UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of the Unfolded Protein Response in Human Breast Cancer and Trastuzumab Resistance

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by

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The Thesis of Sreekanth Kumandan is approved and it is accepted in quality and form for publication on microfilm and electronically:

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I dedicate this to my mother, Usha Kumandan.

You made me who I am.

I miss you every day.

I also dedicated this to my father, Krishna Kumandan.

Each day I grow up to be a little more like you.

And I thank God for it.

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ABSTRACT OF THESIS

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The HER2 growth receptor is upregulated in approximately 30% of human breast cancer, which remains the leading cause of cancer deaths in women. The HER2 subtype is more aggressive than others, and less responsive to traditional treatments, including chemotherapy and hormone therapy. Due to this decreased responsiveness, trastuzumab, a humanized antibody that blocks HER2 activity, is used. Although trastuzumab treatment is initially very successful, it has been shown clinically that the effectiveness of trastuzumab becomes severely diminished in a majority of patients after approximately one year of treatment.

This occurrence is known as trastuzumab/HER2 resistance, a phenomenon that severely impacts survival rates and treatment options.

The tumorigenic role of HER2 in breast cancer has been linked to its activation of the phosphoinositol-3-kinase (PI3K)/AKT signaling pathway, a critical regulator of cell proliferation, and to lipocalin 2 (LCN2), an onco-protein correlated with tumorgenesis and changes in tumor stage. Previous research in this lab has shown that activation of the unfolded protein response (UPR), a microenvironmental stress adaptation mechanism, results in the increased expression of LCN2. Using $HER2^{+}$ SKBr3 human breast cancer cells, we found that induction of the UPR abrogated trastuzumab-mediated LCN2 downregulation. Furthermore, through use of PI3K/AKT inhibitors, we discovered that the upregulation of LCN2 by the UPR was AKT-dependent. Taken together, this suggests that the UPR may serve to reactivate tumorigenic signaling pathways downstream of HER2, thus initiating trastuzumab resistance, making it a possible novel target in therapy in HER2 positive breast cancer.

Introduction

Human Epidermal Growth Receptor 2 (HER2) is a 185-kilodalton glycoprotein cell surface receptor of the tyrosine kinase family. Its functions are linked to signal transduction pathways important to cell growth and proliferation.[1] 20% to 30% of all patients that are diagnosed with breast cancer are of the HER2 subtype[2] and overexpression of HER2 has been correlated with almost a 20% decrease in both 10-year disease free survival and overall survival.[3,4] In one study of 185 primary breast carcinomas, HER2 positivity was found to be a better prognostic indicator of relapse-free and overall survival than even ER status, tumor size, and histological grade.[5] HER2+ breast cancer is characterized by being a particularly aggressive form, and one that is unresponsive to traditional hormone therapy.[6] In lieu of these therapies, direct targeting of HER2 has been shown to be a very effective method of treatment for this breast cancer subtype. For this reason, trastuzumab, a monoclonal IgG antibody that targets the HER2 receptor, has become a commonly used drug therapy for this atypical breast cancer subtype. Clinical studies have shown that trastuzumab can be an effective single agent in HER2+ breast cancer patients.[7,8] It has also been found to work synergistically with a wide variety of chemotherapeutic drugs such as doxorubicin, cyclophosphamide, paclitaxel, and many others.[9-13] These successes have resulted in trastuzumab commonly being used in adjuvant therapy when a HER2+ diagnosis is present.

Intensive research has been conducted in the attempt to increase understanding of what makes HER2 such a potent oncogenic protein. As a result of this strong research focus, the critical relationship between AKT and HER2 was discovered. AKT is another onco-protein that has been found to play an important role in cancer progression. As a potent signaling kinase associated with cell cycle regulation, cell survival, and angiogenesis,[14,15] AKT has been linked to tumor proliferation, metastasis, and survival in breast cancer. *Yakes et al*[16] show that the importance of trastuzumab's inhibition of HER2 is that it results in the downregulation of cyclin D1, the upregulation p27 levels, and it decreases the cancer cells ability to form colonies. Using an AKT inhibitor, they found they achieved similar results to that of when the cells were treated with trastuzumab. These findings would suggest that the source of the antibody's effectiveness comes from its ability to inhibit the interaction between HER2 and the PI3K/AKT pathway. This hypothesis was confirmed by *Longva et al*[17] who show that treatment with trastuzumab does not result in a downregulation of HER2 in SKBr3 cells but does inhibit the phosphorylation of AKT. Clinically, overexpression of AKT and HER2 in breast cancer patients leads to the lowest benefit when treated with traditional hormone therapy, suggesting that AKT plays a role in resistance against this type of treatment.[6] Additionally, MCF-7 breast cancer cells shown to highly express HER2 see a corollary activation of AKT, leading to multi-drug resistance against commonly used chemotherapeutic agents.[18]

In attempting to understand what the downstream effectors that HER2 and AKT activate are, a research group discovered that one downstream target of HER2 signaling, as well as consequent AKT activation, is lipocalin 2 (LCN2).[19] LCN2 has become a growing topic of interest in the research community. Its native function is that of a bacteriostatic agent that prevents invading pathogens from scavenging host iron for their own survival and growth.[20,21] *Iannetti et al*[22] have shown that LCN2 plays a role in chemotherapeutic drug resistance in thyroid cancer. They also suggest that LCN2's function of iron sequestration is critical to this protection. Iron metabolism has been shown to be a significant factor in disease progression.[23,24] In clinical studies, patients are often found to be anemic prior to chemotherapy and even more so post-treatment.[25] Research done by the *Berger* lab discovered that knocking out Lcn2 in tumor prone mice led to a significant delay in even the prognosis of cancer. Additionally, these Lcn2-KO mice were found to have less total cancer as a whole versus the control group.[26]

In recent years a relationship between LCN2 and human breast cancer has begun to emerge. A survey of urine samples saw the presence of LCN2 in over 86% of the breast cancer patients tested versus the healthy control group.[27] This was confirmed by *Bauer et al*[28], which found that in 68 breast carcinomas that contained NGAL, the secreted protein was correlated with negative receptor status, poor histologic grade, and HER2/neu overexpression. Using a univariate survival analysis, NGAL was associated with decreased disease specific survival

and decreased disease free survival in the entire cohort. In MCF-7 cells, one group found that LCN2 activated the EMT pathway via the ERα/Slug axis.[29]

Though highly effective in treating the difficult HER2 subtype initially, a current problem plaguing the use of trastuzumab is that over time, a majority of patients develop resistance or insensitivity to the drug, a condition referred to as HER2 resistance. When looking back at various clinical studies, it is found that primary resistance to trastuzumab as a single agent is 66% to 88%.[8,9,30-34] In one of these studies, where HER2+ patients had not yet received chemotherapy, although the median time to disease progression was greater in those who received a combination treatment of chemotherapy and trastuzumab versus trastuzumab alone, it still only delayed progression by 7.4 months.[9] Trastuzumab only treatment provided a median disease progression delay of 4.6 months. This would suggest that even when trastuzumab provides a clinical benefit for breast cancer patients, the duration of its efficacy is only around 1 year. This begs the question, what is causing this?

