

# Trastuzumab signaling in ErbB2-overexpressing inflammatory breast cancer correlates with X-linked inhibitor of apoptosis protein expression

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## Abstract

Inflammatory breast cancer (IBC) patients show poor survival and a significant incidence of epidermal growth factor receptor-2 (ErbB2) overexpression. A distinct mechanism involving increased expression of X-linked inhibitor of apoptosis protein (XIAP) and survivin, key members of the inhibitor of apoptosis protein (IAP) family, was observed post-trastuzumab (an ErbB2 monoclonal antibody) treatment in an ErbB2-overexpressing, estrogen receptor negative, IBC cellular model, SUM190PT, isolated from a primary IBC tumor. In contrast, a decrease in the IAP expression was observed in the non-IBC, ErbB2-overexpressing SKBR3 cells in which trastuzumab treatment also decreased p-AKT and cell viability. Further, in SUM190PT cells, therapeutic sensitivity to GW583340 (a dual epidermal growth factor receptor/ErbB2 kinase inhibitor) corresponded with XIAP down-regulation and abrogation of XIAP inhibition on active caspase-9 release. Specific small interfering RNA-mediated XIAP inhibition in combination with trastuzumab caused decrease in inactive procaspase-9 and inhibition of p-AKT corresponding with 45% to 50% decrease in cell viability in the SUM190PT cells, which have high steady-state p-AKT levels. Further, embelin, a small-molecule inhibitor that abrogates binding of XIAP to procaspase-9, caused significant decrease in SUM190PT viability. However, embelin in combination with trastuzumab failed to affect SUM190PT viability because it has no direct effect on XIAP, which is induced by trastuzumab treatment. These data have identified a novel functional link between ErbB2

signaling and antiapoptotic pathway mediated by XIAP. Blockade of the IAP antiapoptotic pathway alone or in combination would be an attractive strategy in IBC therapy. [Mol Cancer Ther 2008;7(1):38–47]

## Introduction

Inflammatory breast cancer (IBC) is an aggressive, highly invasive tumor, with the worst clinical outcome among breast cancers that was first described in 1924 (1). IBC is defined by the invasion and growth of tumor emboli in dermal lymphatics. Contrary to the earlier belief that IBC is simply a neglected locally advanced breast cancer, it is now regarded clinicopathologically as distinct from neglected locally advanced breast cancer based on aggressive features such as rapid clinical progression (2) and resistance to chemotherapy and radiotherapy (3, 4). This is attributed to estrogen receptor negativity, which constitutes 80% of IBC tumors (5), and ErbB2 positivity (42.3% IBC versus 17.5% non-IBC; ref. 5). Loss of estrogen receptor and expression of ErbB2 have both been previously shown to correlate with lower disease-free survival (6, 7). Prognosis for women diagnosed with IBC is poor with only a 26.7% 10-year overall survival rate compared with 44.8% for non-IBC locally advanced breast cancer tumors (8). Furthermore, women are often diagnosed at a younger age (58 years old for IBC versus 63 years old for non-IBC), have higher-grade tumors at diagnosis (risk ratio of 3.1 for IBC versus 1.6 in non-IBC), and have more lymph node involvement (risk ratio of 4.7 versus 2.5; ref. 9). Even with neoadjuvant and multimodality treatment, disease-free survival is still poor (10, 11) and there is an unmet clinical need for new therapeutic strategies.

Increased overexpression of ErbB2 in IBC patients has made it an attractive therapeutic target. ErbB receptor signaling is largely regulated by a family of peptide ligands (epidermal growth factor family members) that bind to their cognate ErbB receptors (ErbB1, ErbB3, and ErbB4), with ErbB2 lacking an exogenous ligand (12). Binding of ligand to its cognate ErbB receptor(s) triggers the formation of receptor homodimers or heterodimers (with ErbB2 the preferred heterodimeric partner) and induces autophosphorylation of tyrosine residues within the cytoplasmic tail of the receptor. Lacking an exogenous ligand, ErbB2 is autophosphorylated and transactivated by its heterodimeric partner. ErbB2 contains six tyrosine autophosphorylation sites that serve as docking sites for phosphotyrosine-binding domain and/or Src homology 2-containing proteins, which in turn activate downstream mitogen-activated protein kinase (MAPK)-Erk1/2 and phosphatidylinositol 3-kinase (PI3K)-Akt proliferation and survival signaling pathways (12). Trastuzumab (Herceptin)

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is a Food and Drug Administration–approved humanized anti-ErbB2 monoclonal antibody for treating patients with ErbB2-overexpressing breast cancers (13) and is used in various combinations, with objective response rates between 15% and 26% (13, 14). However, subsets of women with ErbB2-overexpressing tumors do not respond and resistance is common (15, 16). Lapatinib (GW583340/GW572016/Tykerb), a dual inhibitor of the oncogenic ErbB2 and ErbB1 receptor tyrosine kinases, blocks ErbB2 tyrosine autophosphorylation with consequential downstream inhibitory effects on MAPK-Erk1/2 and PI3K-Akt growth/survival signaling in tumor cell lines, tumor xenografts, and in patients, notably those with ErbB2-overexpressing breast cancers where lapatinib induces tumor cell apoptosis (17–19). In a recent clinical trial, lapatinib showed a 50% clinical response rate in ErbB2-overexpressing IBC patients compared with ErbB2 overexpressing non-IBC patients (20); however, acquired resistance is a common outcome even in those patients who show an initial clinical response.

It is clear that deregulation of most growth-promoting factors triggers apoptosis in a “normal cell” and this fundamental characteristic is lost in cancers (21, 22). In part, this failure is due to defects in caspase activation, the execution phase of apoptosis. X-linked inhibitor of apoptosis protein (XIAP) is one of the most potent and versatile caspase inhibitors and is part of a family of proteins called BIRC/IAP proteins (23). This family consists of proteins with 1 to 3 baculovirus IAP repeats, which are highly conserved domains necessary for function. BIRC/IAP homologues have been found across many species, including *Caenorhabditis elegans*, yeast, insects, and other non-primate mammals. In addition to XIAP/BIRC4, the most potent caspase inhibitor to date, this family currently includes livin/BIRC7, survivin/BIRC5, human inhibitor of apoptosis protein-1 (c-IAP1/BIRC2), human inhibitor of apoptosis protein-2 (cIAP-2/BIRC2), neuronal apoptosis inhibitory protein (NAIP/BIRC1), hILP-2/BIRC8, and Apollon/BIRC6. These proteins have many different functions, including binding to and inhibiting caspases, regulating cell cycle progression, ubiquitination of proteins, and receptor-mediated signaling (24).

