X-Linked Inhibitor of Apoptosis Protein Inhibits Apoptosis in Inflammatory Breast Cancer Cells with Acquired Resistance to an ErbB1/2 Tyrosine Kinase Inhibitor

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Abstract

Inflammatory breast cancer (IBC) is a highly aggressive subtype of breast cancer that is often characterized by ErbB2 overexpression. ErbB2 targeting is clinically relevant using trastuzumab (anti-ErbB2 antibody) and lapatinib (small-molecule ErbB1/2 inhibitor). However, acquired resistance is a common outcome even in IBC patients who show an initial clinical response, which limits the efficacy of these agents. In the present study, using a clonal population of GW583340 (lapatinib analogue, ErbB1/2 inhibitor)–resistant IBC cells, we identified the overexpression of an antiapoptotic protein, X-linked inhibitor of apoptosis protein (XIAP), in acquired resistance to GW583340 in both ErbB2-overexpressing SUM190 and ErbB1-activated SUM149 cell lines derived from primary IBC tumors. A marked decrease in p-ErbB2, p-ErbB1, and downstream signaling was evident in the GW583340-resistant cells (rSUM190 and rSUM149) similar to parental counterparts treated with the drug, suggesting that the primary mechanism of action of GW583340 was not compromised in resistant cells. However, rSUM190 and rSUM149 cells growing in GW583340 had significant XIAP overexpression and resistance to GW583340-mediated apoptosis. Additionally, stable XIAP overexpression using a lentiviral system reversed sensitivity to GW583340 in parental cells. The observed overexpression was identified to be caused by IRES-mediated XIAP translation. XIAP downregulation in rSUM190 and rSUM149 cells using a small-molecule inhibitor (embelin), which abrogates the XIAP/procaspase-9 interaction, resulted in decreased viability, showing that XIAP is required for survival of cells with acquired resistance to GW583340. These studies establish the feasibility of development of an XIAP inhibitor that potentiates apoptosis for use in IBC patients with resistance to ErbB2-targeting agents. Mol Cancer Ther; 9(5); 1432–42. ©2010 AACR.

Introduction

Apoptotic dysregulation is a fundamental characteristic of cancer that allows transformed cells to survive and proliferate (1, 2). In part, this is due to defects in caspase activity, the execution phase of apoptosis. The inhibitors of apoptosis proteins (IAP) are one of the major protein families that regulate caspase activation and programmed cell death (3). The family currently consists of eight members that are characterized by the presence of one or more baculoviral IAP repeat (BIR) domains and are highly conserved among mammalian and nonmammalian species (4).

In particular, one of the IAP proteins, X-linked inhibitor of apoptosis protein (XIAP), has been identified as the most potent caspase inhibitor to date (4). XIAP can bind and inhibit the activation of procaspase-9, procaspase-7, and procaspase-3 (5). This leads to the inhibition of both intrinsic (mitochondrial) and extrinsic (death receptor mediated) pathways of apoptosis (3), which is not evident with another prominent antiapoptotic protein Bcl-2, which inhibits cytochrome c release from the mitochondria but does not directly bind to caspases (6). In addition, XIAP mRNA has an internal ribosomal entry sequence (IRES; ref. 7), which has been identified to be upregulated during cellular stress (8–10). XIAP is expressed in almost all tissues and cell types (11); however, it is often overexpressed in tumors versus normal tissue (12), including breast cancer (13), and has been strongly linked to therapeutic resistance in cervical, ovarian, and prostate cancers (14, 15). In addition to its caspase-binding function, XIAP has been observed to regulate the activity of key survival factors such as AKT, NF-κB, and another IAP family member, survivin (16). Therefore, there is a growing interest in targeting XIAP and inhibitors of XIAP are currently being developed to help overcome resistance to mainstay therapies (17).