Multiple mechanisms are thought to be behind HER2 resistance.[30,31] One possible source of this problem is the membrane-associated glycoprotein MUC4. MUC4 has been an interesting target for a myriad of reasons, including its ability to activate HER2, inhibit immune recognition of cancer cell, suppress apoptosis, and promote proliferation.[35] Additionally, MUC4 has the ability to interact with HER2 in such a way that it can sterically hinder the binding of the trastuzumab antibody to the receptor.[35,36] Another suggested mechanism includes

compensatory signaling by other members of the Her receptor family. Since trastuzumab binds a domain that is not involved in the dimerization with non-HER2 partners this antibody does not interfere with HER3-HER2 dimerization in SKBr3 cells.[37] Yet another possible source of resistance that also holds substantial weight is alternative downstream signaling of targets normally induced by HER2 activation. There is strong evidence supporting the importance of this. *Chan et al*[38] were able to produce trastuzumab resistant BT474 human breast cancer cells, which had much higher levels of phosphorylated AKT versus their non-resistant counterpart. Additionally, when given a PI3K inhibitor, the trastuzumab resistant cells were phenotypically more like their trastuzumab sensitive counterparts.

If it is HER2s activation of AKT and AKTs potent effectors such as LCN2 that makes it critically important, this suggests that active upregulation of these targets via alternate pathways may lead to what is perceived as HER2 resistance. Looking at alternative pathways that activate LCN2, this lab has shown that LCN2 can indeed be upregulated by the cellular event known as endoplasmic reticulum stress (hereafter referred to as ER stress) in a NF-kb dependent manner.[39] When a cell is met with the conditions of hypoxia, pH imbalance, or nutrient deficiency, these *noxae* can lead to protein misfolding within the endoplasmic reticulum of the cell, a physiological occurrence that is known as ER stress. Mammalian cells activate a protective mechanism when this occurs known as the unfolded protein response (UPR). This cell signaling

pathway(s) is beneficial to the cell in that it will induce cytoprotective measures in order to protect the cell from apoptosis while it attempts to correct the protein imbalance. However, these cytoprotective mechanisms of the UPR have been shown to play a pro-tumor and pro-inflammatory role in cancer progression.[40] The unfolded protein response is compromised of three main cell signaling arms/pathways: ATF6, IRE1, and PERK. All three signaling proteins are bound in an inactive state through endoplasmic reticulum to GRP78, the initial sensor of the UPR. When unfolded/misfolded proteins accumulate in the ER, GRP78 dissociates from these signaling proteins and acts as a chaperone protein in order to reduce the stress.[41] The environmental factors that activate the UPR in healthy cells are analogous to the microenvironmental state found in most solid tumors. Conditions such as hypoxia and nutrient deprivation due to poor vascularization are common in tumors.[42-47] ER stress response proteins such as GRP78, ATF4, and spliced XBP-1 have been shown to be upregulated in a variety of primary human tumors, including breast cancer.[48-54]

It is the UPR's link to inflammation in the tumor microenvironment that has driven research into its role in cancer. The correlation between inflammation in the microenvironment and the consequent tumor outgrowth is well established.[55,56] Critical inflammatory regulators, such as NF-kB and STAT3 as well as some of their downstream targets have been indicated as key players in the tumorigenicity of cancer cells. ER stress and the UPR have already been linked to several conditions that are characterized by chronic inflammation.[57,58]

All three major arms of the UPR – ATF6, PERK, and IRE1a – have been linked to the induction of NF-kB.[59-62] Combining this with knowledge of the cancer tumor microenvironment, a place of frequent hypoxia, nutrient deprivation, and oxidative stress due to hyper proliferation and poor vascularization, the role that ER stress may play in cancer becomes rather significant.

Statement of Hypothesis

Breast cancer is the second most common diagnosis for women in America. Of the five possible subtypes of breast cancer, HER2 positive breast cancer is a dominant form with 25-30% of patients identified.[2] With HER2+ breast cancer being unresponsive to traditional hormone and chemotherapy, doctors and researchers have targeted the unique signaling pathways that underlie this cancer subtype. The humanized IgG antibody trastuzumab can specifically target HER2 receptor domain IV, inactivating it.[63] When used in conjunction with other forms of therapy, the use of trastuzumab has been shown to be extremely effective in improving a remission diagnosis, as well as reducing the probability of recurrence.[64] However, it has become apparent that the initial effectiveness of trastuzumab's ability to stave off cancer progression wears off after a year or so, leaving patients with very few remaining viable treatment therapies to use after resistance has occurred. The frequency of clinical cases of HER2 resistance has led to the creation of alternative drugs such as Tykerb. Tykerb, or Lapatinib, works intracellularly by reversibly binding the phosphorylation sites of both HER2 and EGFR.[65] In trying to understand the cell signaling that underlies HER2 resistance, several theories of its root problems have been suggested. These include the participation of other homologous receptor subunits of the Her family such as HER3 and HER4. A possible source of HER2 resistance comes from the alternative activation of downstream mediators and effectors normally initiated by the HER2 receptor. One such effector is LCN2, a small protein whose native

function is proposed to be that of a bacteriostatic agent against bacterial iron scavenging. It does this by forming a complex with bacterial iron siderophores. LCN2 has been shown to be a downstream effector of HER2 stimulation via the AKT/PI3K pathway.[19] This lab has shown that the activation of the unfolded protein response (UPR) through ER stress can also lead to the activation of LCN2. I hypothesize that ER stress and the consequent UPR response can lead to the activation of HER2 downstream targets such as AKT and LCN2, showing that a possible source of HER2 resistance is the alternative stimulation of critical mediators and effectors generally attributed to HER2 signaling (**Fig. 1**). To test this, I will treat SKBr3 cells with trastuzumab with or without the presence of ER stress looking to see if ER stress leads to the induction of LCN2 expression even in the face of inhibition with trastuzumab. Following this, the cells will be treated with Phenyl Butyric Acid (PBA) in order to see if ER stress inhibition can rescue the upregulation of LCN2 by the UPR. Additionally, SKBr3 cells will be treated with a PI3K inhibitor while in the presence of ER stress to verify if the upregulation of LCN2 is being done in an AKT dependent manner. AKT phosphorylation will be examined at the Ser473 site in order to understand how AKT activity correlates to LCN2 expression. If ER stress and the UPR do indeed abrogate the effects that trastuzumab has on LCN2 by alternatively signaling the PI3K/AKT pathway, this could delineate a new therapeutic target for adjuvant therapy in HER2+ breast cancer patients where resistance has become a serious factor.

