Blockade of Epidermal Growth Factor Receptors Chemosensitizes Breast Cancer Cells through Up-Regulation of Bnip3L

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Abstract

Epidermal growth factor receptor-1 (EGFR) and EGFR-2 (HER2) have become major targets for cancer treatment. Blocking antibodies and small-molecule inhibitors are being used to silence the activity of these receptors in different tumors with varying efficacy. Thus, a better knowledge on the signaling pathways activated by EGFR and HER2 may help unravel novel therapeutic targets and molecular markers of response. Here, we show that treatment of breast cancer cell lines with blocking antibodies against EGFR (cetuximab) or HER2 (trastuzumab) promotes the specific induction of proapoptotic Bnip3L and chemosensitization. Moreover, we found that the Bnip3L gene is transcriptionally activated by FoxO3a. Trastuzumab-mediated induction of Bnip3L and nuclear translocation of FoxO3a was also shown in pleural effusion cells from a breast cancer patient. Transfection of breast cancer cells with constitutively active FoxO3a or with Bnip3L promotes sensitization to chemotherapy-induced apoptosis. On the contrary, blockade of Bnip3L expression by a small interfering RNA strategy significantly diminished the chemosensitizing effect of cetuximab. We found also an inverse correlation between EGFR and Bnip3L expression in surgical specimens from patients with breast cancer. Therefore, blocking EGFR or HER2 specifically up-regulates Bnip3L, which is required for chemosensitization of breast cancer cells. This novel pathway provides also the rationale for therapeutic strategies aimed to induce the expression of Bnip3L. (Cancer Res 2005; 65(18): 8151-7)

Introduction

Human epidermal growth factor receptor-2 (EGFR-2, HER2) belongs to a family of four transmembrane receptor tyrosine kinases (EGFR/HER1, HER2, HER3, and HER4; ref. 1) involved in signal transduction pathways that regulate cell survival and proliferation. Overexpression of HER2 occurs in about 30% of human breast cancers and is associated with a poor clinical outcome including short survival time and short time to relapse (2). Additionally, high expression of EGFR in breast carcinoma confers a growth advantage to the tumor cells (3, 4). EGFR is involved in survival signaling, cell migration, metastasis formation, angiogenesis, and reduced responses to chemotherapy. Consistently, preclinical data support the concept that EGFR may be an optimal target for treatment with receptor blocking antibodies in breast cancer, either alone or in combination with chemotherapy (5).

HER2 and EGFR activate a number of signal transduction pathways of which Ras-mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3’-kinase (PI3K)-Akt are the best studied (6). However, down-regulation of HER2 protein by blocking antibodies or antisense oligonucleotides decreased Akt kinase activation but not MAPK activation (7). Furthermore, inhibition of EGFR-induced MAPK signaling has no effects on cell growth of breast cancer cell lines (8). However, inhibition of Akt kinase activity resulted in enhancement of tumor necrosis factor–related apoptosis-inducing ligand–mediated apoptosis. Additionally, a recent study that evaluated the association between phosphorylation status of Akt and MAPK and activity of an EGFR kinase inhibitor in patients with advanced lung carcinoma concluded that the inhibitor was most effective in patients with basal Akt activation (6). Thus, a better understanding of the molecular mechanisms involved in EGFR- or HER2-signaling pathways, mostly those mediated by Akt, may help unravel novel therapeutic targets.