Recent studies (25–28) in ErbB2-overexpressing non-IBC cell lines and tumor samples have shown dysregulation in the apoptotic pathway in response to trastuzumab and lapatinib. Inhibition of ErbB2 phosphorylation and downstream PI3K-Akt and MAPK-Erk1/2 signaling, although perhaps necessary for lapatinib antitumor activity, is not sufficient (28, 29). Instead, this led to a unique observation that ErbB2 regulates a member of the BIRC/IAP protein family, survivin, and that the antitumor effects of lapatinib were more closely related to lapatinib-induced down-regulation of survivin.

In the present study, we evaluated the immune-mediated function of trastuzumab and compared its signaling effects with GW583340 (composition similar to lapatinib) on survival signaling and expression of antiapoptotic proteins XIAP and survivin in ErbB2-overexpressing IBC and

non-IBC cells. We have identified that response to trastuzumab and GW583340 inversely and distinctly correlates with expression of XIAP and survivin in the breast cancer cell lines. Further, down-regulation of XIAP expression using small interfering RNA (siRNA) in combination with trastuzumab sensitized the ErbB2-overexpressing IBC cells by abrogating the dual function of XIAP on caspase inactivation and increased p-AKT survival signaling.

## Materials and Methods

### Cell Culture

SUM149PT (human IBC, primary tumor), SUM159PT (human anaplastic carcinoma of the breast, primary tumor), SUM44PE (human pleural effusion), and SUM190PT (human IBC, primary tumor) cells were obtained from Asterand, Inc. SKBR3 (human adenocarcinoma of the breast, pleural effusion), MCF-7 (human adenocarcinoma of the breast, pleural effusion), MDA-MB-231 (human adenocarcinoma of the breast, pleural effusion), and BT474 (human ductal carcinoma) cells were obtained from American Type Culture Collection. Ham's F-12 nutrient mixture, McCoy's 5a medium, Eagle's MEM, and Leibovitz's L-15 medium were purchased from Life Technologies. DMEM was purchased from Mediatech. Insulin, hydrocortisone, HEPES, penicillin, streptomycin, ethanolamine, transferrin, sodium selenite, and epidermal growth factor were all purchased from Sigma Chemical Co. All SUM lines were cultured as per the manufacturer's instructions and as described previously (30). SKBR3 cells were routinely cultured in modified McCoy's 5a medium with 1.5 mmol/L L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate and 10% fetal bovine serum. MCF-7 cells were routinely cultured in MEM (Eagle) with 2 mmol/L L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate, and supplemented with 0.01 mg/mL bovine insulin and 10% fetal bovine serum. BT474 cells were routinely cultured in DMEM and 10% fetal bovine serum. MDA-MB-231 cells were routinely cultured in Leibovitz's L-15 medium with 2 mmol/L L-glutamine and 10% fetal bovine serum. Cells were kept in a 5% CO<sub>2</sub> and 95% air humidified incubator at 37°C.

### Cell Viability and Proliferation Assays

Cell proliferation assay was done as described previously (31). Absorbance was read at 550 nm in a Bio-Rad plate reader. In some experiments, cell viability was also determined by the trypan blue exclusion assay as described previously (31).

### Western Immunoblot Analysis

Cells were harvested and immediately lysed in NP40 cell lysis buffer (BioSource) with fresh protease inhibitor cocktail (Sigma) and 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentration was determined by the Pierce BCA Protein Assay Kit. Equal amounts of cell lysates were then subjected to SDS-PAGE under reducing

conditions. Before loading onto the gel, all lysates were boiled for 5 min and immediately cooled on ice. The protein was transferred onto Immobilon-P membrane (Millipore) previously soaked in methanol and transfer buffer by the TRANS-BLOT SD semidry transfer cell (Bio-Rad). After the transfer process was complete, the membranes were allowed to dry, re-soaked in methanol, and incubated with blocking buffer (5% dry nonfat milk in  $1 \times$  TBS-0.1% Tween 20) for 1 h at room temperature. Membranes were incubated with primary antibody against XIAP (1:2,000; BD Bioscience), actin (1:1,000; Santa Cruz Biotechnology), procaspase-9 (1:2,000; NeoMarkers), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2,000; Santa Cruz Biotechnology) for 1 h at room temperature and survivin (1:2,000; R&D Systems), p-MAPK (1:1,000; Cell Signaling), MAPK (1:1,000; Cell Signaling), p-AKT (1:1,000; Cell Signaling), AKT (1:1,000; Cell Signaling), p-ErbB2 (1:1,000; Cell Signaling), and ErbB2 (1:1,000; Cell Signaling) overnight at 4°C. Membranes were washed thrice with wash buffer ( $1 \times$  TBS-0.1% Tween 20) for 10 min each and subsequently incubated with appropriate secondary antibody conjugated with horseradish peroxidase (1:2,000 dilution) for 1 h at room temperature. Membranes were again washed thrice in wash buffer for 15 min each, and bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Signals were developed after exposure to Kodak BioMax XAR X-ray film (X-Omat films, Eastman Kodak Co.). Actin or GAPDH immunodetection was conducted to serve as a loading control. This was done by stripping the same membrane as described previously (31) Densitometric analysis was done using the NIH ImageJ software.<sup>4</sup>

#### Trastuzumab Binding Assay

Cells were collected and washed in 100  $\mu$ L 1% bovine serum albumin-PBS (per  $3 \times 10^5$  cells) and subsequently incubated in 10  $\mu$ g/mL trastuzumab on ice for 30 min. Cells were washed twice with 1% bovine serum albumin-PBS in 3.5 mL and suspended 100  $\mu$ L of 1% bovine serum albumin-PBS. Cells were then incubated with 3  $\mu$ L of anti-human IgG-phycoerythrin on ice for 30 min. Cells were washed twice as before and resuspended in 250  $\mu$ L of 1% bovine serum albumin-PBS for analysis by flow cytometry. Twenty-five thousand events were collected on a FACScalibur flow cytometer (Becton Dickinson) and analyzed using Cellquest software (Becton Dickinson).

#### Antibody-Dependent Cell Cytotoxicity Assay

Healthy human peripheral blood mononuclear cells were obtained under a Duke Institutional Review Board-approved protocol and used as effector cells. The effector cells were incubated with interleukin 2 (1,000 units/mL) overnight at 37°C. Target cells ( $1 \times 10^6$ ) were labeled with 100  $\mu$ Ci chromium for 1 h at 37°C and washed thrice with PBS and incubated with 10  $\mu$ g/mL trastuzumab for 1 h at 37°C. Target cells were then

incubated with effector cells at a effector to target ratio of 80:1 for 4 h at 37°C. After incubation, cells were spun down and supernatant radioactivity was quantified using a liquid scintillation and luminescence counter (model: 1450 MicroBeta Trilux; Wallac).