Recently, we reported a novel functional link between the epidermal growth factor receptor 2 (ErbB2) signaling pathway and XIAP in SUM190 cells, an ErbB2-overexpressing inflammatory breast cancer (IBC) cell line resistant to...
trastuzumab (an ErbB2-targeting monoclonal antibody; ref. 18). IBC is an aggressive, fast-growing, and highly invasive cancer that is clinicopathologically distinct from a neglected locally advanced breast cancer (19). IBC tumors are often resistant to chemotherapy and radiotherapy and therefore disease-free survival is poor (20, 21). ErbB2 is commonly overexpressed in IBC tumors; (22) however, the development of acquired resistance to Food and Drug Administration–approved agents, trastuzumab (humanized ErbB2 monoclonal antibody) and lapatinib (a dual ErbB1/2 tyrosine kinase inhibitor), limits the clinical efficacy of these anti-ErbB2 therapeutic strategies (23–25). Clinical trials using lapatinib as a mono-therapy have shown that it is effective in patients with ErbB2-overexpressing breast cancer that have been heavily pretreated with other therapeutics including trastuzumab (26, 27) with response rates ranging from 7% to 35% (28). Interestingly, in IBC patients, lapatinib has a greater efficacy with response rates ranging from 50% to 100% (29, 30). However, clinical studies with lapatinib as a monotherapy also indicate that clinical responses are generally short lived in breast cancer patients (31) and acquired resistance is common. Previously reported mechanisms of lapatinib resistance include the activation of estrogen receptor signaling (25), upregulation of the antiapoptotic protein MCL-1 (32), and potentially the modulation of cancer cell metabolism (33). In the present study, we evaluated XIAP action in a model of acquired resistance to a lapatinib analogue (GW583340) in both ErbB2-overexpressing and ErbB1-activated IBC cell lines in which cells were chronically exposed to GW583340, similar to patients receiving daily doses of lapatinib when given as a monotherapy. Continuous exposure to GW583340 for >3 months converted the parental GW583340-sensitive IBC cells to being resistant to the apoptotic-inducing and growth-inhibitory effects of the inhibitor. We identified XIAP overexpression to be the key difference between the parental GW583340-sensitive and GW583340-resistant IBC cell lines studied here. This overexpression was shown to be mediated through the translation of XIAP using its IRES in its 5′ untranslated region (UTR). Additionally, stable XIAP overexpression using a lentiviral system reversed the sensitivity to GW583340 in parental cells. Further, XIAP downregulation using embelin (a small-molecule inhibitor that interrupts the interaction between XIAP and procaspase-9; ref. 34) caused the reversal of GW583340 resistance in the acquired resistant IBC cellular models. The present study supports the observation that XIAP is required for the survival of IBC cells with acquired GW583340 resistance.

Materials and Methods

Cell culture. SUM149 and SUM190 cells were obtained from Asterand, Inc. All cell lines were cultured as previously described (18). Laboratory grade lapatinib analogue (GW583340; Sigma) was dissolved in DMSO. Acquired resistance to GW583340 was selected in SUM190 and SUM149 cells (referred to as rSUM190 and rSUM149) by culturing cells in normal growth medium supplemented with increasing concentrations of GW583340 (0.25–2.5 and 0.25–7.5 μmol/L, respectively). Initially, marked cell death and decrease in cell growth was observed in the cells. However, after 2 weeks of each increase in drug concentration, small colonies of viable cells were observed, which were cultured until confluence before the next increase in drug concentration. This was continued for a minimum of 3 months. From then on, both rSUM190 and rSUM149 cells were routinely cultured in 2.5 and 7.5 μmol/L GW583340, respectively.

Generation of stable XIAP-overexpressing IBC cell lines. SUM149 cells stably expressing wtXIAP and FG9 green fluorescent protein vector control were generated using a lentiviral expression system (kindly provided by Dr. Colin Duckett, University of Michigan, Ann Arbor, Michigan). Briefly, HEK293T cells were transfected using polyethyleneimine with 5 μg of pHCMV, pRRE, and pR5Srev (35), which drive the expression of lentiviral structural proteins, and 5 μg of pFG9 E1A XIAP wild-type silent mutation hyg or pFG9 E1A hygro green fluorescent protein (as a control; ref. 36). Twenty-four hours posttransfection, the medium was changed. Forty hours posttransfection, the virus-containing medium on the HEK293T cells was collected and filtered through a 0.45-mm Millex HV polyvinylidene difluoride filter unit (Millipore) onto cells [with 25 mmol/L polybrene (Sigma)]. After 4 hours, fresh medium was added and cells were incubated for an additional 48 hours at 37°C, 5% CO2. Stable cells were selected by the addition of hygromycin B (Invitrogen; 200 μg/mL).