Both EGFR and HER2 activate cell survival pathways, which represents an advantage for tumor cells as they may become resistant to chemotherapy-induced apoptosis. The Bcl-2 family of proteins constitutes a critical intracellular checkpoint in the intrinsic pathway of apoptosis (9). Mammals possess an entire family of Bcl-2 proteins that includes proapoptotic as well as antiapoptotic members. Among the proapoptotic proteins, those belonging to the BH3-only subfamily serve as upstream sentinels that selectively respond to apoptotic stimuli inducing activation of other members, mainly bax and bak, required for execution of cell death. Blockade of expression or activation of BH3-only proteins has been associated with cancer cell survival. To this end, it has been shown that mice deficient in Bad progress, with aging, to diffuse large B-cell lymphoma (10), and that Bcr-Abl supports leukemic cell survival in part through down-regulation of Bim (11). Additionally, Hrk is methylated in colorectal and gastric cancer cell lines, which correlates with loss of expression of this proapoptotic gene. Furthermore, restoration of Hrk promotes apoptosis and enhances Adriamycin-induced cell death (12). We previously described that Hrk expression is silenced in hematopoietic progenitors by a growth factor–dependent transcriptional repres sor mechanism that avoids inappropriate apoptosis in these cells (13). Therefore, inactivation or transcriptional repression of BH3-only proteins may represent an advantage for tumor cells to escape chemotherapy-induced apoptosis.

We report here that EGFR and HER2 control the expression of the BH3-only protein Bnip3L. Blockade of these receptors activates FoxO3a, which functions as a transcriptional activator of Bnip3L.
Although expression of this gene is not sufficient to promote apoptosis of breast cancer cells, it is required for chemosensitization. Finally, the expression of Bnip3L is inversely correlated with the levels of EGFR in surgical specimens of breast cancer, which may help predict the response of tumor cells to target-based or conventional chemotherapy.

Materials and Methods

**Cells.** Human breast cancer cell line MB231 and human epidermal kidney cells HEK293T were maintained in RPMI 1640 (Biochrom KG, Berlin, Germany) supplemented with 10% FCS (Flow Laboratories, Irvine, CA). SUM159 breast cancer cells, derived from a primary tumor with metaplastic carcinoma, were incubated in DMEM/Ham's F-12 (Biochrom) supplemented with 5% FCS, 5 µg/mL insulin, and 1 µg/mL hydrocortisone (both from Sigma, St. Louis, MO).

When indicated, cells were incubated with 50 µg/mL trastuzumab (F. Hoffmann-La Roche Ltd., Basel, Switzerland), 2 µg/mL cetuximab (Merck KGaA, Darmstadt, Germany), 20 µmol/L LY294002 (Calbiochem, San Diego, CA), 10 µmol/L etoposide (VP-16), 0.5 µmol/L paclitaxel (both from Sigma), or 1 µmol/L Adriamycin.

**Confocal microscopy.** Freshly isolated pleural effusion cells were obtained with consent from a 42-year-old female patient with a primary invasive ductal carcinoma. The patient was lymph node positive at primary surgery and had relapsed with metastatic disease at multiple sites within 1 year, after successive rounds of chemotherapy, including antracyclins and taxanes, and treatment with trastuzumab. The malignant cells were separated from the reactive mesothelial cells in the effusion by a 2-hour attachment in tissue culture medium. Isolated breast cancer cells were cultured in DMEM/Ham's F-12 supplemented with 2% FCS, 5 µg/mL insulin, 1 µg/mL hydrocortisone, and 10 ng/mL of EGF (Sigma) in the presence of trastuzumab or LY294002 for 24 hours. Following treatment, cells were assayed for the expression and subcellular localization of Bnip3L and FoxO3a by confocal microscopy with an MRC-1024 laser scanning microscope (Bio-Rad, Hercules, CA) equipped with an Axiosvert 100 invert microscope (Carl Zeiss, Inc., Jena, Germany), as previously described (14). Cells were incubated overnight with rabbit anti-Bnip3L (Oncogene Research, San Diego, CA) or anti-FoxO3a (Upstate Biotechnology, Lake Placid, NY) antibodies followed by a 45-minute incubation with Texas red–conjugated goat anti-rabbit (Jackson ImmunoResearch, Cambridgeshire, United Kingdom) secondary antibodies. Tumor cells were also labeled with FITC- or Texas red–conjugated mouse monoclonal anti-Ki-67 antibody (Vitro SA, Madrid, Spain).

