Evidence for cytoprotective autophagy in response to HER2-targeted monoclonal antibodies

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Abstract: The advent of HER2-targeted monoclonal antibodies such as trastuzumab has significantly improved the clinical outcomes for patients with breast cancer overexpressing HER2, and more recently also for gastric cancers. However, the development of resistance, as is frequently the case for other antineoplastic modalities, constrains their clinical efficacy. Multiple molecular mechanisms and signaling pathways have been investigated for their potential involvement in the development of resistance to HER2-targeted therapies, among which is autophagy. Autophagy is an inherent cellular mechanism whereby cytoplasmic components are selectively degraded to maintain cellular homeostasis via the generation of energy and metabolic intermediates. Although the cytoprotective form of autophagy is thought to predominate, other forms of autophagy have also been identified in response to chemotherapeutic agents in various tumor models; these include cytotoxic, cytostatic, and non-protective functional forms of autophagy. In this review, we provide an overview of the autophagic machinery induced in response to HER2-targeted monoclonal antibodies, with a focus on trastuzumab and trastuzumab-emtansine, in an effort to determine whether autophagy targeting or modulation could be translated clinically to increase their effectiveness and/or overcome resistance development.

Significance statement: This manuscript is one in a series of papers that investigate the different roles of the autophagic machinery induced in response to versatile anti-neoplastic agents in various cancer models. This series of papers was designed in an effort to interrogate whether autophagy targeting or modulation is an effective adjuvant strategy to increase the efficacy of chemotherapeutic agents. In this review, we explore the relationship between the autophagic machinery and HER2 targeted therapies.
Keywords: Autophagy; Cytoprotective; Trastuzumab; Trastuzumab-emtansine; HER2
1. Introduction

This manuscript follows a series of papers published by our research group in an effort to evaluate the role(s) of autophagy in response to various anti-neoplastic modalities. Our previous publications investigated the nature of autophagy in tumor cells in response to radiation (Patel et al., 2020), cisplatin (Xu and Gewirtz, 2022), microtubule poisons (Xu et al., 2022), hormonal therapies in estrogen-positive breast cancer (Finnegan et al., 2022b), PARP inhibitors (Elshazly et al., 2022), topoisomerase I poisons (Elshazly et al., 2023b), temozolomide (Elshazly and Gewirtz, 2023c), BET family inhibitors (Elshazly and Gewirtz, 2023b), BRAF-targeted therapies (Elshazly and Gewirtz, 2023a) and, most recently, androgen-targeted therapies (Elshazly and Gewirtz, 2023d). This review addresses the role of the autophagic machinery in response to HER2-targeted monoclonal antibodies; other HER2-targeted therapies such as HER2 tyrosine kinase inhibitors will be discussed in a separate article. The primary goal of this series of papers is to determine whether there are particular therapeutic modalities where the preclinical data, and where available, clinical trials, support the inclusion of autophagy inhibition or modulation as an adjuvant approach.