#### Treatment of Cells with Trastuzumab and GW583340 for Determination of Cell Proliferation

Cells were treated for 72 h in normal growth medium in a 96-well plate (Corning Incorporated) with trastuzumab (0–100  $\mu$ g/mL; Genentech) or for 24 h with GW583340 (0–50  $\mu$ mol/L; Sigma). Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability was determined by trypan blue exclusion assay.

#### Treatment of Cells with Trastuzumab and GW583340 for Determination of Signaling Pathway

Cells were seeded in their respective medium in 24-well plates (Corning Incorporated) and allowed to reach 70% to 80% confluency. Upon reaching correct confluency, culture medium was removed and replaced with serum-free DMEM (Life Technologies) for 24 h. Trastuzumab (15  $\mu$ g/mL; ref. 32) was made in MEM (Life Technologies) with 0.1% bovine serum albumin (Sigma) for the 1 h experiment and normal growth medium without serum for the 48 h experiment. GW583340 was made in normal growth medium without serum. At time of treatment, the medium was aspirated and treatments were dispensed into wells. Cells were treated with trastuzumab for 1 or 48 h and with GW583340 for 48 h. DMSO was used as a vehicle control for lapatinib. Cells were harvested and lysed as per antibody manufacturer's instructions.

#### Real-time PCR

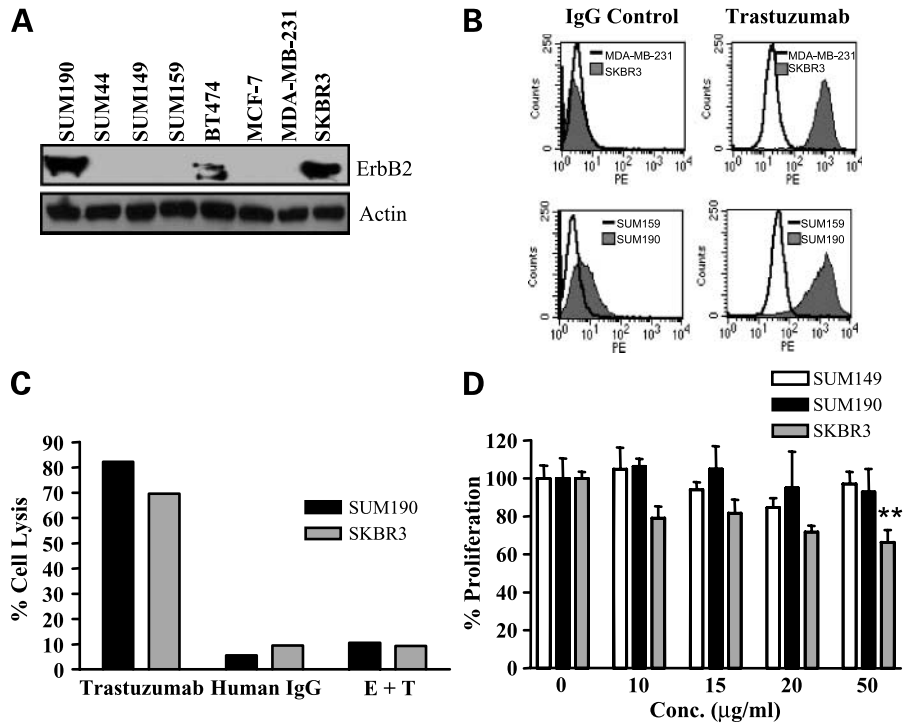
RNA was prepared from different cells using the RNeasy minikit (Qiagen) with a DNase step. Two micrograms of RNA in 20  $\mu$ L were converted to cDNA using iScript cDNA Synthesis (Bio-Rad). Quantitative real-time PCR was done in a 20  $\mu$ L reaction mixture containing 10  $\mu$ L  $2 \times$  Power SYBR Green PCR Master Mix (ABI Applied Biosystem), 200 nmol/L of each primer, and 2  $\mu$ L cDNA product. Amplification was carried out in ABI 7300 real-time PCR instrument. After initial denaturation (50°C 2 min, 10 min at 95°C), amplification was done with 40 cycles of 15 s at 95°C and 60 s at 60°C.  $\beta$ -Actin was used as an internal control.  $\Delta\Delta C_T$  shows the difference between *actin* control and *survivin* or *XIAP*. Folds ( $2^{-\Delta\Delta C_T}$ ) represent changes normalized to a separate reference cell line (SKBR3). The primers were designed to target *XIAP*: 5'-GCAGGGTT-TCTTTATACTGG-3', 5'-TGTCCTTCTGTTCTAACAG-3', *survivin*: 5'-GCATGGGTGCCCGACGTTG-3', 5'-GC-TCCGGCCAGAGGCCTCAA-3', and  $\beta$ -actin: 5'-TCACC-CACACTGTGCCCATCTACGA-3', 5'-CAGCGGAA-CCGCTCATTGCCAATGG-3'.

#### Transfection of Survivin siRNA into Cells for Determination of Cell Viability

Survivin-specific siRNA and its appropriate nonsilencing control were designed as previously reported (29) and synthesized by Qiagen. RNA interference was transfected into cells with Lipofectamine 2000 (Invitrogen) as

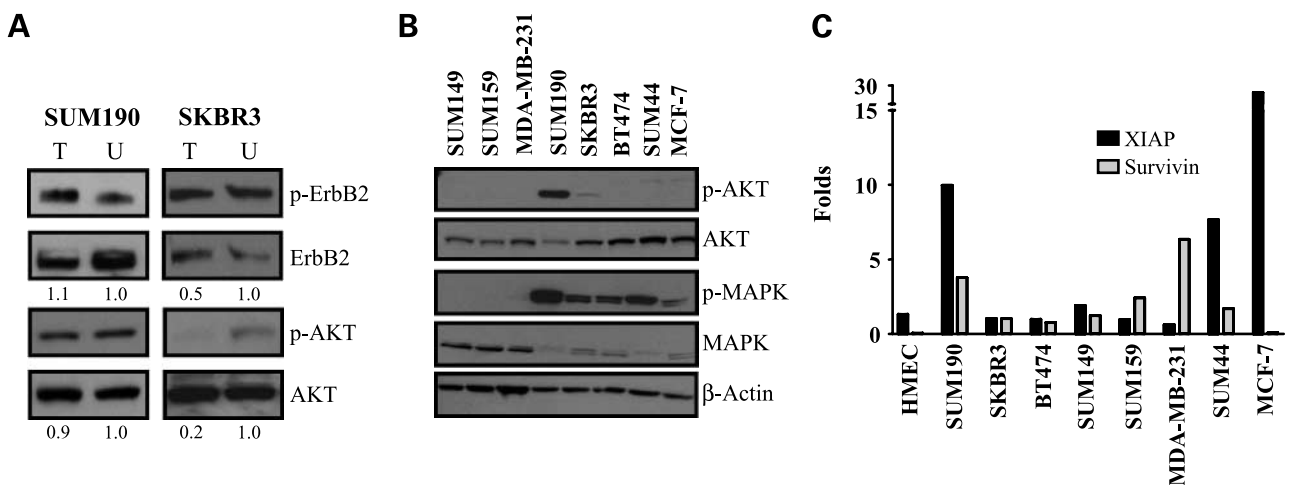
<sup>4</sup> <http://rsb.info.nih.gov/ij/>