**Tissue microarray and immunohistochemistry.** A total of 35 cases of breast carcinomas were selected from the surgical pathology archives of the Hospital Universitario Marques de Valdecilla. All paraffin-embedded donor tissue blocks were sampled with 0.6-mm punches using a Beecher tissue microarray instrument (Beecher Instruments, Inc., Sun Prairie, WI). Paraffin tissue array blocks containing arrayed core cylinders were subjected to H&E and immunohistochemical staining with mouse monoclonal antibodies against EGFR and HER2 (Novocastra Lab., Newcastle, United Kingdom) and rabbit anti-Bnip3L and anti-Bim antibodies (Calbiochem). Briefly, antigen retrieval was done by boiling sections in citric acid buffer for 90 seconds in a pressure cooker. The EnVision kit (DAKO, Glostrup, Denmark) was used as a visualization system according to the manufacturers’ instructions, in a Technmate 500-220 automatic immunostainer (Biotek, Santa Barbara, CA). Diaminobenzidine was used as the chromogen.

The staining was graded using the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. Specimens that were not interpretable were excluded from the analysis. The tissue microarrays were scored separately by two experienced pathologists (J.C. and J.G-R.), with a very high correlation between scores. A consensus score was determined for spots with discrepant scoring between the two observers. The correlation between the different expression scores was done by the χ² test using the Analysie-It software (Analysie-It, Ltd., Leeds, England).

**Analysis of apoptosis.** Apoptosis was routinely determined by both an enzyme-immunoassay method that quantifies the histone-associated DNA fragments present in the cytosol (Roche, Mannheim, Germany) as described previously (15), and a flow cytometry analysis using a phycoerythrin-labeled Annexin V antibody (Becton Dickinson, Madrid, Spain).

**Electrophoretic mobility shift assay.** Cells were lysed and total nuclear fractions were resuspended in 20 mmol/L HEPES (pH 7.9), 420 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 20% glycerol. Nuclear extracts (5-10 µg of total protein) were incubated with a 32P-labeled double stranded DNA probe from the promoter region of the Bnip3L gene (5'-CTTCCCTGTGTTCCCGTATGG-3'). Samples were run on a 5% non-denaturing polyacrylamide gel in 200 mmol/L Tris-borate, 2 mmol/L EDTA. Gels were dried and visualized by autoradiography. Supershifts were done using rabbit polyclonal antibodies specific for FoxO3a and signal transducers and activators of transcription 3 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Quantitative real-time PCR.** Total RNA was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA). To assess mRNA expression, a quantitative real-time PCR was done as previously described (16). The generated cDNA was amplified by using primers for human Bad (15), Bid (5'-GATGAGCTGACGACTGATGCG-3' and 5'-GATGCTACGGTCCTACGTGTC-3'), Bnip3 (5'-CAGGGCCGGCTTGTAAGACTG-3' and 5'-GCTCTTGAGGACTCTCCTGC-3'), Bik (5'-CACACCAAGTCACTGGCT-3'), Bim (5'-TTGCTGAGTGCTCTG-3'), Bnip3L (5'-TGGTGAATGCGACAGCGC-3' and 5'-CTTGAGGGGGAATCCTG-3'), and β-actin (5'-GGCGGAAACCTCGTGCAGACC-3' and 5'-GGAGGATGTTAGGCTGTTCTG-3').

**Western blot analysis.** Protein expression was determined by Western blotting as previously described (17). Proteins (30-60 µg) were separated on a 12% polyacrylamide gel and transferred to nitrocellulose. Blots were blocked with 3% bovine serum albumin and incubated with rabbit anti-Bnip3L, and anti-HA (Santa Cruz Biotechnology) antibodies, or mouse anti-HER2 (Oncogene Research), anti-EGFR (Becton Dickinson), and anti-β-Tubulin (Sigma) antibodies, and then incubated with goat anti-rabbit or anti-mouse antibodies conjugated to alkaline phosphatase (Sigma). Bound antibody was detected by a chemiluminescence system (Applied Biosystems, Foster City, CA).