2. HER-2 and autophagy

HER2 (ERBB2) is transmembrane tyrosine kinase receptor belonging to the ERBB family, which is comprised of epidermal growth factor receptor 1 (EGFR) (ERBB1/HER1), HER3 (ERBB3), and HER4 (ERBB4) (Garrett and Arteaga, 2011; Elshazly and Gewirtz, 2022). The HER2 receptor consists of three primary components: the N-terminal domain, α-helix domain, as well as an intracellular domain with tyrosine kinase activity (Garrett and Arteaga, 2011; Elshazly and Gewirtz, 2022).
HER2 does not bind directly to any ligand (Citri and Yarden, 2006), whereas the homo- and heterodimeric interactions between the ERBB receptors induce autophosphorylation of the intracellular tyrosine kinase domain (Hsu and Hung, 2016). These phosphorylated residues acts as docking sites for various adapter and scaffolding proteins, including Grb2, guanine exchange factor, and SOS, triggering a variety of downstream signaling pathways, including PI3K/AKT, Ras/MEK/ERK, JAK/STAT, and PLCγ/PKC, that regulate diverse cellular behaviors such as cell survival, proliferation, and differentiation (Hsu and Hung, 2016). Despite the crucial role of HER2 in cellular development, HER2 dysregulation, particularly overexpression, may lead to increased signaling pathways activity, particularly the MAPK and PI3K/AKT pathways, ultimately contributing to tumor development (Wong and Hurvitz, 2014). Various HER2-targeted therapies have been developed including monoclonal antibodies, which are the scope of this review, as well as tyrosine kinase inhibitors. HER2 monoclonal antibodies include trastuzumab, pertuzumab, as well as margetuximab (Wong and Hurvitz, 2014; Schlam et al., 2022). Although these agents have markedly improved the prognosis of HER2-overexpressed breast cancer patients, resistance development, as the case with other chemotherapeutic agents, often limits their efficacy. Of note, 70% of HER2-overexpressing breast cancer patients either are intrinsically resistant to trastuzumab or develop trastuzumab resistance within one year of initiation of treatment (Yang and Klionsky, 2009; Wong and Hurvitz, 2014; Luque-Cabal et al., 2016; Elshazly and Gewirtz, 2022; Schlam et al., 2022). Various molecular mechanisms have been investigated in efforts to restore sensitivity to HER2-targeted therapies (Luque-Cabal et al., 2016; Elshazly and Gewirtz, 2022), among which is that of autophagy.
Autophagy is an intrinsic cellular machinery for recycling damaged and misfolded proteins and other cellular organelles such as endoplasmic reticulum and mitochondria to maintain cellular homeostasis as well as generating energy and metabolic intermediates necessary for cell survival. Autophagy is induced in response to various stimuli including starvation, hypoxia, ER stress, damaged organelles, DNA damage as well as, critically, chemotherapeutic agents (Yang and Klionsky, 2009; Feng et al., 2014; Galati et al., 2019; Xu et al., 2022). The autophagic machinery proceeds through a number of now well-established steps, beginning with the generation of the phagophore, followed by formation of the autophagosomes, fusion between autophagosomes and lysosomes, forming autolysosomes in which the cellular cargo is degraded (Yang and Klionsky, 2009; Feng et al., 2014; Xu et al., 2022; Elshazly and Gewirtz, 2023d). The detailed mechanism for autophagic machinery has been discussed in detail in our previous publication (Finnegan et al., 2022b).

The scientific literature describing autophagy in response to chemotherapeutic modalities as well as radiation generally focuses on the cytoprotective functional form of autophagy. Cytoprotective autophagy is implicated in tumor cell survival, and in certain cases contribute to resistance development, where its inhibition, pharmacologically and/or genetically, results in sensitization of the tumor cell to the anti-neoplastic agents. As an example, resistance to tamoxifen has been associated with the induction of autophagy (Liu et al., 2019). The cytoprotective form has been investigated in multiple clinical trials in an attempt to increase the efficacy of distinct chemotherapeutic modalities by using pharmacological autophagy inhibitors such as hydroxychloroquine (HCQ) (Elshazly and Gewirtz, 2023c; Elshazly and Gewirtz, 2023a). However, three other functional forms of autophagy have been identified,
specifically cytotoxic, cytostatic and non-protective autophagy. In the case of cytotoxic autophagy, autophagy induction contributes to cell death and may be associated with apoptosis (Sharma et al., 2014b); conversely, its inhibition results in improved cell survival; an example is the autophagy induced by chlorpromazine in glioblastoma cells (Gewirtz, 2014; Matteoni et al., 2021; Xiao et al., 2021; Xu et al., 2022). The cytostatic form, as the name indicates, is associated with cellular growth arrest and potentially with senescence, although this linkage is not firmly established (Gohe et al., 2012; Sharma et al., 2014a; Elshazly and Gewirtz, 2023b; Elshazly et al., 2023a). As an example, cytostatic autophagy is induced by vitamin D and the vitamin D analog, EB 1089, in non-small cell lung cancer cells when combined with radiation (Sharma et al., 2014a). The non-protective form of autophagy is not implicated in either cell survival or cell death, as its inhibition neither results in sensitization or loss of drug sensitivity. As an example, in the study by Finnegan et al. (Finnegan et al., 2022a), the combination of Fulvestrant plus Palbociclib induced autophagy in ER+ breast cancer, but autophagy inhibition did not result in either significant sensitization to the drug treatment or a reduction in cell sensitivity to the drug treatments; this form of autophagy is less understood than the cytoprotective and cytotoxic forms since it is unclear to what purpose the tumor cell would engage this mechanism. Although these functions of autophagy are quite distinct, currently the only approach for distinguishing one from the other is to evaluate the impact on cell/drug sensitivity upon pharmacologic or genetic inhibition since no other distinguishing biological or molecular characteristics have been identified.

Mechanistically, HER2 is clearly associated with autophagy induction, as one of the signaling pathways activated by HER2 is mTOR (Janser et al., 2019), which is a negative regulator of autophagic flux (Jung et al., 2010). Furthermore, several studies
have reported that autophagy is associated with HER2 resistance and tumor progression (Vazquez-Martin et al., 2009; Zambrano and Yeh, 2016; Hao et al., 2021). Therefore, in an effort to determine whether autophagy targeting or modulation could be an effective strategy to increase the therapeutic benefits of HER2-targeted therapies, we analyze the nature of the autophagy induced in response to HER2-targeted monoclonal antibodies, (Figure 1).