Material and Methods

Cell Culture and Treatments

Human SKBr3 cells were grown in RPMI containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone #SH3036.03), 1% non-essential amino acids, 1% sodium pyruvate, 1% HEPES, 1% penicillin/streptomycin/L-glutamine, and .005% b-ME. The timing of drug treatments is indicated in the figure legends. Trastuzumab (Herceptin, Genentech) was a gift from the Moores Cancer Center Pharmacy. Thapsigargin (Enzo Life Sciences #BML-PE180-0001) was used at 300 nM. 4-phenyl butyric acid (SIGMA #P21005-25G) was used at 30 mM. LY294002 (SIGMA #PHZ1144) was used at concentrations ranging from 5 μM to 20 μM. NVP-BEZ235 (Selleck Chemicals # S1009-5mg) was used at concentrations ranging from .1 μM to 5 μM.

RT-qPCR

RNA was isolated from cells using Nucleopsin II Kit (Machery-Nagel #740955). Concentration and purity of RNA was quantified the NanoDrop (ND-1000) spectrophotometer (Thermo Scientific) and analyzed with NanoDrop Software v3.8.0. cDNA was obtained using the High Capacity cDNA Synthesis kit (Life Technologies/Applied Biosystems #4368813), and RT-qPCR was performed on an ABI StepOne system using TaqMan reagents for 50 cycles using universal cycling conditions. Target gene expression was normalized to β-actin, and analyzed using the –ΔΔCt relative quantification method. Validated FAM-labeled Human LCN2, HSPA5 (GRP78), DDIT3 (CHOP), and VIC-labeled Human β-actin

TaqMan primer/probe sets (Life Technologies/Applied Biosystems, #4331182) were used.

Western Blot Analysis

After treatment, SKBr3 cells were washed with ice cold PBS and suspended with the RIPA Lysis Buffer system: 1X RIPA buffer and cocktail of protease inhibitors (Santa Cruz Biotechnologies #sc-24948). Cell lysates were centrifuged at 4 °C at 16,000xg for 15 min and the supernatants were extracted. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific #23335). Samples were denatured and equal concentrations of protein (70-95 μg) were electrophoresed on a Bio-Rad 4-20% Mini-PROTEAN TGX Precast Gels (Bio-Rad #456-1094). The proteins were transferred at 4°C using the Novex XCell II Blot system (Invitrogen #EI0002) for 2 hrs at 30V onto 0.2 μM PVDF membrane. The membranes were probed with goat polyclonal anti-human Lipocalin-2/NGAL antibody (R&D Systems #AF1757), and rabbit monoclonal anti-human P-AKT (Ser473) and Pan-AKT antibodies (Cell Signaling #4060 and #4691, clones: D9E and C67E7). β-actin was used as a loading control and blots were probed with polyclonal goat anti-human β-actin (Abcam #ab8229). Blots were revealed using either HRP-conjugated donkey antibody to goat IgG (Santa Cruz Biotechnology #sc2020) or HRP-conjugated goat antibody to rabbit IgG (Cell Signaling #7074). Bands were visualized using Pierce ECL Blotting Substrate (Thermo Scientific #32106). All samples were normalized to β-actin.

Secreted LCN2 Quantification

Supernatant was extracted from treated SKBr3 cells and concentrated ~40 fold. Concentrated sample were than analyzed using the hLipocalin2 Quantikine ELISA Kit (R & D Systems, #DLCN20).

Results

Trastuzumab Effectiveness on Non-Stressed SKBr3 Cells

In order to confirm previous research showing that trastuzumab downregulates LCN2 in HER2+ breast cancer, SKBr3 cells were plated at 250,000 cells per well in C-DMEM or C-RPMI in duplicate. Cells were either exposed to trastuzumab at 10 or 20 ug/ml, a vehicle control of PBS, or an IgG isotype control; the concentrations used were decided on based on prior research.[19] Following others' work with trastuzumab and SKBr3, an exposure time of 48 hours was used in order to examine the effect of the drug on *LCN2* expression. Analysis by qPCR revealed that trastuzumab causes a 38% reduction in basal *LCN2* expression at 48 hours versus controls (**Fig. 2A**). The effect of trastuzumab on *GRP78* and *CHOP* expression was negligible (**Fig. 2A**). This result confirms that trastuzumab inhibits basal *LCN2* expression in HER2+ cancer cells.

Thapsigargin Abrogates Trastuzumab inhibition of LCN2

After establishing that in non-stressed SBKr3 cells trastuzumab inhibits the basal expression of *LCN2*, the effects of thapsigargin on trastuzumab inhibition were then examined. Thapsigargin is a sesquiterpene lactone that acts as a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) class of enzymes. This allows it to impede the pumping of calcium into the Endoplasmic Reticulum leading to protein misfolding and ER stress. Cells were plated at 500,000 cells per well at a 48-hour total exposure time using

Gamma-1 as the isotype control. Based on previous work done in this lab,[39] thapsigargin was introduced at 30 hours by media replacement resulting in a total exposure time of 18 hours to the cells. Analysis shows a large increase in expression of *GRP78* and *CHOP* in the SKBr3 cells exposed to ER stress via thapsigargin (**Fig. 2B**). This is consistent with the activation of the UPR during ER stress. Looking at *LCN2*, when ER stress is introduced there is over a 80-fold increase in *LCN2* expression versus the Gamma-1 control condition (**Fig. 2B**). This shows that ER stress via thapsigargin may have the ability to override the inhibitory effects of trastuzumab on *LCN2* expression.

ER stress can override the effects of Trastuzumab on LCN2

In order to validate that it is thapsigargin's induction of ER stress that caused the abrogation of trastuzumab inhibition on *LCN2* in SKBr3 cells, PBA was introduced. 4-Phenylbutyric Acid, or PBA, acts as an additional molecular chaperone to GRP78 in times of unfolded protein accumulation, making it an ER stress inhibitor. Using the cell culture conditions from the previous experiment, SKBr3 cells were additionally exposed to PBA for the last 18 hours of the 48-hour treatment time. Examining the expression of *GRP78* and *CHOP* (**Fig. 3**), a large increase in their gene expression is seen when the SKBr3 cells are exposed to thapsigargin, a result that is once again indicative of ER stress. However, when cells are additionally treated with PBA, there is a downregulation of these ER stress response proteins in line with the direct aid given by PBA to the unfolded protein response. This confirms that PBA does in fact act as an ER stress

inhibitor on SKBr3 cells. Looking to the effect of PBA on *LCN2* (**Fig. 3**), a significant decrease in expression is seen in stressed SKBr3 cells when also dosed with the ER stress inhibitor. At 30 mM of PBA, a complete rescue of trastuzumab function on *LCN2* expression is seen. Taken together, these results show that ER stress is capable of overriding the effectiveness of trastuzumab on *LCN2* expression. This data suggest that ER stress may be a possible cause of trastuzumab resistance that occurs in patients with HER2+ breast cancer. Using 2-Site Capture ELIZA, the secretion of LCN2 protein by these cells was examined (**Fig. 4**). In agreement with the gene expression analysis, trastuzumab is able to decrease basal secretion of LCN2 in these breast cancer cells while activation of the UPR via thapsigargin not only abrogates trastuzumab's inhibitory effect but potently upregulates secretion.