**Transfections and gene reporter assays.** A genomic PCR fragment of 767 bp from the promoter region of Bnip3L (Bnip3L-p1), was cloned into Kpn1 and HindIII sites of the plg2-L2 basic Luciferase reporter vector (Promega Corp., Madison, WI). The authenticity of the construct was confirmed by sequencing. HEK293T cells were cotransfected with 1 µg of plg2-Bnip3L-p1 and 50 ng of pRSV-β-gal by lipofection using Superfect (Qiagen, Hilden, Germany). When indicated, cells were cotransfected with plg2-Bnip3L-p1 and 1 µg of a vector containing FoxO3a (GenScript), a constitutively active form of FoxO3a, in which all three inhibitory phosphorylation sites were mutated to alanine (18). Twenty-four hours post-transfection, cell extracts were prepared and analyzed for the relative luciferase activity by a dual-light reporter gene assay system (Applied Biosystems). Results were normalized for transfection efficiency with values obtained with pRSV-β-gal. Site-directed mutagenesis of the FoxO3a consensus site in the plg2-Bnip3L-p1 vector was carried out by using the QuickChange Site-directed mutagenesis kit (Stratagene, CA) with the following primers: 5'-GGCTTTCCTGGCCCGCGTGATGG-3' and 5'-CCATACGGCCGGCCAGGAGG-3'. The Bnip3L-p1 DNA insert was sequenced to verify the mutation. Transfection of cells with the pCNA3 vector containing either HA-tagged FoxO3a (GenScript) or HA-tagged Bnip3L, or with the vector alone were done using Superfect (Qiagen).

**Gene silencing.** Two 21-base oligonucleotides (sense and antisense linked by a loop sequence) of the open reading frame of Bnip3L (5'-ACAACAAGCCTGGAGGAAA-3' and 5'-GACAGTGACTATCTCTG-3') were designed using small interfering RNA (siRNA) design guidelines (GenScript Corp., Piscataway, NJ). The oligonucleotides were cloned into the green fluorescent protein (GFP)–containing pBNA1-U6/LNeo vector (GenScript). MB231 cells were cotransfected with siRNA constructs or empty vector using LipofectAMINE2000 (Invitrogen). Stably expressing cell lines were selected using 12 µg/mL neomycin followed by selection of cells with the highest expression of GFP by cell sorting.
Results

Specific induction of Bnip3L following blockade of HER2 and epidermal growth factor receptor in breast cancer cells. HER2 and EGFR are usually overexpressed in cancer cells and protect them against chemotherapy-induced apoptosis. On the other hand, the execution of apoptosis is mainly mediated by BH3-only members of the Bcl-2 family. Thus, we argued that survival signals triggered by EGFR receptors could impede the expression of proapoptotic BH3-only genes, conferring resistance to apoptosis upon treatment with chemotherapy. To approach this, we selected breast cancer cell lines that express both EGFR and HER2 proteins (Fig. 1A). MB231 cells were treated with either trastuzumab (19) or cetuximab (20) to block HER2 or EGFR, respectively, and the expression of BH3-only genes were then analyzed by real-time quantitative PCR. We showed that blocking these receptors specifically induced the expression of Bnip3L (Fig. 1A). Both treatments provoked at least a 4-fold induction of Bnip3L mRNA levels compared with untreated cells. Furthermore, we observed a time-dependent induction of Bnip3L protein following treatment with trastuzumab or cetuximab for 24 to 72 hours, as assessed by Western blot analysis (Fig. 1A). If a proapoptotic protein were overexpressed in breast cancer cells, one would expect a direct induction of apoptosis or alternatively a sensitization to apoptotic stimuli. Treatment of MB231 cells with cetuximab did not induce apoptosis; however, it clearly enhanced the apoptotic response of cells cotreated with VP-16 (Fig. 1B). Similar results were obtained with another breast cancer cell line, SUM159, which shows an expression pattern of EGFR and HER2 similar to that in MB231 cells (Fig. 1A). SUM159 cells were treated with blocking antibodies and after 24 hours the mRNA levels of Bnip3L were found to increase >3-fold over untreated cells (Fig. 1C). We also showed that neither cetuximab nor trastuzumab promoted apoptosis of these cells (Fig. 1D). In addition, moderate apoptosis was detected in SUM159 cells treated with VP-16. However, cotreatment with blocking antibodies and VP-16 notably enhanced the apoptotic response of tumor cells (Fig. 1D). These data suggest that expression of Bnip3L may be related to chemotherapy sensitization rather than to induction of apoptosis in breast cancer cells.