3. Trastuzumab and autophagy

Trastuzumab, a recombinant HER2-targeting monoclonal antibody, was the first biological drug approved for the adjunctive treatment of HER2-overexpressing breast cancer (Maximiano et al., 2016). Although other available anti-HER2 agents include pertuzumab and lapatinib, trastuzumab remains the gold standard for treatment of this disease subtype (Maximiano et al., 2016). In addition to its utilization for the treatment of patients with HER2-positive early-stage and metastatic breast cancer (Gao et al., 2021), trastuzumab is also utilized in the treatment of HER2-overexpressing gastric carcinoma (Bang et al., 2010). Trastuzumab targets the HER2 extracellular domain with high affinity. De et al. (De et al., 2013) reported that trastuzumab acts at three main molecular levels, specifically the inhibition of HER2–HER3 hetero-dimerization (Way and Lin, 2005), prevention of the proteolytic cleavage of the HER2 extracellular domain and the subsequent formation of the active p95HER2 fragment (Parra-Palau et al., 2014), as well as promoting antibody-dependent cellular cytotoxicity by engaging with Fc receptors on immune effector cells, and phagocytosis toward HER2-positive tumors (Petricovic et al., 2013). Furthermore, trastuzumab has been shown to suppress signaling pathways involved in cellular division, including PI3K/AKT, MAPK pathways
(De et al., 2013), thereby suppressing cell cycle progression, and ultimately halting cell growth and proliferation (Vu and Claret, 2012). Trastuzumab can also be utilized in an antibody-drug conjugate form, whereby the antibody-drug conjugate is selectively and specifically delivered to the target tumor cells. Comprised of a cytotoxic component as well as a linker region, this complex allows for the delivery followed by the internalization of the drug-antibody conjugate, and the subsequent release of highly active cytotoxic agent within cancer cells, ultimately causing cell death (Barok et al., 2014). The first drug-antibody conjugate approved to be utilized in HER2-overexpressing breast cancer is trastuzumab-emtansine, where trastuzumab is conjugated with emtansine, a potent microtubule inhibitory and cytotoxic derivative of maytansine (Dhillon, 2014). Another drug-antibody conjugate approved for HER-positive breast cancer is trastuzumab-deruxtecan, where trastuzumab is conjugated with deruxtecan, a topoisomerase I inhibitor (Barok et al., 2014; Modi et al., 2020). These agents have improved the overall survival in the second and third-line settings with manageable side effect profiles in HER2-positive breast cancer patients (Rassy et al., 2022). Despite the superior clinical activity for trastuzumab either alone or in a conjugation with cytotoxic moieties, resistance development, as is often the case with other chemotherapeutic agents, constrains their efficacy. Various molecular mechanisms contributing to resistance development for HER2-targeted therapies involving trastuzumab have been identified (Elshazly and Gewirtz, 2022) including signaling from other HER receptors, such as HER3 or epidermal growth factor receptor (Garrett and Arteaga, 2011), insulin-like growth factor receptor (Nahta, 2012), activation of PI3K/AKT/mTOR (Saal et al., 2005), overexpression of c-MET (Shattuck et al., 2008) or loss of PTEN (Saal et al., 2005). In addition, autophagy, due to its cytoprotective role in response to various chemotherapeutic agents, may hinder their therapeutic efficacy (Gewirtz, 2014).
3.1. Breast cancer

Vazquez-Martin et al. (Vazquez-Martin et al., 2009) studied the potential contribution of autophagy in the development of resistance to trastuzumab. The protein autophagy marker, LC3II, was significantly elevated in the trastuzumab resistant cell line, Tzb-resistant SKBR3 cells, as compared to the parental SKBR3 cell line. Elevation of LC3II in the resistant cells was confirmed by the immunofluorescence of LC3 puncta. In addition, an increase in the levels of the lysosomal protein, LAMP-1, also shown by immunofluorescence staining, was indicative of increased lysosomal function, consistent with autophagy induction. The induction of autophagy was further confirmed by elevated p62/SQSTM1 degradation in the Tzb-resistant SKBR3 cells as compared to the parental cells.