ER stress increases the phosphorylation of AKT

In order to determine the possibility that AKT played a role in the upregulation of LCN2 by ER stress, AKT phosphorylation was examined 2, 4, and 6 hours after the introduction of thapsigargin to trastuzumab pretreated SKBr3 cells. As seen in **Figure 5**, trastuzumab does indeed downregulate the activation of AKT at Ser473. However, the thapsigargin induced ER stress robustly reactivates AKT even with pretreatment with trastuzumab. These results suggest that the unfolded protein response mediates the secretion of LCN2 through the PI3K/AKT pathway.

ER stress Signals LCN2 in an AKT-dependent manner

To elucidate the cell signaling mechanisms involved in ER stress activation of LCN2, the AKT/PI3K Pathway inhibitors LY294002 and NVP-BEZ235 were used. It has been previously shown that HER2 activates LCN2 via AKT activation.[19] This lab has shown that there is relationship between LCN2 and NF-kB, a major downstream target of the unfolded protein response. Recent findings have suggested that a link exists between AKT and NF-kB.[59] As with thapsigargin and PBA, LY294002 or NVP-BEZ235 was introduced to SKBr3 cells for the final 18 hours of the 48-hour experiment. LY294002 is a morpholine derivative of quercetin where NVP-BEZ235 is an imidazoquinoline derivative. Both act by inhibiting phospho-oinositide-3-kinases, such as PI3K. As highly selective inhibitors of PI3K, they have, unsurprisingly, a negligible effect on the ER stress proteins *CHOP* and *GRP78* (**Fig. 6A and 6B**). When examining *LCN2* however, a decrease in expression correlated with exposure to either of the AKT inhibitors is seen (**Fig. 6A and 6B**). Looking at SKBr3 cells exposed to trastuzumab, thapsigargin, and LY294002 at 10 mM, we see a 72% reduction in *LCN2* levels versus cells exposed solely to trastuzumab and thapsigargin. NVP-BEZ235 is even more effective at inhibiting the unfolded protein response's upregulation of *LCN2*, reducing its expression by 99.9%. This suggests that ER stress activation of LCN2 is AKT-dependent, similar to activation by HER2. Taken together, this suggests a possible source of trastuzumab resistance as well as a potential solution: inhibition of ER stress in the tumor microenvironment could possibly lead to restoration of trastuzumab's effectiveness against human

breast cancer proliferation. In order to confirm that the effects seen in *LCN2* transcription levels are due to changes in AKT activity, cells were concurrently stressed with thapsigargin and treated with a PI3K inhibitor 9 (**Fig. 7**). The results show that thapsigargin does not activate AKT when PI3K activity is blocked by NVP-BEZ235. This confirms that upregulation of *LCN2* in SKBr3 cells by ER stress is mediated by the PI3K/AKT pathway.

Discussion

The research conducted throughout this study has shown that trastuzumab reduces basal level expression of *LCN2* in SKBr3 human breast cancer cells. When these same cells are additionally exposed to ER stress, the consequent activation of the UPR resulted in a complete abrogation of trastuzumab's inhibition of LCN2. Through the use of an UPR inhibitor, PBA, it is shown that the activation of *LCN2* is indeed ER stress/UPR specific. The success of LY294002 and NVP-BEZ235 treatment in dampening the induction of *LCN2* by ER stress is indicative of AKT/PI3K playing role in this cell-signaling pathway.

Trastuzumab and LCN2 expression

In corroboration with the protein work done by *Leng et al*,[19] here it is seen that trastuzumab has a definitive effect on the onco-protein LCN2 in unstressed human breast cancer cells. This presents another possible benefit of specifically targeting the HER2 receptor. LCN2's link to iron aggregation may provide a critical nutrient supply for aggressive HER2+ breast cancer as well as be a driver of metastasis. Trastuzumab's inhibition of LCN2 expression in breast cancer may one of the reasons it delays disease progression. This correlation between HER2+ breast cancer and LCN2 may also to apply to other cancers where HER2 overexpression plays a role, such as gastric cancers and glioblastomas.[66-68] This suggests that other HER2+ tumor lines should be examined for the LCN2 expression.

ER stress and Trastuzumab's Interplay

This body of research outlines an important new relationship between ER stress and breast cancer. Many researchers are currently trying to delineate the mechanisms behind trastuzumab resistance. HER2 dimerization with HER3 or HER4 has been indicated as a possible alternate pathway for PI3K/AKT activation, making the blocking of the HER2 receptor obsolete. Other mechanisms for resistance have also been suggested, including MUC4 disruption, IGF-1 compensatory signaling, and altered signaling due to PTEN.[35,69,70] Shown in this research is a completely new pathway by which drug resistance may be occurring. The ability of the UPR to abolish trastuzumab inhibition of LCN2 via upregulation of AKT shown here both by protein analysis and gene expression indicates that persistent ER stress in the tumor microenvironment may interfere with the effectiveness of clinical treatment with this antibody.

ER stress Inhibition as a Therapeutic Target

The introduction of PBA to SKBr3 cells confirmed that the high levels of LCN2 expression observed during exposure to thapsigargin were indeed due to the chemical's effect on the unfolded protein response. Additionally, it showed that inhibiting the UPR could restore the effectiveness of trastuzumab on LCN2 inhibition that was lost by the induction of ER stress in these cells.

In current clinical treatment, trastuzumab is without question the first line treatment for HER2 positive breast cancer. When combined with chemotherapy,

it regresses tumor suppression in 30-35% of patients treated.[8] It is been shown to be effective in both early state and late stage cancer.[71,72] Hence, the situation of disease progression during trastuzumab treatment is a serious one. The loss of this most potent therapeutic treatment outside of chemotherapy results in afflicted patients having little recourse. One of the only treatments available to those immune to trastuzumab is lapatinib. Lapatinib is a specific kinase inhibitor that specifically targets HER2 and EGFR. In current clinical use of trastuzumab, the loss of efficacy after long-term treatment is commonly tested for[73] and when found to be occurring, use of trastuzumab is replaced with an adjuvant therapy such as additional treatment with lapatinib.[65] However, this drug has not shown the success that trastuzumab has demonstrated in treating early stage breast cancer. Looking at late stage treatment, the effectiveness it has at delaying disease progression versus trastuzumab is small.[74] Additionally, it has been suggested that lapatinib is not cost effective in terms of pricing.[75] This work suggests that lapatinib's mechanism of action, inhibiting the intercellular phosphorylation site of HER2 thereby preventing PI3K docking and activation, may be indicative of its short-term success in patients. If pathways such as the unfolded protein response are still alternatively activating critical targets of PI3K, such as AKT and LCN2, then this could explain why the overall effect of lapatinib treatment, much like trastuzumab, diminishes over time.