**Figure 1.** Effect of trastuzumab on IBC and non-IBC cell lines. **A**, immunoblot analysis of breast cancer cell line panel at basal conditions with antibody against ErbB2 (185 kDa).  $\beta$ -Actin (42 kDa) was used as a loading control. **B**, non-IBC and IBC cell lines were assessed for trastuzumab binding. Cells were incubated for 30 min with 10  $\mu$ g/mL trastuzumab and binding was assessed by flow cytometry. IgG was used as an internal negative control. **C**, SUM190PT and SKBR3 cells were incubated for 1 h with 10  $\mu$ g/mL trastuzumab and assessed for ADCC with an effector to target ratio of 80:1 (*E*, effector cells; *T*, target cells). **D**, SUM149PT, SUM190PT, and SKBR3 cells were treated with indicated concentrations of trastuzumab for 72 h. Cell proliferation was monitored by MTT assay. *Columns*, mean of triplicate ( $n = 3$ ); *bars*, SE. \*\*,  $P < 0.01$  for SKBR3 50  $\mu$ g/mL versus untreated control.

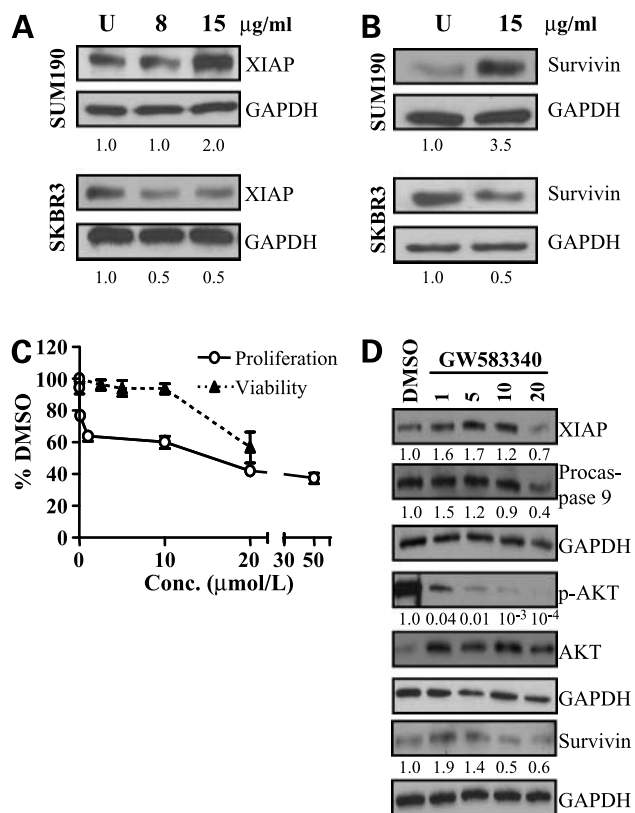


per manufacturer's instructions. Briefly, cells were seeded into 12-well plates (Corning Incorporated) and allowed to reach 50% confluence. At that time, medium was removed and replaced with 500  $\mu$ L fresh normal growth medium. In separate tubes, 100  $\mu$ L serum-free Opti-MEM (Life Technologies) medium was mixed with 2  $\mu$ L transfection reagent and 100  $\mu$ L serum-free Opti-MEM medium was

mixed with 100 nmol/L siRNA (29) and incubated at room temperature for 5 min. After incubation, tubes were mixed and further incubated for 20 min at room temperature. Mixtures were added directly to wells, and plates were gently swirled to mix. Cell viability was determined by trypan blue exclusion assay 48 h after transfection.



**Figure 2.** Trastuzumab-mediated signaling in ErbB2-overexpressing IBC and non-IBC cell lines. **A**, immunoblot analysis of untreated cells (*U*) or cells treated with 15  $\mu$ g/mL trastuzumab (*T*) for 1 h with antibodies against p-ErbB2 (185 kDa) and p-AKT (60 kDa). Phospho blots were stripped and reprobed for corresponding total protein. Numbers represent densitometric analysis of p-ErbB2 and p-AKT expression normalized to corresponding total protein. **B**, immunoblot analysis of cells in basal conditions treated with antibodies against p-AKT and p-MAPK (42/44 kDa). Phospho blots were stripped and reprobed for corresponding total protein.  $\beta$ -Actin (42 kDa) was used as a loading control. **C**, RT-PCR analysis of *XIAP* and *survivin* mRNA expression in a panel of breast cancer cell lines.  $\beta$ -Actin was used as an internal control.



**Figure 3.** Effect of ErbB2-targeting agents on BIRC/IAP protein expression in ErbB2-overexpressing IBC and non-IBC cell lines. **A**, XIAP (57 kDa) immunoblot analysis of SUM190PT and SKBR3 cells treated for 48 h with 0 to 15 µg/mL trastuzumab. GAPDH (37 kDa) was used as a loading control. Numbers represent densitometric analysis of XIAP normalized to GAPDH. **B**, survivin (19 kDa) immunoblot analysis of SUM190PT and SKBR3 cells treated for 48 h with 15 µg/mL. GAPDH (37 kDa) was used as a loading control. Numbers represent densitometric analysis of survivin expression normalized to GAPDH. **C**, SUM190PT cells were treated with indicated concentrations of GW583340 for 24 h. Cell proliferation was determined by MTT assay and cell viability was determined by trypan blue exclusion assay. **D**, immunoblot analysis of cells treated with 0 to 20 µmol/L GW583340 for 48 h with antibodies against XIAP (57 kDa), procaspase-9 (43 kDa), p-AKT (60 kDa), and survivin (19 kDa). The phospho blots were stripped and reprobbed for corresponding total protein. GAPDH (37 kDa) was used as a loading control. Numbers represent densitometric analysis of XIAP, procaspase-9, and survivin expression normalized to GAPDH. Densitometric analysis of p-AKT expression was normalized to corresponding total protein.

#### Transfection of XIAP siRNA into Cells for Determination of Cell Viability

XIAP siRNA (Cell Signaling) was transfected into cells as per manufacturer's instructions. Briefly, cells were seeded into 12-well plates (Corning Incorporated) and allowed to reach 50% confluence. At that time, the medium was removed and replaced with 500 µL fresh normal growth medium. In separate tubes, 100 µL serum-free medium was mixed with 2 µL transfection reagent (Mirus) and incubated at room temperature for 5 min. XIAP (100 nmol/L) siRNA or negative control siRNA (100 nmol/L) was added to tubes, mixed, and incubated

at room temperature for 5 min. Mixtures were added directly to wells and plates were gently swirled to mix. Cell viability was determined by trypan blue exclusion assay 48 h after transfection.