Bnip3L gene is transcriptionally activated by FoxO3a. To analyze the transcriptional pathway that leads to Bnip3L expression, we searched for transcription factor recognition sites in the Bnip3L gene promoter and found a putative binding sequence for FoxO3a, 453 bases upstream from the transcription start site (Fig. 2A), that closely matched the 8-bp consensus sequence described previously (21). Then, a 717-bp fragment from the promoter region of Bnip3L, encompassing the transcription start site and 36 nucleotides of exon 1, was cloned into a promoterless luciferase reporter vector (Bnip3L-Luc). We found that the levels of luciferase activity in HEK293T cells cotransfected with Bnip3L-Luc and a constitutively active FoxO3a mutant, FoxO3a(A3), were at least 15-fold higher than those in cells transfected with the reporter vector alone (Fig. 2B). Then, we made sequential deletions of the promoter and analyzed their capacity to trans-activate the luciferase gene in response to FoxO3a(A3) expression. A first deletion to nucleotide −525 that maintained the FoxO3a site did not decrease significantly the induction of luciferase activity. However, all other deletions that removed this consensus sequence abrogated the transcriptional activation induced by FoxO3a(A3) (Fig. 2B). Furthermore, activation of the Bnip3L promoter was abolished when a mutagenesis strategy changed four bases within the FoxO3a site (Fig. 2C). The activity of FoxO3a is blocked by phosphorylation through the serine-threonine kinase Akt, a well-known downstream mediator of PI3K (22, 23). Thus, inhibition of this pathway would allow the activation of endogenous FoxO3a. As shown in Fig. 2C, the PI3K inhibitor LY294002, increased about 5-fold the luciferase activity. Based on these data, we determined whether FoxO3a mediated the endogenous expression of Bnip3L. MB231 cells were transfected with FoxO3a(A3), and then the expression of Bnip3L mRNA was analyzed by real-time PCR. After 24 hours of transfection, the mRNA levels of Bnip3L were significantly up-regulated (about 2.5-fold) and increased to almost 4-fold by 48 hours (Fig. 3A). The increased levels of FoxO3a may be related to...
indicated the expression of the mutant transcription factor during this time interval (Fig. 3A).

To directly prove the binding of FoxO3a to the Bnip3L promoter, nuclear extracts from MB231 cells were incubated with a radiolabeled probe containing the FoxO3a site and then subjected to electrophoretic mobility shift assay. We detected a protein-DNA complex that was partly supershifted in the presence of antibodies against FoxO3a but not in the presence of an irrelevant antibody (Fig. 3B). Overall, these data indicate that FoxO3a specifically binds to the sequence found in the Bnip3L promoter and induces transactivation of this gene.

Activation of FoxO3a-Bnip3L transcriptional pathway sensitizes breast cancer cells to chemotherapy-induced apoptosis. To translate our previous results to a more physiologically relevant model, we isolated pleural effusion cells from a patient with breast cancer and cultured them in the presence of trastuzumab or LY294002. After 24 hours of treatment, isolated cancer cells were assayed for the expression and subcellular localization of Bnip3L and FoxO3a by confocal microscopy. We observed that following treatment with either trastuzumab or LY294002, FoxO3a translocates to the nucleus and Bnip3L is up-regulated in the cytoplasm of breast cancer cells (Fig. 3C), which indicates that blockade of HER2 or inhibition of PI3K activates the FoxO3a-Bnip3L transcriptional pathway in *in vitro* treated primary tumor cells.