The role of autophagy in this experimental system was investigated using the pharmacological autophagy inhibitors 3-MA, LY294002 and bafilomycin A1. 3-MA, an inhibitor of early stages of autophagy as showed by Wu et al. (Wu et al., 2010), reduced the viability of Tzb-resistant SKBR3 cells, without affecting that of the parental cells. Similar results were obtained with LY294002, where the trastuzumab resistant cell line proved to be exquisitely sensitive to this agent, which blocks phosphatidylinositol 3-kinase activity and prevents autophagic sequestration as demonstrated by Xing et al. (Xing et al., 2008), when compared to SKBR3 parental cells, suggesting a cytoprotective role of autophagy in the resistant cell line. These results were further validated with bafilomycin A1, which prevents the fusion between autophagosomes and lysosome as showed by Klionsky et al. (Klionsky et al., 2008), demonstrating markedly greater cytotoxicity in Tzb-resistant SKBR3 cells than the SKBR3 cells. The
The cytoprotective function of autophagy in this model was supported by a genetic approach using ATG8/LC3-targeted siRNA. Genetic knockdown of ATG8 sensitized Tzb-resistant SKBR3 cells to trastuzumab treatment, confirming the role of autophagy in the development of resistance to trastuzumab.

Vazquez-Martin et al. (Vazquez-Martin et al., 2009) also showed that trastuzumab treatment promoted autophagy in HER2-dependent BT474 breast carcinoma cells without affecting autophagy levels in HER2-negative MCF-7 cells, as assessed by LC3 expression, lysosomal function and p62/SQSTM1 expression. These data reinforce the premise that HER2 expression is a required element for autophagy induction following exposure to the anti-HER2 monoclonal antibody, trastuzumab.

Rodríguez et al. (Rodríguez et al., 2015) studied the effect of trastuzumab and its relationship with autophagy using 2D and 3D cultures of HER2-overexpressing BT474 cells and HER2-low expressing MCF-7 cells. These investigators showed that trastuzumab treatment mildly increased the percentage of the BT474 cells undergoing G0/G1 cell cycle arrest, with no apoptosis induction, while MCF-7 cells were largely unaffected by trastuzumab treatment (as would be expected in cells that are HER2 low or negative). Trastuzumab treatment increased the expression of LC3 II/I in both 2D and 3D cultures of the BT474 cells, suggesting an active autophagic flux. The latter was confirmed by autophagosome formation detected by immunofluorescence. Further studies of autophagy inhibition in trastuzumab resistant BT474 cell line as well as in the parental cells demonstrated that the combined treatment of trastuzumab together with the autophagy inhibitor 3-MA reduced the viability of both cell lines as compared to each drug alone accompanied by the promotion of apoptosis, consistent with a cytoprotective role for the autophagic flux induced by trastuzumab.
Cufí et al. (Cufí et al., 2013) investigated the potential of autophagy inhibition for restoring sensitivity to trastuzumab in HER2-positive breast cancer cells using trastuzumab-refractory JIMT-1 as well as trastuzumab-sensitive SKBR3 cells. Initially immunoblotting indicated that JIMT-1 cells have higher levels of LC3II than in the SKBR3 cell line, suggesting a higher level of basal autophagic flux in the refractory cells; this observation was confirmed using confocal imaging with intense punctate LC3 fluorescence in the refractory cells. The active autophagic flux in the refractory cells was further validated by the utilization of a GFP-LC3 reporter, indicating higher GFP-LC3 puncta, together with a greater degree of p62/SQSM1 degradation in the refractory cell line. Trastuzumab treatment in the JIMT-1 cells further increased GFP-LC3 puncta, suggesting that trastuzumab promoted autophagic flux. Of note, the dynamics/autophagy induction of the autophagic machinery in the refractory JIMT-1 cells was notably comparable to that observed in SKBR3 cells made resistant to trastuzumab by continued exposure for 10 months, modeling the primary resistance to trastuzumab.

These investigators then proceeded to determine whether the autophagy induced in the refractory cells played a cytoprotective role via genetic inhibition of autophagy using ATG8-targeted shRNA. Genetic autophagy inhibition in combination with trastuzumab resulted in a small but significant reduction in the viability of JIMT-1 cells as compared to the controls, suggesting a possible cytoprotective role for the autophagic machinery induced in this system. Of note, autophagy inhibition increased the efficacy of other HER2-targeted therapies including gefitinib, erlotinib and lapatinib (Cufí et al., 2013). Pharmacologic inhibition of autophagy using CQ in combination
with trastuzumab synergistically reduced the viability of JIMT-1 cells as shown by an MTT assay together with apoptosis induction validated by annexin V staining. These findings were further confirmed by determination of clonogenicity, where the combination of CQ and trastuzumab markedly suppressed the frequency of surviving cells, whereas each drug alone did not affect the clonogenic capacity of JIMT-1 cells. Genetic inhibition of autophagy using shRNA directed to ATG8, ATG5 or ATG 12, or pharmacological inhibition of autophagy using CQ in combination with trastuzumab resulted in upregulation of cleaved caspase-3 levels as compared to either therapy alone, indicative of apoptosis induction. These data rigorously support the premise that the autophagic machinery induced in this model plays a cytoprotective role, participating in the development of resistance to HER2-targeted therapies including trastuzumab, indicating that autophagy targeting could be a viable strategy for tumor cell sensitization. Cufí et al. (Cufí et al., 2013) further validated their results in vivo using JIMT-1 xenograft animal models. The combination of CQ and trastuzumab drastically reduced the tumor volume as compared to each drug alone, confirming the cytoprotective role played in this system. Furthermore, the combination increased the expression of the pro-apoptotic protein, BAX, while reducing that of the anti-apoptotic protein, BCL2, suggesting that inhibition of the autophagic machinery induced apoptotic cell death in this experimental model.