#### Treatment of Cells with siRNA or Embelin in Combination with Trastuzumab for Determination of Cell Viability and Signaling Pathway

Cells were transfected with XIAP or survivin siRNA as described above or with 20 µmol/L embelin. For the combination, trastuzumab (15 µg/mL) was added directly to wells, and plates were swirled to mix. Cell viability was determined by trypan blue exclusion assay at 48 h after transfection. Cells were then collected and lysed as per manufacturer's instructions.

#### Statistical Analysis

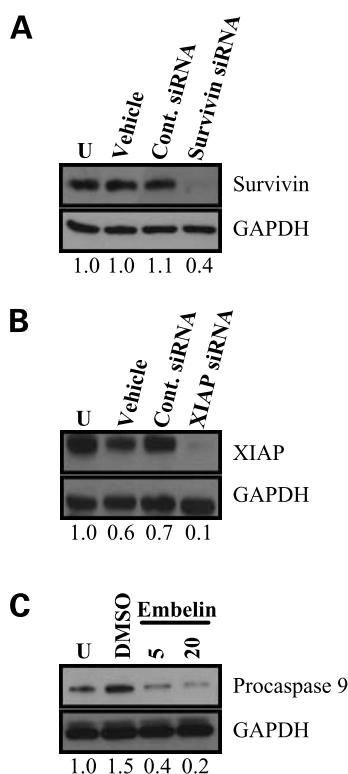
The statistical analyses were done using Graphpad InStat Student's two-tailed *t* test and ANOVA (Turkey-Kramer multiple comparison test). Differences were considered significant at  $P < 0.05$ .

## Results

### Trastuzumab Elicits an Antibody-Dependent Cell Cytotoxicity Response, But Does Not Directly Affect Cell Proliferation in ErbB2-Overexpressing IBC Cells

The ability of trastuzumab to bind cell surface ErbB2, elicit antibody-dependent cell-mediated cytotoxicity (ADCC), and its effect on tumor cell proliferation were tested in an ErbB2-overexpressing IBC cell line (SUM190PT) and compared with the ErbB2-overexpressing non-IBC cell line SKBR3 (Fig. 1). Incubation of the breast cancer cells with trastuzumab reveals specific binding to cell surface ErbB2 in both SKBR3 and SUM190PT cells as shown in Fig. 1B (mean fluorescent intensities of 897.32 and 1197.09, respectively) compared with insignificant cell surface binding to the non-ErbB2-overexpressing non-IBC (MDA-MB-231 and SUM159PT) cells. ADCC has been reported as a prominent immune-mediated effect of trastuzumab in ErbB2-overexpressing breast cancer cells (15). Data in Fig. 1C show that trastuzumab caused a strong ADCC response in the SUM190PT cells (80% cell lysis), which is comparable with the non-IBC SKBR3 cells (70% cell lysis). In contrast, in cell proliferation assays (Fig. 1D), SUM190PT was insensitive to trastuzumab (80–90% proliferation at 50 µg/mL up to 72 h), which was similar to the IBC cell line SUM149PT, isolated from an aggressive primary IBC tumors that are estrogen receptor negative and express constitutively active EGFR but do not express any other active ErbB family members (33). Trastuzumab caused a dose- and time-dependent decrease in cell proliferation in ErbB2-overexpressing non-IBC SKBR3 cells (20–25% at 48 h, 35–40% at 72 h), which was consistent with previously published studies (32, 34).

The effect of trastuzumab on ErbB2 downstream signaling has been reported in various breast cancer cells, including SKBR3, and was compared here with the SUM190PT IBC cells. Incubation of cells for 1 h with 15 µg/mL trastuzumab slightly decreased p-ErbB2



**Figure 4.** Effect of inhibition of BIRC/IAP proteins ErbB2-overexpressing IBC cells. **A**, survivin (19 kDa) immunoblot analysis of SUM190PT cells transfected for 48 h with survivin siRNA. GAPDH (37 kDa) was used as a loading control. Numbers represent densitometric analysis of survivin normalized to GAPDH. **B**, XIAP (57 kDa) immunoblot analysis of SUM190PT cells transfected for 48 h with XIAP siRNA. GAPDH (37 kDa) was used as a loading control. Numbers represent densitometric analysis of XIAP expression normalized to GAPDH. **C**, procaspase-9 (43 kDa) immunoblot analysis of SUM190PT cells treated for 48 h with 20  $\mu\text{mol/L}$  embelin. DMSO was used as a vehicle control. GAPDH (37 kDa) was used as a loading control. Numbers represent densitometric analysis of procaspase-9 expression normalized to GAPDH.

expression in SKBR3 cells, but did not alter ErbB2 phosphorylation or total ErbB2 expression in the SUM190PT cells. A significant down-regulation of p-AKT post-trastuzumab treatment was observed in SKBR3 cells, consistent with that reported by Longva et al. (32) and Asanuma et al. (25). In contrast, trastuzumab did not alter p-AKT in SUM190PT cells (Fig. 2A). This may in part be due to high constitutive expression of activated AKT and MAPK in SUM190PT cells as shown in Fig. 2B. In addition, evaluation of the antiapoptotic proteins *XIAP* and *survivin* by reverse transcription-PCR analysis revealed a relatively high steady-state level of *XIAP* in the SUM190PT cells compared with other IBC and non-IBC cell lines tested (Fig. 2C).

#### Trastuzumab Causes Up-regulation of BIRC/IAP Proteins, XIAP and Survivin, in ErbB2-Overexpressing IBC Cells

Evaluation of the effect of trastuzumab on the antiapoptotic signaling pathway revealed a distinct mechanism of

up-regulation of XIAP and survivin expression in trastuzumab-resistant SUM190PT cells as shown in Fig. 3A and B. In contrast, a decrease in XIAP and survivin expression was observed in SKBR3 cells post-trastuzumab treatment (Fig. 3A and B).

We then studied the effect of another ErbB2-targeting agent, GW583340, which has similar composition as the clinical compound lapatinib/Tykerb and has been shown to be more effective compared with trastuzumab in inhibiting ErbB2 signaling and inducing tumor apoptosis in ErbB2-overexpressing advanced metastatic breast cancer patients (17) and breast tumor lines (18, 29, 35, 36). Immunoblot analysis of the cell lysates at 48 h post-GW583340 treatment revealed a significant inhibition of p-AKT expression at the lowest concentration of 1  $\mu\text{mol/L}$  with complete inhibition at higher concentrations (Fig. 3D). However, analysis of cell proliferation by MTT and viability by trypan blue exclusion assays (Fig. 3C) revealed that >50% cell killing response to GW583340 treatment occurred only at the higher 20  $\mu\text{mol/L}$  concentration, which correlated with down-regulation of XIAP and survivin expression (Fig. 3D). Prolonged treatment up to 7 days showed a similar trend in effects of GW583340 on signaling and viability (data not shown). This is consistent with prior reports in breast cancer patients and BT474, a non-IBC cell line (28, 29), that inhibition of ErbB2 phosphorylation and downstream PI3K-Akt, MAPK-Erk1/2 signaling, although perhaps necessary for lapatinib antitumor activity, is not sufficient and requires down-regulation of the antiapoptotic proteins.