Treatment of cells with agents for the determination of cell counts and signaling. Cells were seeded in six-well plates (Corning Incorporated) and allowed to reach 70% confluence. Cells were treated for 24 hours to 7 days in regular growth media with GW583340 (Sigma) or 48 hours with embelin (Sigma, dissolved in DMSO) or phos- phoinositide 3-kinase (PI3K) inhibitor, LY294002 (40 μmol/L, Sigma, dissolved in DMSO). DMSO (at the same concentration as drug treatments) was used as a vehicle control in all experiments. Cell counts were determined by trypan blue exclusion.

Determination of cell death. Cells were treated as described above and then stained for Annexin V and propidium iodide (PI) using the Annexin V Biotin kit (Beckman Coulter) as per the manufacturer’s instructions. Total cell death is presented as the sum of Annexin V+/PI−, Annexin V+/PI+, and Annexin V−/PI− cells. Alternatively, for some experiments, cell death was ascertained by staining with 7-AAD (Invitrogen) for 30 minutes. At least 25,000 events were collected on a FACScalibur flow cytometer (Becton Dickinson) and analyzed using the Cellquest software (Becton Dickinson).

Western immunoblot analysis. Western immunoblot analysis was carried out as previously described (18). Cells were harvested for Western immunoblot analysis at 24 hours (GW583340) or 48 hours (embelin,
LY294002) posttreatment. Membranes were incubated with primary antibodies against XIAP (BD Biosciences), pro-caspase-9 (NeoMarkers), actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), FOXO3a, Bcl-2 (Santa Cruz), survivin (R&D Systems), p-akt (Ser473), AKT, p-ErbB2 (Tyr877), ErbB2, phosphorylated mito-gen-activated protein kinase (p-MAPK; Thr202/Tyr204), MAPK, ErbB1 (Cell Signaling), and total phosphotyrosine clone 4G10 (Upstate) overnight at 4°C. Detection of total protein was carried out in all experiments by stripping the same membrane as previously described (15). Densitometric analysis was done using the NIH ImageJ software (http://rsb.info.nih.gov/ij/).

**Immunofluorescent microscopy.** Cells were seeded onto coverslips (VWR) in dishes and allowed to reach 70% confluence. Cover slips were washed once with PBS and fixed with 100% methanol at −20°C for 20 minutes. After fixation, cells were blocked with 1% bovine serum albumin/PBS at 37°C for 30 minutes and incubated with XIAP antibody (BD Biosciences) for 1 hour at room temperature. Cells were then washed thrice with PBS for 5 minutes and incubated with R-PE-labeled secondary antibody (Southern Biotech) for 1 hour at room temperature. For a nuclear counterstain, cells were incubated for 1 minute with 0.1 µg/mL Hoechst 33258 (Sigma). Finally, cells were washed thrice with PBS and coverslips were inverted onto slides, sealed, and imaged on a Zeiss Axio Observer inverted widefield fluorescence microscope using a ×63/1.40 differential interference contrast microscopy Plan Apochromat objective. Images were captured on a Hamamatsu ORCA ER CCD camera (Hamamatsu Corp.). The system was controlled by the MetaMorph Software (Molecular Devices). XIAP staining intensity was measured by using the NIH ImageJ software.

**Real-time PCR.** Quantitative real-time PCR was done as previously described (18). β-Actin was used as an internal control. ΔΔCT shows the difference between actin control and XIAP. Folds (2−ΔΔCT) represent changes normalized to the parental IBC cell. The primers designed to target XIAP and β-actin were previously described (18).