When combining this with a chronically stressed tumor microenvironment[76,77] it paints a picture showing that in the long term, ER stress

in breast cancer tumors may deter the efficacy of trastuzumab's inhibition of AKT mediated LCN2 expression. In fact, it has been shown that HER2 overexpression is correlated with increased angiogenesis and lower levels of hypoxia in breast tumors.[78] This would suggest that inhibiting HER2 via trastuzumab would actually increase the hypoxic state of the cancer. One study found that this was indeed the case, and found more specifically that trastuzumab had a negative impact on the ability of the EGFR inhibitor gefitinib to reduce the percentage of hypoxia viable breast cancer cells, actually abrogating its ability to do so.[79] This increase in hypoxia as a side effect of trastuzumab treatment could then create ER stress and consequently induce the UPR.[80] Given that trastuzumab is initially an effective treatment for HER2 positive cancer, concurrent administration of an ER stress inhibitor could prolong trastuzumab's potency, providing a new type of adjuvant therapy.

There is strong drive in the field of cancer drug research to find new adjuvants to be used with trastuzumab to boost its efficacy. The UPR may provide a new novel target for this type of research. Eventual resistance to trastuzumab has shown to be an unfortunate consequence of treatment. Any and all alternative targets and pathways should be examined for the ability to stave off the onset of resistance.

AKT as Role Player in the UPR-LCN2 Pathway

AKT has been indicated as a mediator for LCN2 expression.[19,81] However, it remains unclear what other cell signaling pathways activate its expression,

specifically in cancer cells. Prior work in this lab has shown that ER stress alone can induce LCN2 expression in a NF-kB dependent manner. Here I present a relationship between LCN2 and ER stress where it appears that AKT is an important mediator. Taken together, this suggests a possible interplay between AKT and NF-kB in the activation of LCN2 by the ER stress response. A relationship between AKT and NF-kB has been suggested before.[82-86] It has even been suggested that NF-kB --an inflammatory mediator that is strongly tied to the UPR response-- actually behaves in an AKT dependent manner.[59] Their observations paint a relationship between AKT activation and activity and the UPR that these experimental findings reinforce. It is the activation of AKT/PI3K pathway that is thought to be why targeting HER2 in cancer cells is so effective.[6,16,17] Further study will be needed in creating a clearly defined relationship between the unfolded protein response, NF-kB and the AKT/PI3K pathway.

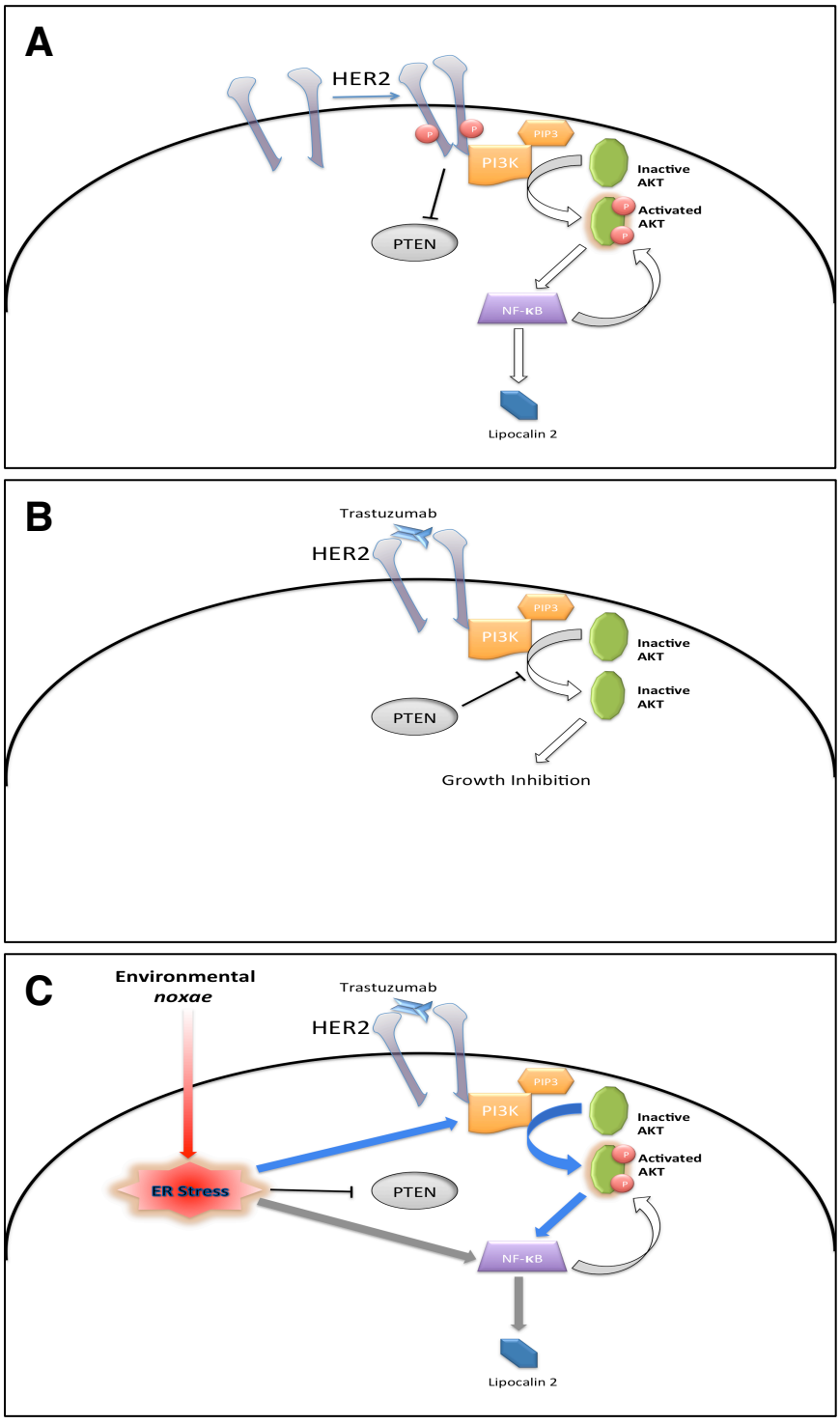
The UPR as a Mechanism of Resistance

Drug resistance is a common outcome of cancer treatment with single agents. In variety of these cases redundant activation of PI3K/AKT pathway has been suggested as a source of resistance.[87,88] The importance of AKT as an escape route for cells against single agent mediated apoptosis would suggest that the importance of the UPR's activation of AKT could extend to cases outside of HER2+ breast cancer and even outside of breast cancer itself. As hypoxia and other stressors that create ER stress are common to solid tumors of all types, the

UPR has been found to be upregulated in a myriad of cancers. Many currently used drugs attempt to fight tumors through the use of a variety of growth inhibitor such as gefitinib and vemurafenib in attempt curb rampant growth, similar to trastuzumab. However, the effectiveness of these treatments may be compromised by the UPR that acts as a redundant activator of the PI3K/AKT pathway and its many targets, such as LCN2. More work must be done to examine if the UPR has the same ability to reactivate important growth signaling pathways in other tumor types where drug resistance is a common occurrence.