#### XIAP Small-Molecule Inhibitor that Releases XIAP Inhibition on Caspase-9 Activity Causes Decreased Cell Viability in IBC Cells

The effect of XIAP siRNA, survivin siRNA, and embelin, a nonpeptidomimetic XIAP small-molecule inhibitor that inhibits the formation of XIAP complex with procaspase-9, causing release of active caspase-9 (37, 38), were evaluated as single agents in a panel of IBC cell lines. siRNA targeting survivin and XIAP (Fig. 4B) decreased target proteins in the IBC cells tested. Representative immunoblots (Fig. 4) of SUM190PT cell lysates treated for 48 h with various siRNAs are shown in Fig. 4A and B. Similarly, representative immunoblots (Fig. 4C) show that embelin decreased procaspase-9 levels (Fig. 4C) and caused 40% to 50% decrease in cell viability in the SUM190PT (Fig. 5A;  $P < 0.025$ ) cells. In contrast, inhibition of XIAP and survivin protein expression alone by respective siRNA did not have a significant change in SUM190PT cell viability (Fig. 5A).

#### Specific Inhibition of XIAP mRNA in Combination with Trastuzumab Causes Decrease in Cell Viability Corresponding to Decreased Procaspase-9 and p-AKT in ErbB2-Overexpressing IBC Cells

Because our data (Fig. 3A and B) show that trastuzumab treatment caused an increase in XIAP and survivin protein expression in SUM190PT cells, we evaluated the combination of trastuzumab with inhibition of XIAP or survivin using XIAP siRNA, embelin, or survivin siRNA (Fig. 5B). Treatment of cells with trastuzumab + XIAP

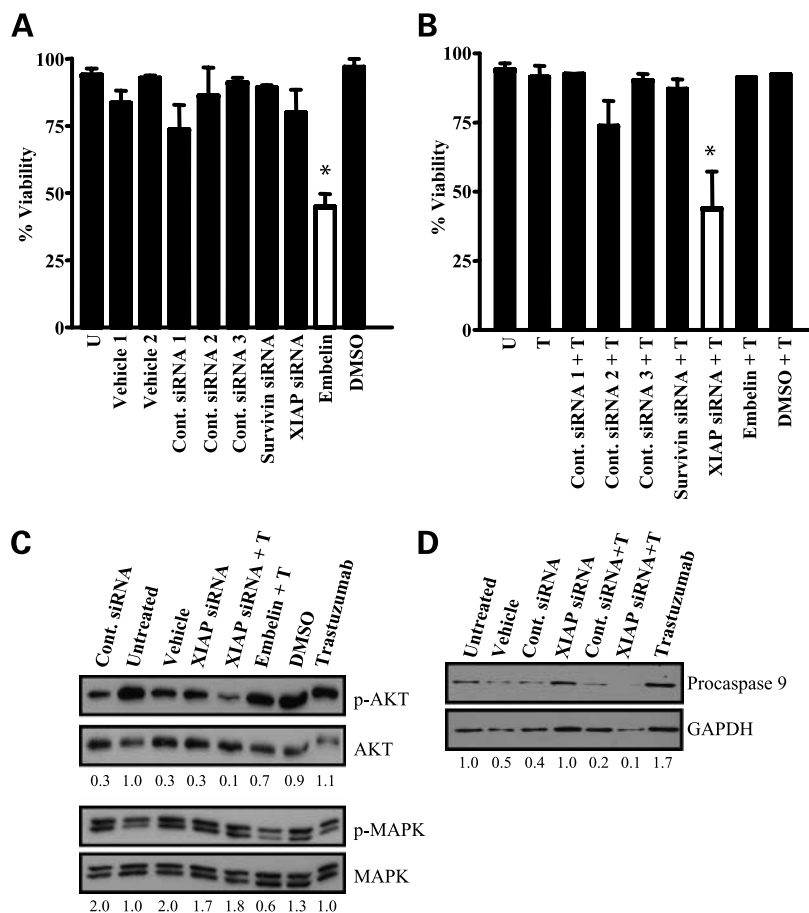
siRNA in combination caused a significant decrease ( $P < 0.025$ ) in cell viability (45–50% decrease compared with controls). This inhibitory effect on cell viability was not observed in trastuzumab + control siRNA treatment or in trastuzumab + survivin siRNA-treated cells. Further, although embelin as a single agent decreased SUM190PT cell viability, embelin in combination with trastuzumab was similar to trastuzumab alone, suggesting that increased XIAP expression post-trastuzumab treatment (Fig. 3A) overrides the effect of embelin, which unlike siRNA has no effect on XIAP translation, but rather inhibits XIAP interaction with procaspase-9, a downstream event. Moreover, in SKBR3 cells wherein trastuzumab treatment causes a decrease in XIAP expression (Fig. 3A), further inhibition of XIAP using siRNA in combination with trastuzumab had no significant potentiation of decrease in cell viability (data not shown) over that seen with trastuzumab alone, suggesting a need for treatment-dependent specific increase in XIAP for a combination strategy involving specific XIAP inhibition to be effective. Data in Fig. 5D show that the decrease in viability seen in the trastuzumab + XIAP siRNA combination corresponded with decreased procaspase-9. Further, a significant decrease in p-AKT levels (Fig. 5C)

was seen only in the combination of XIAP inhibition along with trastuzumab treatment in the SUM190PT cells, which have constitutively activated p-AKT (Fig. 2B) that is unaffected by trastuzumab treatment alone.