**Construction of XIAP IRES luciferase plasmid and transfection of cells.** The luciferase construct was constructed by deleting the sequence between the [pGL3-XIAP.IRES (cont 1) and pGL3-XIAP .IRES (cont 2)] of the parental IBC cell. The primers designed to target XIAP and β-actin were previously described (18).

**Luciferase activity assay.** Cells were lysed for 15 minutes in 500 µL luciferase lysis buffer [35 mg/mL Tris base, 5 mg/mL CDTA, 10% glycerol, and 0.5% Triton-X 100 (pH 7.8)] and 25 µL of the lysate was added to a 96-well plate (Greiner Bio-One). Luciferase activity was determined using a luminometer (Turner Biosystems). Firefly or Renilla luciferase substrate [1 mmol/L Luciferin or colelentrerin (Gold Biotechnology) in 15 mmol/L MgSO4, 15 mmol/L K2HPO4, 4 mmol/L EGTA, 1 mmol/L DTT, and 0.1 mmol/L ATP] was added (100 µL) to wells and luciferase activity was read after 10 seconds.

**Caspase-9 activity assay.** Cells were seeded in six-well plates (Corning Incorporated), and the next day, cells were treated with embelin (50 µmol/L) for 4 hours in regular growth media. DMSO (at the same concentration as embelin) was used as a vehicle control. After incubation, caspase-9 activity was determined in 3 µg total cell lysates using the Caspase-Glo Assay (Promega) as per the manufacturer's instructions. Peak light intensity of treatment wells was normalized to DMSO.

**Nucleosome enrichment assay.** Cells were seeded in 96-well plates (Corning Incorporated). Embelin (50 µmol/L) and staurosporine (5 µmol/L) were made in regular growth media. DMSO (at the same concentration as the drug treatments) was used as a vehicle control. After 20 hours of incubation, nucleosome enrichment was determined by the Cell Death Detection ELISA PLUS (Roche Applied Science) as per the manufacturer's instructions. Nucleosome enrichment was calculated as (mU sample – Blank)/(mU nontreated – Blank) *100.

**Statistical analysis.** The statistical analyses were done using the GraphPad InStat Student's two tailed t test. Differences were considered significant at P < 0.05.

**Results**

**Development of a model of acquired resistance of IBC cells to an ErbB1/2 tyrosine kinase inhibitor.** The effect of a laboratory grade lapatinib analogue (GW583340) on cell growth and cell death was characterized in two well-established IBC cell lines isolated from primary IBC tumors (37): SUM190 (ErbB2 overexpressing) and SUM149 (ErbB1 activated). Because resistance to lapatinib monotherapy in patients treated with daily doses of lapatinib is commonly seen (31), GW583340-resistant lines (referred to here as rSUM190 and rSUM149) were established by chronic exposure of the parental SUM190 and SUM149 cells to increasing concentrations of GW583340 for >3 months (see Materials and Methods). GW583340 treatment at concentrations in which the rSUM149 and rSUM190 cells were established for acquired resistance to this agent caused significant growth inhibition in the parental SUM149 (P < 0.005; Fig. 1A) and SUM190 (P < 0.05; Fig. 1B) cells. However, the growth curves indicate that rSUM149 and rSUM190 cells have similar doubling times to their parental counterparts [14.1 hours (SUM149) versus 14.9 hours (rSUM149); 39.6 hours (SUM190) versus 40.1 hours (rSUM190)]. Analysis of cell
death as measured by Annexin/PI staining showed an increase in total dead cells 24 hours posttreatment in the parental cells compared with rSUM149 (P < 0.05; Fig. 1C, left) and rSUM190 (P < 0.05; Fig. 1D, left) cells. In addition, exposing rSUM149 and rSUM190 cells (which are maintained in 7.5 and 2.5 μmol/L GW583340, respectively) to increasing concentrations of GW583340 (up to 20 μmol/L for 24 hours) caused only a modest increase (10–20%) in cell death as measured by 7-AAD viability stain (Fig. 1C and D, right, left Y-axis) and no decrease in cell proliferation (MTT assay; Fig. 1C and D; right, right Y-axis) compared with 70% to 80% cell death and decrease in proliferation in the parental SUM149 and SUM190 cells. It should be noted that the effect of GW583340 on decreasing cell proliferation in parental SUM149 and SUM190 cells saturates to 50% to 60% at 10 to 20 μmol/L at 24 hours and only increase in time of treatment causes further significant growth inhibition. These data support the establishment of two IBC cell models (rSUM149 and rSUM190) with acquired resistance to GW583340.