As FoxO3a induces the expression of Bnip3L and Bnip3L seems to correlate with chemosensitization, we asked whether activated FoxO3a could sensitize breast cancer cells to chemotherapy. We stably expressed HA-tagged FoxO3a(A3) in SUM159, as detected by Western blot with anti-HA antibodies (Fig. 4A), and found increased levels of Bnip3L mRNA (about 3-fold) compared with cells transfected with the empty expression vector (Fig. 4A).

**Figure 2.** Bnip3L promoter region responds to activated FoxO3a transcription factor. A, scheme of the Bnip3L promoter showing the DNA sequence of wild-type and mutant FoxO3a site (modified nucleotides are underlined). B, HEK293T cells were transfected with a luciferase reporter vector containing sequential deletion fragments of the Bnip3L promoter either in the presence or in the absence of a constitutively active FoxO3a mutant [FoxO3a(A3)]. Following 24 hours of transfection, cell extracts were prepared and analyzed for the relative luciferase activity. Results were normalized for transfection efficiency with values obtained with pRSV-β-gal. C, cells transfected with the reporter vector were cultured in the presence of the PI3K inhibitor LY294002 for 6 hours and then nuclear lysates were prepared and analyzed for luciferase activity. Reporter vector containing a mutant FoxO3a site (marked with an x) was introduced into HEK293T cells and luciferase activity in response to activated FoxO3a was determined. Columns, means of three independent experiments; bars, ±SD.

**Figure 3.** FoxO3a-Bnip3L transcriptional pathway in cell lines and primary breast cancer cells. A, MB231 cells were transfected with FoxO3a(A3), and then the mRNA levels of Bnip3L and FoxO3a were analyzed by quantitative reverse transcription-PCR. Columns, means of triplicate analyses; bars, ±SD. B, formation of FoxO3a-DNA binding complexes was determined by an electrophoretic mobility shift assay using a radiolabeled probe from the Bnip3L promoter containing the FoxO3a site. Nuclear extracts were preincubated with 100-fold molar excess of unlabeled Bnip3L-specific probe (100 × SP) or an irrelevant nonspecific probe (100 × NSP). Anti-FoxO3a and irrelevant anti-signal transducers and activators of transcription 3 (Stat3) antibodies were used to show the specific binding of FoxO3a. C, pleural effusion-derived breast cancer cells were cultured in the presence of trastuzumab or LY294002 and the intracellular localization and expression of FoxO3a (green) and Bnip3L (red) was determined by confocal microscopy. The proliferation marker Ki-67 was detected in the nuclei of tumor cells (red staining in the top and green staining in bottom).
Interestingly, treatment with Adriamycin, VP-16 or paclitaxel promoted a stronger apoptotic response in cells transfected with FoxO3a(A3) than in control cells (Fig. 4B). Moreover, we transiently transfected HEK293T cells with HA-tagged Bnip3L to study whether expression of this single gene is enough to sensitize cells to chemotherapy-induced apoptosis. HEK293T is a highly efficient cell model for transient transfection experiments, and as shown in Fig. 4C, these cells clearly expressed Bnip3L protein after 24 hours of transfection. Consistent with our previous data, Bnip3L was not able per se to induce cell death. However, it enhanced the response to chemotherapy about thrice over levels detected in vector-transfected cells (Fig. 4D).

We further confirmed the relevance of Bnip3L in chemotherapy sensitization by using an RNA interference approach to block Bnip3L expression. A double-stranded siRNA-like specifically targeted against Bnip3L was stably expressed in MB231 cells using a mammalian expression vector. Specific siRNA severely impaired the expression of Bnip3L when cells were treated with cetuximab, trastuzumab, or LY294002, compared with cells transfected with the empty vector (Fig. 5A). Interestingly, blockade of Bnip3L expression reduced the chemosensitizing activity of cetuximab. As shown in Fig. 5B, cotreatment with cetuximab and VP-16 promoted much lower levels of apoptosis in siRNA-transfected MB231 cells than in vector-transfected cells. These results suggest that activation of the FoxO3a-Bnip3L transcriptional pathway leads to chemotherapy sensitization in breast cancer cells.