Figures

Figure 1. The UPR overrides trastuzumab inhibition of HER2 in cancer cells
(**A**) HER2 homodimerization in unstressed cancer cells inhibits PTEN and activates PI3K/AKT signaling, which in turn leads to the activation of NF-kB, which drives the upregulation of LCN2.
(**B**) Trastuzumab disables HER2 homodimerization inhibiting PI3K docking and derepressing PTEN. The net effect of trastuzumab inhibition is to stop tumor cell growth.
(**C**) Upon induction of the UPR several events occur: (1) the reactivation of the PI3K/AKT axis; (2) the inhibition of PTEN; and (3) the activation of NF-kB. Cumulatively, these three events result in LCN2 upregulation, and reactivation of tumor cell growth despite continuous HER2 inhibition. Notably, UPR-activated NF-kB can also independently contribute to AKT phosphorylation, hence further contributing to LCN2 expression.


A
HER2
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Inactive AKT
PTEN
Growth Inhibition
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NF-κB
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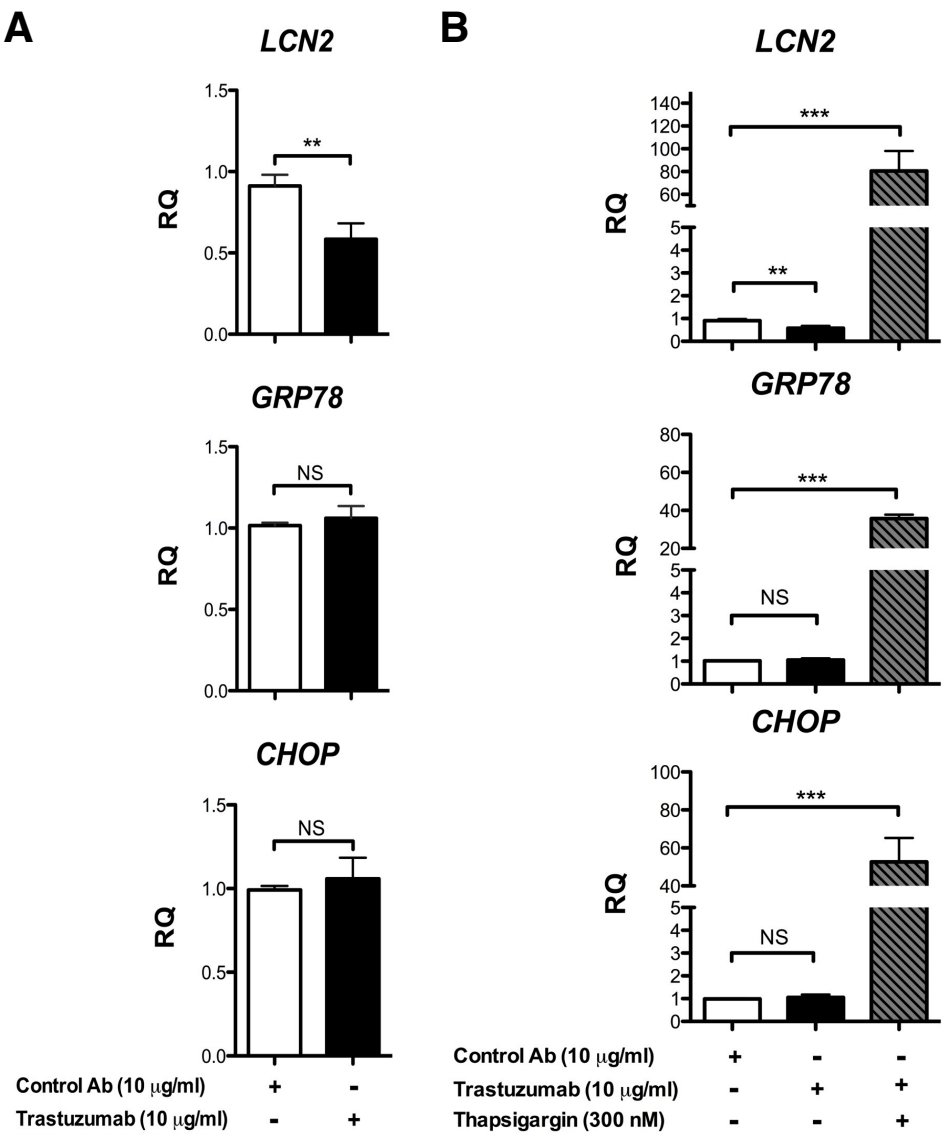

Figure 2. Induction of ER stress in SKBr3 cells rescues inhibition of *LCN2* by trastuzumab under basal conditions

(A) SKBr3 cells were treated with trastuzumab or an isotype control antibody for 48 hrs, and then analyzed by RT-qPCR for *LCN2* transcription and UPR activation.

(B) SKBr3 cells were treated with trastuzumab or an isotype control antibody for 48 hrs. Tg was introduced during the final 18 hrs of treatment. Tumor cell mRNA was isolated and analyzed by RT-qPCR for *LCN2* transcription and UPR activation. Columns indicate fold increase in expression levels (RQ) for each treatment condition. The value of the isotype control was set to 1. Error bars represent the SEM of biological replicates pooled from 5 or more independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, NS = not significant (unpaired, two-tailed *t* test.)

