydroxychloroquine, gefitinib	I/II	NCT00809237

DCIS: Ductal carcinoma *in situ*.