### Bnip3L expression in breast cancer tissues correlates with the levels of epidermal growth factor receptor

Based on our data with cell lines, we investigated whether the expression of Bnip3L protein correlates with the levels of EGFR and HER2 in breast tumors by immunohistochemical staining. Tissue sections of 35 primary breast carcinomas were analyzed simultaneously on a tissue microarray, of which 24 (68%) were ductal carcinomas, five (15%) were lobular carcinomas, and six (17%) were infiltrating carcinomas. We found a highly significant ($P < 0.0001$) inverse correlation between the expression of EGFR and Bnip3L (Fig. 6A and B). Nineteen tissue specimens (54.3%) were EGFR positive and Bnip3L negative, whereas 13 (37.1%) were EGFR negative and Bnip3L positive. Only three samples were negative for both EGFR and Bnip3L and no double-positive samples were found. On the contrary, no correlation was established between Bnip3L and HER2 (data not shown).

Recently, it has been described that Bim, another proapoptotic BH3-only member of the Bcl-2 family, is transactivated by FoxO3a in sympathetic neurons (24) and paclitaxel-treated MCF7 cells (25). Therefore, as shown in Fig. 6, Bim not only shows a different expression pattern than Bnip3L but positively correlates with EGFR ($P < 0.003$).

### Discussion

The varying efficacy of cytotoxic drugs has driven the development of target-based strategies for cancer treatment. Activation of EGF receptors triggers a network of signaling pathways that promote tumor cell proliferation, migration, angiogenesis, and inhibition of apoptosis. As a result of these findings, a number of strategies against EGFR and HER2, including kinase inhibitors and blocking antibodies, have been developed to block downstream signaling events and, consequently, tumorigenesis (26). However, their clinical use is still hampered by modest activities or nonoptimal combination regimens (27, 28). Despite these therapeautic approaches, a range of potential targets exists within the signaling pathways triggered by EGF receptors.

Here we have shown that the BH3-only protein Bnip3L is specifically up-regulated by blocking EGF or HER2 in breast cancer cells. Expression of Bnip3L is not enough to promote...
apoptosis but greatly sensitizes cells to chemotherapy-induced apoptosis. Recently, it has been proposed a model for BH3 domains by which Bid-like BH3 domains are effective inducers of apoptosis, whereas Bad-like BH3 domains sensitize cells against apoptotic stimuli (29). Consistently, Bnip3L BH3 shares a higher degree of similarity with Bad BH3 than with Bid BH3 domain (30).

We have also shown that the Bnip3L gene is transactivated by FoxO3a, a forkhead transcription factor that has been associated with apoptosis. It has been described that activation of FoxO3a in a mouse pre-B cell line promoted up-regulation of the BH3-only protein Bim and apoptotic cell death (18). Moreover, hematopoietic progenitors isolated from Bim-deficient mice exhibited reduced levels of apoptosis following activation of FoxO3a. Thus, expression of Bim seems to promote apoptosis rather than sensitization to apoptotic stimuli in this hematopoietic cell system. Consistently, Bim BH3 is a Bid-like BH3 domain and according to the model explained above Bim activates the mitochondrial apoptotic pathway.