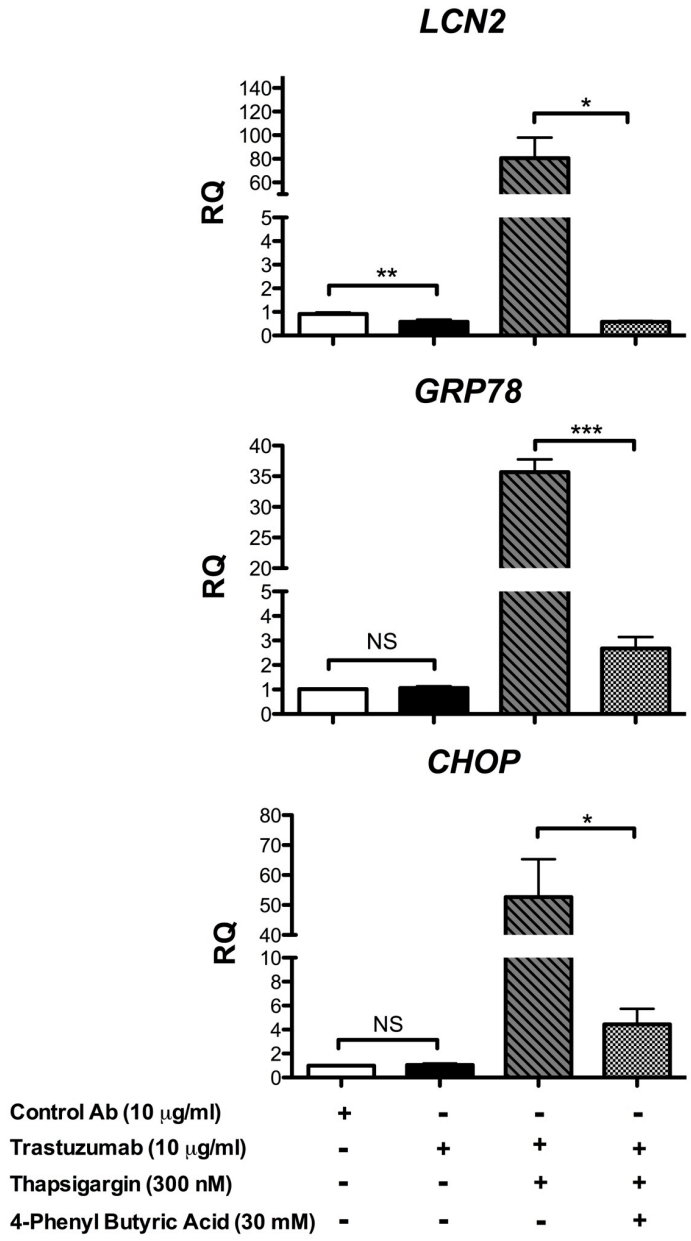

Figure 3. ER stress amelioration restores trastuzumab sensitivity leading to downregulation of *LCN2*

SKBr3 cells were treated with trastuzumab or an isotype control antibody for 48 hrs. Tg and 4-phenyl butyric acid (PBA) were introduced during the final 18 hrs of treatment. Tumor cell mRNA was isolated and analyzed by RT-qPCR for *LCN2* transcription and UPR activation. Columns indicate fold increase in expression levels (RQ) for each treatment condition. The value of the isotype control was set to 1. Error bars represent the SEM of biological replicates pooled from 4 or more independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, NS = not significant, (unpaired, two-tailed *t* test.)

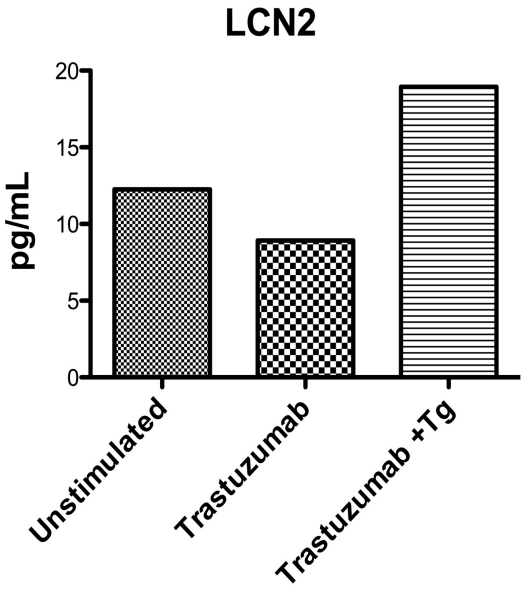

Figure 4. ER stress induction of the UPR increases LCN2 protein secretion negating trastuzumab inhibition

SKBr3 cells were treated with or without trastuzumab for 38 hrs and Tg was introduced during the final 8 hrs of treatment. Cell-free supernatants were concentrated ~40-fold and then analyzed for secreted extracellular LCN2 protein by 2 site capture ELISA. Concentrations were then normalized for cell number.

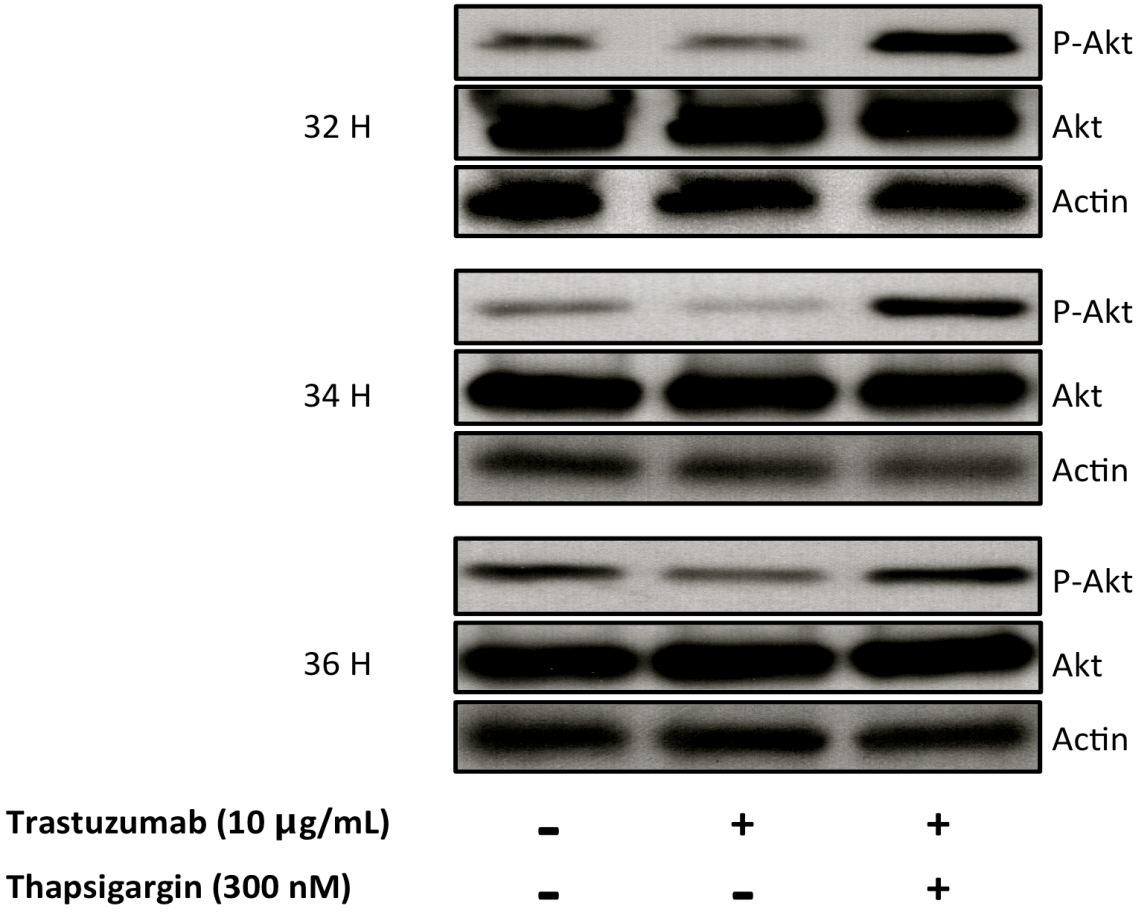

Figure 5. Activation of the UPR by ER stress leads to the reactivation of AKT
SKBr3 cells were treated with or without trastuzumab for 36 hrs and Tg was introduced during the final 6 hrs of treatment. Cells from each condition were extracted at 32, 34, and 36 hrs, lysed, and then interrogated for protein expression by Western blot.