Dysregulation of the apoptotic pathway and not inhibition of ErbB signaling contributes to acquired resistance to GW583340. Evaluation of the effect of GW583340 on the ErbB signaling pathways in SUM149 and SUM190 IBC cells revealed that treatment with GW583340 caused a marked and comparable down-regulation of p-ErbB1 in both parental SUM149 and rSUM149 cells compared with nontreated parental cells (Fig. 2A). Similarly, expression of p-ErbB2, p-AKT, and p-MAPK were inhibited in the GW583340-treated SUM190 and rSUM190 cells. However, an increase in

Figure 1. Effect of GW583340 on cell growth, proliferation, and death in parental and resistant IBC cells. Nontreated SUM149 cells were compared with SUM149 and rSUM149 cells treated with 7.5 μmol/L GW583340 (A) and nontreated SUM190 cells were compared with SUM190 and rSUM190 cells treated with 2.5 μmol/L GW583340 (B). Cell growth was assessed after 1, 2, 3, and 7 days of continuous exposure to GW583340 by trypan blue exclusion assay. Cell count is presented as the total cell number. **, P < 0.005, rSUM149 versus SUM149 treated with 7.5 μmol/L GW583340 (n = 2–3); *, P < 0.05, rSUM190 versus SUM190 treated with 2.5 μmol/L GW583340 (n = 2). Cell death and proliferation of rSUM149 cells and SUM149 cells (C) and rSUM190 and SUM190 cells (D) treated for 24 hours with GW583340. Cell death was assessed by Annexin V/PI (left) or 7-AAD (right, left Y-axis) staining and proliferation was determined by MTT assay (right, right Y-axis). Columns, mean of the total dead cell count relative to the DMSO control or percentage proliferation relative to the DMSO control (*, P < 0.05; **, P < 0.005; n = 2); bars, SEM.
total MAPK expression was observed in the rSUM190 cells (Fig. 2B).

To evaluate the effect of direct inhibition on PI3K (downstream effector in ErbB signaling), SUM190 and rSUM190 cells were treated with a PI3K inhibitor (LY294002). LY294002 treatment inhibited p-AKT as per its mechanism of action and increased cell death of SUM190 cells (P < 0.05; Fig. 2C). In contrast, the rSUM190 cells were not affected by the direct inhibition of PI3K signaling using LY294002. This insensitivity potentially indicates that in ErbB2-overexpressing rSUM190 cells, GW583340 retains the ability to inhibit p-ErbB2, but the cells no longer rely on signaling downstream of the ErbB2 receptor. These data suggest that the primary mechanism of GW583340 action as a dual ErbB1/2 kinase inhibitor (38) is not compromised in the rSUM149 and rSUM190 cells and thereby does not explain the decreased sensitivity to GW583340-induced cell death observed in the rSUM149 and rSUM190 cells.