Cufí et al. (Cufí et al., 2012) also investigated which specific autophagy-related genes might be contributing to the development of resistance to trastuzumab in HER2 breast cancer. Initially, they showed that trastuzumab refractory JIMT1 cells have significantly upregulated expression of ATG-12 by qRT-PCR analysis. Furthermore, upon addition of trastuzumab to JIMT1 cells, autophagy-related genes including
PRKAA2, CDKN2A/p16, DAPK1 and ATG12 were further upregulated, consistent with the role for autophagy in response to trastuzumab. To further confirm these results, these investigators analyzed the ATG-12 gene prolif across the Adai (GSE1090) gene expression dataset (includes 56 breast cancer cell lines) as well as across the Neve's gene expression data set (includes 54 widely used breast cancer cell lines), where they classified the cell lines according to whether they were trastuzumab-sensitive or trastuzumab-refractory. Interestingly, ATG-12 mRNA expression was statistically elevated in the refractory cell lines as compared to the sensitive ones in both data sets. Upon performing ATG-12 knockdown using shRNA in JIMT1 cells, the sensitivity of JIMT1 to the cytotoxic effects of trastuzumab was clearly enhanced, suggesting a cytoprotective role for the autophagy induced by trastuzumab in JIMT1 cells. Similarly, ATG-12 inhibition sensitized the JIMT11 cells to various HER1/2 targeted therapies including gefitinib, erlotinib, and lapatinib. Cufí et al. (Cufí et al., 2012) further investigated the effect of ATG-12 knockdown in vivo using the JIMT1 xenograft animal model. Here, as in the cell culture work, trastuzumab in combination with ATG-12 depletion drastically reduced the tumor volume as compared to each treatment alone, confirming the cytoprotective role of ATG-12 and, indeed, the autophagic machinery in this system.

Recently, Franco et al. (Franco et al., 2023) showed that combining CQ with trastuzumab resulted in a synergistic interaction in reducing in the viability of trastuzumab-resistant HER2-positive JIMT-1 breast cancer cells. However, the conclusions that might be drawn from this study are relatively limited given that the work was lacking in the use of autophagy markers as well as experiments where autophagy was genetically inhibited.
In patient-related studies, Koukourakis et al. (Koukourakis et al., 2014) investigated the changes in various biomarkers in twenty-eight patients diagnosed with locally advanced primary breast cancer who had received one cycle of trastuzumab, followed by a new biopsy on day 21, then treated with taxol/trastuzumab chemotherapy for four cycles prior to surgery. Trastuzumab administration markedly reduced clinical and PET-detected tumor dimensions, proliferation index, GLUT-1 as well as HER2 expression. While the percentage of the LC3A expressing cells as well as LC3A/HER2 expressing cells were upregulated, no change was found in LC3B or LC3B/HER2 expressing cells. These results emphasize on the need for further patients-related studies; however, the difficulties associated with monitoring autophagy and modifications to autophagy in patients makes it extremely difficult to identify biomarkers that could be used to determine whether autophagy inhibition might be useful as a therapeutic strategy (Gewirtz, 2016).

Collectively, these results highlight the cytoprotective role of the autophagic machinery in response to trastuzumab in HER2-positive breast cancer as well as the involvement of autophagy in the development of resistance to HER2-targeted therapies, particularly in the case of monoclonal antibody therapy.