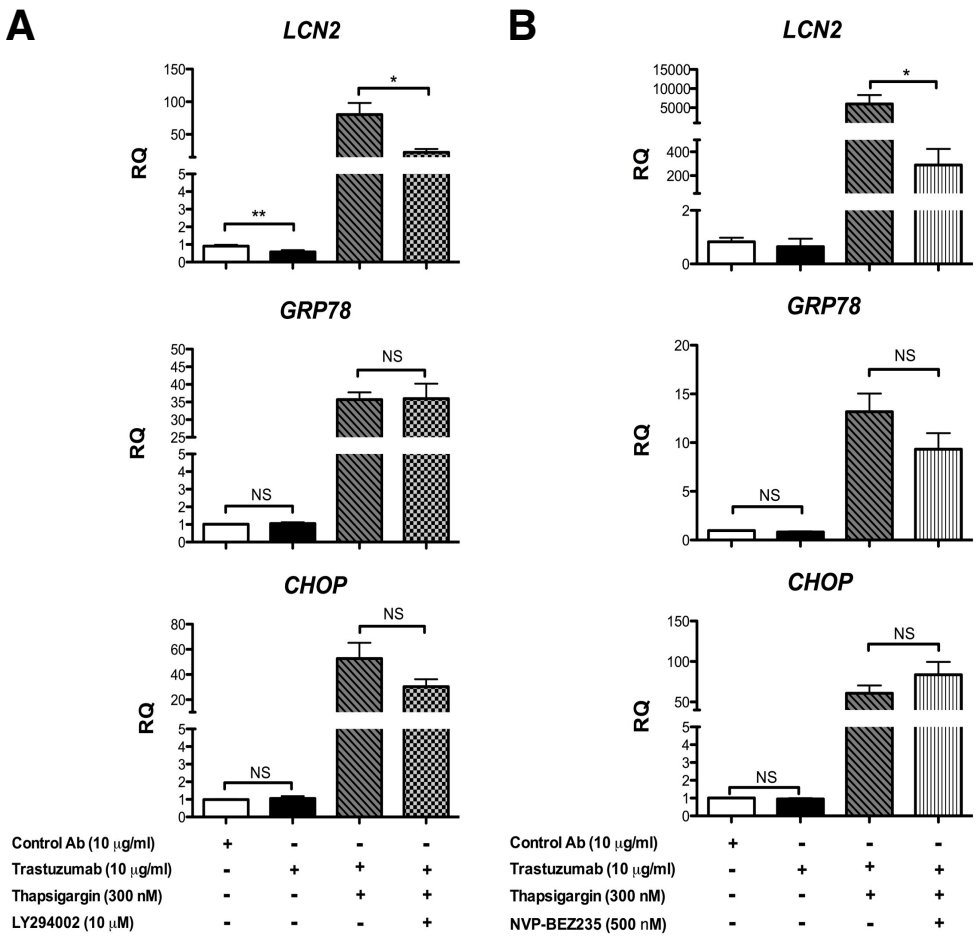

Figure 6. UPR-mediated *LCN2* rescue is partially PI3K/AKT dependent
SKBr3 cells were treated with trastuzumab or an isotype control antibody for 48 hrs. Tg and **(A)** LY294002 or **(B)** NVP-BEZ235 were introduced during the final 18 hrs of treatment. Tumor cell mRNA was isolated, and analyzed by RT-qPCR for *LCN2* transcription and UPR activation. Columns indicate fold increase in expression levels (RQ) for each treatment condition. The value of the isotype control was set to 1. Error bars represent the SEM of biological replicates pooled from 5 or more independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, NS = not significant, (unpaired, two-tailed *t* test.)

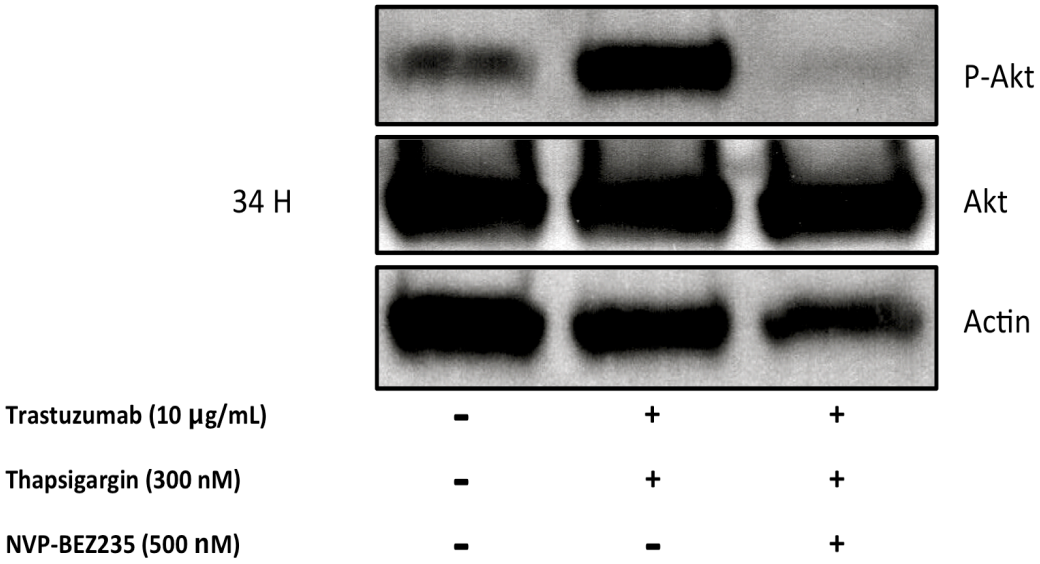

Figure 7. The decrease in LCN2 expression by NVP-BEZ235 is mediated by AKT

SKBr3 cells were treated with or without trastuzumab for 34 hrs. Tg and NVP-BEZ235 was introduced during the final 6 hrs of treatment. Cells from each condition were extracted, lysed, and then interrogated for protein expression by Western blot.

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