The effect of GW583340 on apoptotic signaling was thereby interrogated in these cell lines. Western immunoblot analysis of key antiapoptotic proteins revealed sustained survivin (Fig. 3A) and Bcl-2 (Fig. 3B) expression in rSUM149 and rSUM190 cells. Further, immunoblot analysis of Forkhead box O3 (FOXO3a), a transcription factor known to promote proapoptotic gene transcription, revealed a decrease in FOXO3a expression in rSUM149 and rSUM190 cells (Fig. 3C); however, the difference was more marked in the rSUM190 cells. XIAP, one of the most potent caspase inhibitors that can modulate both the mitochondrial and extrinsic apoptotic signaling cascades, was characterized (Fig. 4) in the IBC cells described herein. A 2- to 3-fold overexpression of XIAP protein levels was observed by immunoblot analysis in rSUM149 and rSUM190 cells compared with nontreated parental cells (Fig. 4A). In addition, inhibition of XIAP expression in SUM149 cells and cleavage (data not shown) were observed post-GW583340 treatment, which was similar to previous results from our laboratory in SUM190 cells (18). XIAP immunofluorescence (Fig. 4B) and further quantitative analysis of the mean intensity of XIAP staining per pixel supports the XIAP overexpression in rSUM149 and rSUM190 cells maintained in GW583340.

To determine whether XIAP overexpression is a potential factor in acquired resistance to GW583340, parental SUM149 cells were stably transfected using a lentiviral expression system with exogenous wild-type XIAP and a corresponding vector control as described in Materials and Methods (Fig. 4D, left). Data in Fig. 4D show that exogenous overexpression of XIAP in parental IBC cells reverses the sensitivity of parental SUM149 cells to GW583340-induced cell death, which shows that XIAP expression is sufficient to mediate the resistance of these cells to the ErbB1/2 tyrosine kinase inhibitor.

**XIAP overexpression in IBC cells with acquired resistance to GW583340 is driven by IRES-mediated translation.** To address the mechanism of XIAP upregulation in the GW583340-resistant IBC cells, XIAP transcription and
The translation of XIAP protein were characterized in the IBC cells. Real-time reverse transcription-PCR analysis (Fig. 5A) showed no significant change in XIAP mRNA levels (SUM149 versus rSUM149, P = 0.467; SUM190 versus rSUM190, P = 0.233). XIAP has been identified to have an IRES element in its 5′UTR that can be used as a non-canonical translational start site in times of cellular stress (8–10). To characterize the IRES-mediated translation of XIAP in the IBC cells, a luciferase reporter construct was generated wherein the 5′UTR of XIAP, which contains the IRES, was cloned immediately upstream of the firefly luciferase gene (pGL3-XIAP.IRES). Data in Fig. 5B reveal that both rSUM149 and rSUM190 cells had higher luciferase activity than their parental counterparts when firefly luciferase expression was normalized to the co-transfected Renilla luciferase plasmid (SUM149 versus rSUM149, P < 0.005; SUM190 versus rSUM190, P < 0.005). Additionally, transfection of cells with truncated forms of the XIAP IRES [pGL3-XIAP.IRES (cont 1) and pGL3-XIAP.IRES (cont 2)], which acted as negative controls, did not elicit any luciferase activity. These data show that the upregulation of XIAP in GW583340-resistant cells is predominantly driven by the IRES-mediated translation of XIAP and not increase in XIAP mRNA.

**Inhibition of XIAP function using a small-molecule inhibitor causes apoptosis and overcomes GW583340 resistance.** Translational upregulation of XIAP seems to correlate with acquired resistance to GW583340-induced apoptotic response when cells are chronically exposed to GW583340 (Fig. 5B). Additionally, exogenous overexpression of XIAP caused parental IBC cells to be resistant to the cell death induced by GW583340 (Fig. 4D), which is similar to that seen is the acquired resistance model (rSUM149) with endogenously high levels of XIAP (Fig. 4A and B). Therefore, we evaluated the effect of inhibition of XIAP action. For this purpose, embelin (a small molecular inhibitor that has been shown to prevent binding of XIAP to procaspase-9 and thereby increase caspase-9 activity) was used (34). The mechanism of embelin is shown in Fig. 6A wherein decreased levels of procaspase-9 were observed with increasing concentrations in parental and rSUM149 and rSUM190 cells. This decrease in procaspase-9 after embelin treatment correlated with increased caspase-9 activity (P < 0.005; Fig. 6A) and apoptosis as measured by nucleosome enrichment (SUM149 and rSUM149, P < 0.005; rSUM149, P < 0.05; Fig. 6A). Treatment of another IBC-like cell line (SUM44) with embelin did not decrease procaspase-9 expression (Fig. 6A) and these cells are resistant to the apoptotic-inducing effects of the XIAP inhibitor (data not shown), which shows the specificity of embelin.