3.2. Gastric cancer

In a gastric cancer model, Ye et al. (Ye et al., 2018) studied the relationship between the autophagic machinery and trastuzumab using HER2-positive NCI-N87 parental cells, trastuzumab-resistant NCI-N87 cells, and HER2-negative SGC 7901 cells. Here, trastuzumab treatment reduced the viability of NCI-N87 parental cells, but not SGC 7901 cells based on MTT and CCK-8 assays, consistent with trastuzumab action
specifically against the HER2 receptor. Trastuzumab was also shown to inhibit the colony forming abilities of NCI-N87 parental cells. In contrast to studies described in the breast tumor cells, trastuzumab reduced the expression of LC3 I/II, while promoting p62/SQSM1 accumulation in NCI-N87 parental cells, indicative of autophagy inhibition. Trastuzumab-resistant NCI-N87 cells exhibited an elevation in p62/SQSM1 level as compared to the parental cells, suggesting autophagic flux inhibition. The latter was further confirmed using transmission electron microscopy (TEM), where the resistant cell line exhibited smaller autolysosomes as compared to the parental cells, with no change in the autophagic flux upon trastuzumab addition to the resistant cell line. Interestingly, the autophagy inhibitors, 3-MA, BAF A1 and HCQ reduced the viability of the parental cell line, while only HCQ alone reduced the viability of the resistant cell line whereas no effects were evident by 3-MA or BAF A1 alone. In combination with trastuzumab, they showed that neither BAF A1 nor 3-MA affect the viability of the resistant cells, while HCQ reduced the viability of the resistant cells but to a lower extent than the parental cell line. Mechanistically, they showed that the resistant cell line exhibited an elevation in the phosphorylation levels of Akt and mTOR as compared to the parental cell line, suggesting that Akt/mTOR pathway may play a role in resistance development, as activation of mTOR interferes with autophagy. Therefore, these investigators used everolimus alone, an autophagy inducer, which significantly reduced the viability of both parental and refractory gastric cancer cells to a greater extent than the autophagy inhibitors, as shown by MTT and CCK-8 assays. Furthermore, the levels of both p-mTOR and p-Akt were reduced upon combining trastuzumab with everolimus as compared to trastuzumab alone in the resistant cell line. These results suggested that autophagy inhibition (rather than autophagy induction, as is generally the case) via Akt and mTOR phosphorylation may mediate the resistant pheno-
type in the trastuzumab resistant NCI-N87 gastric cancer cells. Although trastuzumab clearly inhibited the autophagic machinery in this system, no clear conclusion can be built on the nature of the autophagy in this system, especially in the parental cells, within the absence of genetic knockdown studies for autophagy (Klionsky et al., 2021).

It is important to highlight the different roles of trastuzumab in breast and gastric carcinoma regarding the autophagic machinery, which is consistent with the premise that the nature of autophagy is likely to be dependent both on the chemical compound being utilized as well as the cell line/tumor being investigated (Gewirtz, 2014; Elshazly and Gewirtz, 2023d).

4. Trastuzumab-emtansine and autophagy

4.1. Breast Cancer

In addition to trastuzumab in its monoclonal antibody form, the antibody-drug conjugate form, trastuzumab-emtansine, and its relationship with autophagy is worthy of consideration. Liu et al. (Liu et al., 2020) studied the role of the autophagic machinery in the cytotoxicity induced by trastuzumab-emtansine using the HER2-positive human breast cancer cell lines, BT-474 and SK-BR-3 cells. Initially, they showed that trastuzumab-emtansine induced apoptosis in both cell lines based on Annexin V/PI flow cytometric measurements; these outcomes were confirmed based upon the up-regulation of caspase-3/7 activation levels. Trastuzumab-emtansine treatment also induced the autophagic machinery, evidenced by the formation of autophagosomes as
detected by transmission electron microscopy (TEM), LC3 II accumulation, as well as autophagic fluorescent accumulation of Cyto-ID autophagy green dye. Autophagy inhibition using CQ reversed the apoptosis induced in response to trastuzumab-emtansine treatment, indicating that in these studies autophagy had taken on a cytotoxic function. These results were further confirmed using another autophagy inhibitor, LY294002, where trastuzumab-emtansine cytotoxicity was partially suppressed upon autophagy inhibition in both cell lines. Mechanistically, these investigators demonstrated that trastuzumab-emtansine induced the autophagic machinery via the suppression of mTOR phosphorylation. These results were further validated via evidence for the dephosphorylation of both 4E-binding protein 1 (4EBP1) and Protein S6 kinase (p70s6K), downstream targets of mTOR signaling (Jung et al., 2010; Yang et al., 2013). Additionally, the phosphorylation level of Akt was also significantly reduced upon trastuzumab-emtansine treatment. These findings of cytotoxic autophagy with the antibody-drug conjugate likely reflect the activity of the payload and clearly differ from the outcomes where autophagy function was interrogated with trastuzumab alone. At this point, there is relatively limited data relating to autophagy associated with the antibody-drug conjugate.