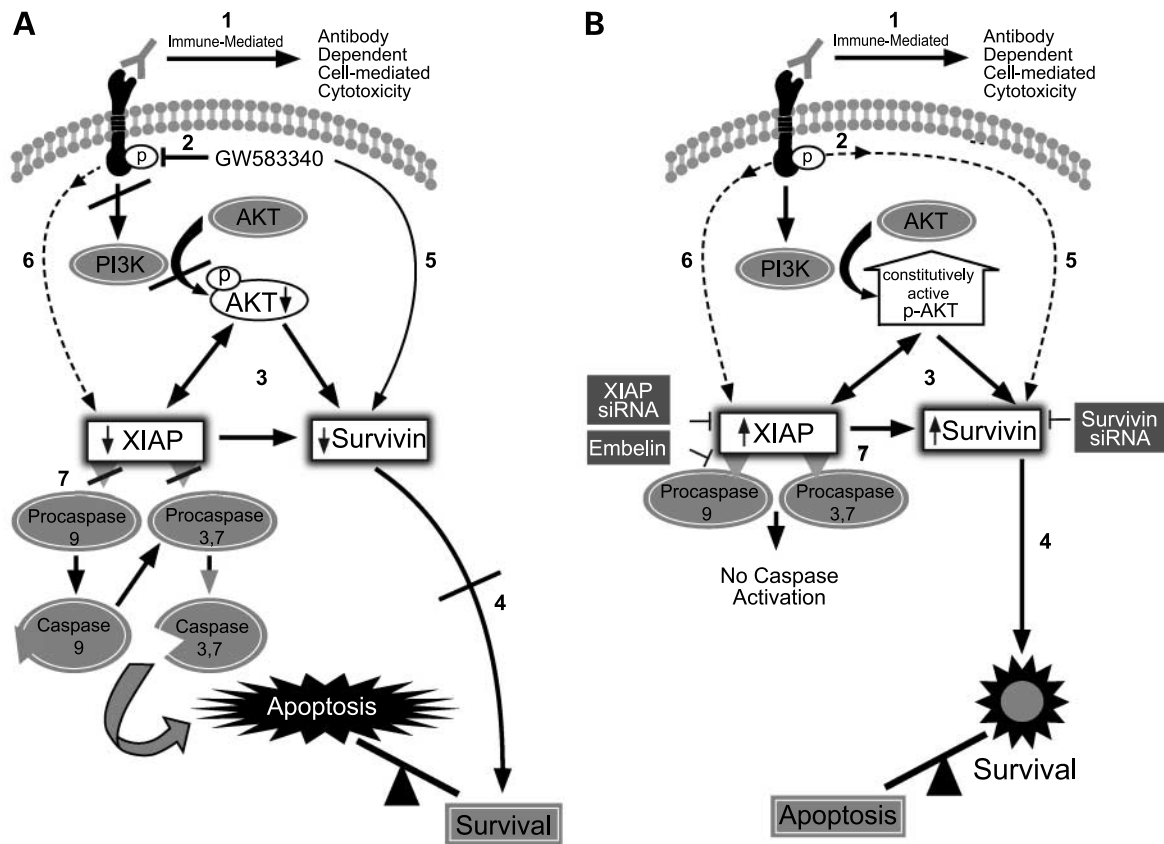
A schematic is presented in Fig. 6 to summarize the current results and potential mechanisms of response and resistance to trastuzumab compared with lapatinib response in breast cancer cells.

### Discussion

We report herein the role of XIAP, an antiapoptotic protein that can inhibit both mitochondrial and extrinsic pathways of apoptosis, to play a key role in therapeutic sensitivity in IBC cells. A distinct mechanism of increased expression of XIAP and survivin, key members of the BIRC/IAP protein family post-trastuzumab treatment, was observed in ErbB2-overexpressing SUM190PT IBC cells, an established ErbB2-overexpressing IBC line isolated from a primary tumor of an IBC patient (30). In contrast, decrease in tumor cell viability in response to treatment with the small-molecule ErbB2-targeting agent, GW583340, correlated with a significant decrease in XIAP and survivin abundance. Further, XIAP inhibition was only seen at concentrations wherein there was >50% cell death.



**Figure 5.** Effect of inhibition of BIRC/IAP proteins alone or in combination with trastuzumab on cell viability and signaling in ErbB2-overexpressing IBC cells. **A**, effect of inhibition of survivin or XIAP alone on cell viability in SUM190PT cells. Cells were transfected for 48 h with 100 nmol/L survivin or XIAP siRNA alone or 20 μmol/L embelin. Cell viability was monitored by trypan blue exclusion assay. Columns, mean ( $n = 3$ ); bars, SE. \*,  $P < 0.05$  for embelin versus control. **B**, effect of inhibition of survivin or XIAP using siRNA or embelin in combination with trastuzumab on cell viability in SUM190PT cells. Cell viability at 48 h posttreatment with various combinations was monitored by trypan blue exclusion assay. Columns, mean ( $n = 3$ ); bars, SE. \*,  $P < 0.05$  for XIAP siRNA + trastuzumab versus trastuzumab alone. **C**, immunoblot analysis of cells transfected for 48 h with 100 nmol/L XIAP siRNA or treated for 48 h with embelin alone or in combination with 15 μg/mL trastuzumab with antibodies against p-AKT (60 kDa) and p-MAPK (42/44 kDa) and **(D)** procaspase-9 (43 kDa). Phospho blots were stripped and reprobed for corresponding total protein. Numbers represent densitometric analysis of p-AKT and p-MAPK expression normalized to corresponding total protein and procaspase-9 expression normalized to GAPDH.



**Figure 6.** Schematic representation showing functional link between BIRC/ IAP expression and response to ErbB2-targeting agents in ErbB2-overexpressing breast cancer cells. **A**, potential mechanisms of trastuzumab and GW583340/lapatinib response in SKBR3. **B**, resistance to trastuzumab-mediated signaling in ErbB2-overexpressing SUM190 IBC cells. 1, trastuzumab (shown as Y) elicits its immune effects by increasing ADCC in both panels. In addition to its immune effects, trastuzumab can effectively bind with ErbB2 receptor with similar affinity in both panels. 2, trastuzumab binding suppresses ErbB2 receptor signaling by inhibiting ErbB2 receptor dimerization and subsequent phosphorylation, which in turn inhibits PI3K activity in **A**, whereas no decrease in p-ErbB2 is seen in SUM190PT cells (**B**). GW583340/lapatinib, a potent ErbB2 kinase inhibitor, acts directly on receptor phosphorylation and inhibits downstream signaling via PI3K, as shown in **A** (3). Down-regulation of PI3K activity by trastuzumab and GW583340 in the sensitive cells inhibits AKT activation, leading to inhibition of downstream survival signaling including survivin abundance (4 and 5). In addition, lapatinib has been identified to destabilize survivin via activation of the ubiquitin-mediated proteosomal pathway (29). The present study reveals that trastuzumab and lapatinib response in the sensitive breast cancer cells correlates with a significant down-regulation of XIAP (6). Overall (**A**), XIAP decrease relieves its inhibitory effect on procaspases leading to their activation and subsequent cellular apoptosis (7). In contrast, in **B** (5 and 6), trastuzumab treatment is observed to further increase survivin and XIAP expression in SUM190PT IBC cells, which have constitutively active p-AKT and high steady-state levels of XIAP and survivin, leading to a cross-talk between p-AKT, survivin, and XIAP to promote cell survival (**B**, 3). p-AKT can stabilize XIAP by phosphorylation (49) and increase expression of survivin, a downstream target. XIAP can also form a complex with survivin, stabilizing survivin and increasing anticaspase activity (50). Whether trastuzumab has a direct effect on increasing these IAP proteins needs to be elaborated. Overall, in **B**, increased XIAP inhibits release of active caspases from the corresponding procaspases leading to a potent suppression of apoptosis (7). The action sites of siRNA targeting XIAP or survivin and embelin are shown.