Because rSUM149 and rSUM190 cells maintained in GW583340 show XIAP overexpression and are resistant to GW583340-mediated apoptosis, experiments were conducted to determine if inhibition of XIAP action using embelin would sensitize the resistant cells to GW583340. Addition of embelin to the rSUM149 and rSUM190 cells growing in GW583340 for 48 hours caused significant cell death compared with GW583340 alone in the absence of embelin (P < 0.005). Additionally, treatment of parental cells with both GW583340 (Figs. 1 and 6B) and embelin (Fig. 6A and B) alone or in combination (Fig. 6B) significantly increased cell death compared with vehicle control cells (SUM149, P < 0.005; SUM190, P < 0.005). Data from our previous study (18) showed that treatment of sensitive parental cells with GW583340 decreases XIAP expression. Taken together, these data indicate that XIAP is a point of failure in both parental and GW583340-resistant IBC cells. Moreover, no synergy was observed between GW583340 and embelin treatment, which is most likely because these drugs affect the same pathway (i.e., decrease in XIAP). In summary, these data show that inhibition of XIAP binding to procaspase-9 using embelin and resultant increase in caspase activity causes apoptosis and potentially overcomes the acquired resistance to cell death in rSUM149 and rSUM190 cells.

**Discussion**

We report herein apoptotic dysregulation correlating with XIAP overexpression in two IBC cell models of acquired resistance to a lapatinib analogue (GW583340). The parental cells, SUM190 (ErbB2 overexpressing) and SUM149 (ErbB1 activated) derived from primary tumors.
of IBC patients (37), were sensitive to GW583340-mediated cell death. A marked decrease in p-ErbB2 or p-ErbB1 and corresponding inhibition of downstream signaling were evident in cells with acquired resistance to GW583340 (rSUM190 and rSUM149, respectively), similar to the parental counterparts treated with the drug, suggesting that the primary mechanism of action of GW583340, a dual ErbB1/2 tyrosine kinase inhibitor, was not compromised in the resistant cells.

Lapatinib is a dual tyrosine kinase inhibitor and is therefore effective in tumors with either ErbB2 expression or ErbB1 expression. Both ErbB2-overexpressing and ErbB1-activated IBC cells were sensitive to the growth-inhibitory and apoptotic-inducing effects of GW583340 (a lapatinib analogue). Evidence from the clinic has shown that IBC tumors are relatively more responsive to lapatinib than other breast cancer types (response rate: 50% in IBC versus <10% in non-IBC; refs. 29, 30); however, the response to lapatinib is often short lived and resistance is common (31). Two recent studies (25, 32) have shown that apoptotic signaling is an important mechanism of lapatinib resistance and the apoptotic pathways have been characterized to be dysregulated in IBC versus other locally advanced breast cancer types (39–42). Xia et al. (25) reported that acquired resistance to lapatinib in the estrogen receptor–dependent non-IBC BT474 cells is due to increased activity of the transcription factor FOXO3a, which regulates estrogen receptor downstream antiapoptotic proteins such as survivin and Bcl-2. The other report showed that MCL-1 (an antiapoptotic member of the Bcl-2 family) is increased in colon cancer cells resistant to lapatinib (32). These studies support the idea that dysregulation of the apoptotic signaling pathway plays a key role in the resistance of cancer cells to lapatinib. In addition, a previous study in our laboratory has shown that XIAP expression correlates with resistance to trastuzumab in the ErbB2-overexpressing SUM190 IBC cells (18), further supporting the hypothesis that the antiapoptotic signaling pathway is dysregulated in response to ErbB2 targeting agents.