4.1. Gastric Cancer

In contrast to the findings with breast cancer, Zhang et al. (Zhang et al., 2021) reported that targeting autophagy in gastric carcinoma improves the therapeutic response to trastuzumab-emtansine, using HER2-positive NCI-N87 cells. These investigators showed that trastuzumab-emtansine reduced the viability of NCI-N87 cells in a dose dependent manner, together with the promotion of apoptosis, as shown by the
annexin V/ PI assay as well as the upregulation of activated caspases 3/9 and PARP levels by immunoblotting. Trastuzumab-emtansine also induced the autophagic machinery, as evidenced by the upregulation of LC3 II and p62/SQSM1 degradation showed by immunoblotting. The latter results were further confirmed via increased punctate autophagosome-like fluorescence by confocal microscopy, using Cyto-ID and LysoTracker double staining as well as the formation and aggregation of autophagosomes visualized by TEM, confirming an active autophagic flux. Pharmacological autophagy inhibition using 3-MA and LY294002 markedly enhanced the cytotoxicity of trastuzumab-emtansine as compared to each drug alone, suggesting that the autophagy induced by trastuzumab-emtansine has a cytoprotective role. These investigators further validated their results in vivo using nude mice subcutaneously injected with NCI-N87 cells, where the combination of trastuzumab-emtansine and LY294002 enhanced anti-tumor effect compared to each drug alone. Furthermore, autophagy blockade by LC3II reduction and p62/SQSM1 accumulation, as well as apoptosis induction, via cleaved PARP and caspase 9/3 upregulation, confirmed the cytoprotective role for the autophagic flux induced in this model by trastuzumab-emtansine. Mechanistically, they showed that trastuzumab-emtansine treatment reduced the phosphorylation of S2448 of mTOR, the phosphorylation of S473 on Akt, an upstream activator of mTOR (Memmott and Dennis, 2009), as well as inhibiting the downstream inducers of Akt/mTOR, 4E-BP1 and p70S6K (Jung et al., 2010; Yang et al., 2013). These data in gastric carcinoma are diametrically opposite to the effect of trastuzumab-emtansine - in breast cancer, in which the cytotoxic role predominates, emphasizing on the need for further investigation to clearly define whether autophagy targeting in gastric carcinoma could be a beneficial strategy to increase the effectiveness of trastuzumab-emtansine.

5. Conclusions
HER2-targeted therapies, including monoclonal antibodies, have improved overall survival in patients with HER2-overexpressed malignancies, specifically breast and gastric cancer (Yang et al., 2023). However, as the case with other anti-neoplastic agents, resistance development often limits and compromises their therapeutic activity in the clinic (Elshazly and Gewirtz, 2022). Therefore, overcoming molecular mechanisms that contribute to resistance, such as autophagy, could serve to improve their clinical utility. Autophagy is fundamentally considered to be both a homeostatic and survival mechanism (Xu et al., 2022; Elshazly and Gewirtz, 2023a), whereby cells respond to stress, including starvation and nutrients deprivation (Xu et al., 2022). In the past decade, many clinical trials have been initiated to investigate the possible targeting of autophagy in order to overcome the development of resistance as well as increasing the efficacy of distinct antineoplastic agents (Elshazly and Gewirtz, 2023a; Elshazly and Gewirtz, 2023c). Four different roles of the autophagic machinery have been identified in different tumor models, specifically cytostatic, non-protective, cytotoxic as well as cytoprotective autophagy, ostensibly the resistance-associated form (Gewirtz, 2014). Of note, it is generally accepted in the literature that the nature of the autophagy induced is dependent on the chemical nature of the inducing compound and the cell line/tumor type being utilized (Gewirtz, 2014; Elshazly and Gewirtz, 2023d).

As summarized in Table 1, the predominant role of the autophagy induced in response to trastuzumab is the cytoprotective form, highlighting the possible translation of autophagy targeting as an adjuvant therapy to increase the effectiveness of trastuzumab or to overcome resistance development in HER-2 overexpressing breast cancer. Conversely, the preclinical data suggested that trastuzumab alone did not induce / inhibit the development of autophagy in HER2 expressing gastric carcinoma. Trastuzumab in its alternative form, as an antibody-drug conjugate, demonstrated es-
sentially two different roles in breast and gastric cancer; cytotoxic and cytoprotective, respectively. The effects of trastuzumab and trastuzumab in the antibody-drug conjugate form on autophagy are likely to differ given that the drug, emtansine, a microtubule poison, is the payload being delivered to the tumor cell. Consequently, instead of the sole interaction of trastuzumab with the HER2 receptor, whatever effects are observed with respect to autophagy will reflect some potentially complex but as yet undefined interaction with both the membrane receptor (trastuzumab) and microtubule function (emtansine).