Our data reveal that trastuzumab can bind effectively to cell surface ErbB2 and elicit a potent ADCC response in both sensitive SKBR3 non-IBC and resistant SUM190PT IBC cells (Fig. 6A and B, 1). Whether this immune-mediated mechanism plays a role in the antitumor activity of trastuzumab in IBC tumors needs to be elucidated in tumor models because insignificant levels of proinflammatory cytokines and thereby fewer host inflammatory cells are typically identified in the tumor stroma (39, 40). Further, Gennari et al. (41) have postulated that because ADCC does not change ErbB2 levels or alter downstream ErbB2 signaling, it may not be the key mechanism of trastuzumab antitumor activity.

In the present study, SUM190PT cells were insensitive to the signaling effects of trastuzumab mediated via its direct inhibitory effect on ErbB2 dimerization and downstream survival signaling (Fig. 6B, 2) through inhibition of PI3K activity and subsequent AKT phosphorylation, a signaling pathway typically inhibited in trastuzumab-sensitive breast cancer cells (25, 32, 42). In addition, SUM190PT cells have high constitutively activated p-AKT (Fig. 6B, 3). Further, trastuzumab treatment was observed to induce overexpression of XIAP and survivin in the trastuzumab-resistant SUM190PT IBC cells compared with the sensitive SKBR3 cells wherein a corresponding decrease in XIAP and survivin was observed (Fig. 6A and B, 5 and 6). In



addition, XIAP and survivin levels were decreased in response to GW583340 (Fig. 6A, 2, 5, and 6), which is a more potent inhibitor of ErbB2 signaling compared with trastuzumab. These data reveal a functional link between ErbB2 signaling and antiapoptotic pathways. Lapatinib, the clinical equivalent of the GW583340 compound, has been shown to cause direct stabilization of survivin from proteosomal degradation in lapatinib-resistant cells (28, 29). Whether trastuzumab and lapatinib have a similar direct effect on XIAP needs to be evaluated.

Specific inhibition of XIAP mRNA using an siRNA strategy (43) in the absence of trastuzumab treatment decreased XIAP protein expression; however, this did not lead to a significant change in viability or apoptosis. This is not surprising as previous reports from our laboratory and others have shown that in many cell lines, inhibiting XIAP by itself does not increase spontaneous apoptosis (31, 44); however, there is a need for an initial insult or stress to the cells like treatment with therapeutic agents. In contrast, a combination of XIAP inhibition along with trastuzumab sensitized the IBC cells to trastuzumab. This suggests the potential mechanism of action of the combination to be able to counteract the effect of trastuzumab to increase XIAP expression, thereby alleviating the inhibitory effect of XIAP on caspase activation and also decreasing AKT phosphorylation. The observed interplay between activated AKT and XIAP (Fig. 6A and B, 3) is consistent with earlier reports (45), which have shown that XIAP overexpression can induce phosphorylation of AKT, thereby stabilizing its activity. A similar mechanism has also been reported in prostate cancer cells in our previous study (31) and in melanoma cells (46) in response to tumor necrosis factor-related apoptosis-inducing ligand, a natural ligand for death receptors that is regulated by the cross-talk between XIAP and p-AKT in potentiating cancer cell survival.

Compared with XIAP siRNA, which works at the level of mRNA translation, direct activation of caspase-9 function by using a small-molecule inhibitor, embelin, that abrogates the binding of XIAP to procaspase-9 significantly decreased IBC cell viability, revealing the inhibitory effect of XIAP on caspase-dependent cell death. However, embelin failed to have any cell killing effect in combination with trastuzumab because embelin has no effect on XIAP translation or stability. XIAP is one of the few eukaryotic cellular mRNAs with an internal ribosomal entry sequence, which allows for XIAP translation during periods of cellular stress (47). The increase in XIAP protein levels that we see after treatment of cells with trastuzumab may be in part due to a cellular stress response increasing translation of XIAP from the internal ribosomal entry sequence, although this needs to be tested in our experimental model.

Similar to XIAP, the role of another key IAP/BIRC member, survivin, has been identified as a potential prognostic marker in breast cancer (48). An inverse correlation of survivin expression and sensitivity to lapatinib and trastuzumab has been observed in non-IBC ErbB2-overexpressing BT474 and SKBR3 cells, respectively (25, 29). In the present study, up-regulation of survivin

expression was observed in the SUM190PT IBC cells within 1 h of trastuzumab treatment. In addition, GW583340 sensitivity in the IBC cells correlated with decrease in survivin and XIAP expression. Although trastuzumab increased survivin expression in SUM190PT cells, inhibition of survivin by siRNA alone or in combination with trastuzumab did not have any significant effect on cell viability compared with trastuzumab alone. This is most likely because survivin is not a direct caspase inhibitor and reveals the dominant role of XIAP in resistance to apoptotic response to trastuzumab in ErbB2-overexpressing IBC cells.

Although trastuzumab is widely used for ErbB2-overexpressing breast cancers, resistance and cardiotoxicity remain major hurdles. To our knowledge, this is the first evidence that trastuzumab treatment causes XIAP up-regulation in trastuzumab-resistant ErbB2-overexpressing breast cancer cells. Resistance mechanisms that have been previously reported include reduced phosphatase and tensin homologue expression, overexpression of the insulin-like growth factor-I receptor (IGF-IR), and down-regulation of p27<sup>kip1</sup> (15). Furthermore, it has been shown that constitutively active AKT can block the cell cycle arrest and apoptotic-inducing abilities of trastuzumab (42). These resistance mechanisms could account for the observation that a large majority of patients with ErbB2-overexpressing metastatic breast cancer rarely respond to trastuzumab as a single agent (13). The ErbB2-overexpressing IBC cell line (SUM190PT) described in this study mimics this phenomenon, because it has low phosphatase and tensin homologue expression (data not shown), activated AKT, and relatively high expression of XIAP, all of which were observed to correlate with resistance. In addition, a previous report has shown the ability of trastuzumab to decrease Bcl-2 protein levels in trastuzumab-sensitive non-IBC cells (26), further supporting the role of antiapoptotic proteins in sensitivity to trastuzumab.

Despite advances in understanding the underlying molecular mechanisms of therapeutic resistance in IBC, minimal treatment options exist for these patients. The present study establishes the feasibility of development of a targeted therapy that potentiates apoptosis in combination with ErbB2-targeting strategies for IBC therapy.

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