Akt has been shown to negatively regulate members of the FoxO family of transcription factors, including FoxO3a (18, 31). Accordingly, we show that inhibition of the PI3K/Akt signaling pathway allows nuclear translocation of FoxO3a and promotes the expression of Bnip3L in primary breast cancer cells. Furthermore, ectopic expression of Bnip3L or active mutant FoxO3a was sufficient to sensitize cells to chemotherapy-induced apoptosis, suggesting that the chemosensitizing effects of blocking antibodies to EGFR and HER2 are mediated by the FoxO3a-dependent expression of Bnip3L. This was further confirmed by a knockdown strategy to selectively silence the Bnip3L gene. Interestingly, activation of Akt by HER2/PI3K plays an important role in conferring a broad-spectrum chemoresistance on breast cancer cells (32, 33). Moreover, we showed that blockade of EGFR did not induce apoptosis of MB231 but clearly enhanced the apoptotic signal of cells treated with VP-16. Consistently, blocking anti-EGFR antibodies have been shown to chemosensitize ovarian, breast, and colon cancer cells by using both cancer xenografts and tumor cell lines (34). Overall, FoxO3a-Bnip3L transcriptional pathway provides the rationale for novel therapeutic strategies aimed to chemosensitize tumor cells.

A number of studies have shown an association of HER2 gene amplification or protein overexpression with prognosis and response to anticancer therapies in breast cancer (35). Moreover, amplification or overexpression of HER2 is the basis of eligibility for trastuzumab therapy. Based on the association that we had established between HER2 and Bnip3L, we asked whether the expression levels of these two proteins were correlated. Analysis of breast cancer surgical specimens on a tissue microarray showed that Bnip3L did not correlate with HER2 but was inversely correlated with EGFR. A likely explanation for this result is that EGFR levels may be rate limiting for signaling. EGFR family members need to dimerize to initiate signal transduction, and the composition of these dimers is thought to influence downstream signaling pathways and to determine the biological response (36). HER2 is a common partner of EGFR and HER3 in breast cancer cells (37). Therefore, taking into account that the majority of samples analyzed in the tissue microarray were highly positive for HER2, the expression of EGFR may be key to the formation of functional heterodimers. In support of this, it has been described that EGFR-HER2

![Figure 5](https://example.com/figure5.png)

**Figure 5.** The expression levels of Bnip3L modify the chemosensitizing effect of cetuximab. A, MB231 cells transfected with a vector expressing a siRNA targeted against Bnip3L were treated with blocking antibodies or with the PI3K inhibitor LY294002, and the expression levels of Bnip3L were determined by quantitative reverse transcription-PCR. B, siRNA-transfected cells were treated with cetuximab or the combination of cetuximab and etoposide and then apoptotic cell death was measured by an enzyme-immunoassay method as described in Fig. 1D. Columns, means of triplicate analyses; bars, ± SD.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Bnip3L expression inversely correlates with the levels of EGFR in breast cancer tissue. A, a tissue microarray containing tissue sections from 35 primary breast carcinomas was analyzed by immunohistochemistry with antibodies against EGFR, Bnip3L, and Bim. B, two representative samples from the tissue microarray showing the inverse correlation between EGFR and Bnip3L. Brown precipitate indicates the presence of the studied protein. Original magnification, ×40.
heterodimers are rate limiting for EGF-mediated proliferation of ovarian cancer cells (38).

A recent study suggested that EGFR mutations may help to predict sensitivity to gefitinib, an EGFR kinase inhibitor, at least in a group of patients with lung carcinoma (39). However, advances in response prediction require a better molecular understanding of the EGFR/HER2 signaling network and improved methods to determine the dependence of individual tumors on EGF receptor signaling pathways for growth and survival advantage. Accordingly, we have delineated a pathway that involves EGFR/HER2, PI3K-Akt, and FoxO3a-Bnip3L in controlling the response to chemotherapy in breast cancer cells.

Together, this study supports an important role for Bnip3L in mediating chemosensitization of breast cancer cells by blockade of EGFR or HER2 and suggests a novel target at which therapeutic interventions could be made to overcome drug resistance.

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References


5. Luna J. Interleukin 3-dependent activation of DREAM is involved in transcriptional silencing of the apoptotic Hrk gene in hematopoietic progenitor cells. EMBO J 2001;20:2286–92.


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