Additional studies confirming the role of autophagy in vivo using different animal models would likely clarify these differences (Klionsky et al., 2021). It is critical to emphasize the major difficulty in extrapolating from preclinical to clinical settings in that no accurate, reliable non-invasive methods are currently available to determine the induction/inhibition of autophagic flux in patients, limiting efforts to measurement of autophagy markers in peripheral blood mononuclear cells (Wu et al., 2018; Bensalem et al., 2021). Regarding the autophagy inhibitors, only HCQ is currently recommended for in vivo studies, as it is the only autophagy inhibitor that is also approved for clinical trials. However, studies by Bristol et al. (Bristol et al., 2013) have argued that the effects of chloroquine and hydroxychloroquine may not be limited to autophagy inhibition. A limited number of preclinical in vivo studies have utilized 3-MA for autophagy inhibition (Bristol et al., 2013; Chude and Amaravadi, 2017). There are other potential autophagy inhibitors being investigated in preclinical studies, for example the ULK inhibitors described by Karmacharya et al. (Karmacharya and Jung, 2023) and Martin et al. (Martin et al., 2018). With regard to the other available monoclonal antibodies, pertuzumab, and margetuximab, as well as trastuzumab-deruxtecan, only
limited data are available regarding the involvement of autophagy in the tumor cell response in either breast cancer or gastric cancer.

6. Data Availability Statement: This article contains no datasets generated or analyzed during the current study.

7. Author Contributions: A.M.E., A.A.E. and D.A.G. contributed to the writing of the manuscript. All authors have read and agreed to the published version of the manuscript.

9. References


8. Conflict of Interest: No author has an actual or perceived conflict of interest with the contents of this article.

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Figure 1. Autophagy induced in response to HER2-targeted monoclonal antibodies. Potential outcomes for the autophagy induced by trastuzumab alone and trastuzumab conjugated with emtansine in breast cancer and gastric cancer.
Table 1. Different forms of autophagy induced in response to HER2-targeted therapies.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Tumor/cell type</th>
<th>Autophagy induction</th>
<th>Role of autophagy</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trastuzumab</td>
<td>Breast cancer; Tzb-resistant SKBR3, SKBR3, HER2-dependent BT474 and HER2-negative MCF-7 cells</td>
<td>Autophagy induced, except in MCF-7 cells</td>
<td>Cytoprotective</td>
<td>(Vazquez-Martin et al., 2009)</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Breast cancer; HER2-overexpressing BT474 cells, HER2-low expressing MCF-7 and resistant BT474 cell line</td>
<td>Autophagy induced</td>
<td>Cytoprotective</td>
<td>(Rodríguez et al., 2015)</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Breast cancer; Trastuzumab-refractory JIMT-1 cells as well as trastuzumab-sensitive SKBR3 cells</td>
<td>Autophagy induced, mainly in the resistant cells.</td>
<td>Cytoprotective</td>
<td>(Cufí et al., 2013)</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Breast cancer; Trastuzumab refractory JIMT1 In vivo using a JIMT-1 xenograft animal model.</td>
<td>Autophagy induced</td>
<td>Cytoprotective</td>
<td>(Cufí et al., 2012)</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Breast cancer; Trastuzumab refractory JIMT1 In vivo using JIMT1 xenograft animal models</td>
<td>Autophagy induced</td>
<td>Cytoprotective</td>
<td>(Franco et al., 2023)</td>
</tr>
<tr>
<td>Compound</td>
<td>Cancer Type</td>
<td>Patients/Cell Lines/Conditions</td>
<td>Autophagy Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------</td>
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</tr>
<tr>
<td>Trastuzumab</td>
<td>Breast cancer</td>
<td>Patients with locally advanced primary breast cancer related studies</td>
<td>The role of autophagy is not established.</td>
<td>(Koukourakis et al., 2014)</td>
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<td></td>
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<td>Gastric cancer; HER-2-positive NCI-N87 cells, trastuzumab-resistant NCI-N87 cells, and HER2-negative SGC 7901 cells</td>
<td>Autophagy inhibited</td>
<td>NA (Ye et al., 2018)</td>
</tr>
<tr>
<td>Trastuzumab-emtansine</td>
<td>Breast cancer</td>
<td>HER2-positive human breast cancer cell lines; BT-474 and SK-BR-3 cells</td>
<td>Autophagy induced</td>
<td>Cytotoxic (Liu et al., 2020)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastric cancer; HER2-positive NCI-N87 cells</td>
<td>Autophagy induced</td>
<td>Cytoprotective (Zhang et al., 2021)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In vivo using nude mice subcutaneously injected with NCI-N87 cells</td>
<td></td>
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