In the present study, a model of acquired resistance to a dual ErbB1/2 tyrosine kinase inhibitor (lapatinib analogue, GW583340) was generated because resistance to lapatinib monotherapy in patients treated with daily doses of lapatinib is commonly seen (31). The GW583340-resistant lines

Figure 4. Effect of acquired resistance to GW583340 on XIAP in IBC cells. A, XIAP immunoblot analysis of rSUM190 and rSUM149 cells. GADPH was used as a loading control. Numbers represent densitometric analysis of XIAP normalized to GADPH. B, representative fluorescent microscopy images of parental and resistant SUM149 and SUM190 cells probed with an XIAP antibody and counterstained with Hoechst. C, mean XIAP staining intensity per pixel in parental and resistant IBC cells. Columns, mean XIAP staining intensity per pixel in over 20 single cells taken from 10 different fields; bars, SEM. D, effect of exogenous, stable XIAP overexpression on viability post-GW583340 treatment. Wild-type XIAP (wtXIAP) or vector control (vector cont.) were stably overexpressed in parental SUM149 cells (left, immunoblot analysis). Actin was used as a loading control and numbers represent densitometric analysis of XIAP normalized to actin. Cells were treated for 24 hours with GW583340 and viability was assessed through trypan blue exclusion (right). Columns, mean (*, P < 0.05; n = 2); bars, SEM.
(rSUM190 and rSUM149) were established by chronic exposure of the parental SUM190 and SUM149 cells to the drug for >3 months. It was shown that the primary mechanism of action of the tyrosine kinase inhibitor remained intact in the GW583340-resistant cellular model, and therefore, we hypothesized that the apoptotic pathway was dysregulated. A significant overexpression of XIAP was observed to be mediated by IRES-dependent translation in the acquired resistant cell models studied here. In addition, it has been reported that FOXO3a, a transcription factor that promotes proapoptotic gene transcription (43), and XIAP expression show an inverse correlation (43). FOXO3a is upregulated by c-jun NH2-terminal kinase (43), which is negatively regulated by XIAP (44). It is therefore not surprising that FOXO3a expression was decreased in the GW583340-resistant IBC cells wherein XIAP was dramatically upregulated. In addition, rSUM149 and rSUM190 cells had sustained Bcl-2 and survivin levels similar to a previous report in a non-IBC cell line resistant to lapatinib (25).

The present data show that XIAP was specifically overexpressed in the acquired resistance IBC model (rSUM149 and rSUM190). Alternatively, exogenous overexpression of XIAP in parental cells reversed the sensitivity to GW583340-mediated apoptosis, revealing the critical role of XIAP in therapeutic resistance. Interestingly, we observed that in contrast to targeting XIAP, small interfering RNA-mediated inhibition of another IAP (survivin) implicated in therapeutic resistance in breast cancer in these cells had no significant effect on viability or apoptosis (data not shown), similar to our previous report in trastuzumab resistance in IBC (18). This is consistent with the role of survivin as a nontraditional inhibitor of apoptosis as it has not been effectively shown to functionally inhibit caspases (45) but is rather a mitotic regulator (46). Interestingly, XIAP has been previously shown to bind and regulate the function of survivin (47), and therefore, it is appealing to speculate that inhibition of both XIAP and survivin may be even more potent than inhibition of these molecules separately.

Embelin, an inhibitor of XIAP’s primary role as a caspase inhibitor (34), was used as a proof-of-principle agent to cause specific abrogation of the inhibitory interaction between XIAP and procaspase-9; treatment of rSUM190 and rSUM149 cells with embelin decreased cell viability and increased apoptosis. This indicates that XIAP is critical for survival of cells with acquired resistant to GW583340.

It is clear that apoptotic dysregulation is a critical factor in acquired lapatinib resistance in breast cancer. In addition, this study is the first to elucidate that XIAP overexpression corresponding with resistance to GW583340-induced apoptosis in the ErbB2-overexpressing and ErbB1-activated IBC cellular models is not due to the increase

![Figure 5. Effect of acquired resistance to GW583340 on XIAP mRNA expression and protein translation in IBC cells.](image-url)
in XIAP transcription but rather due to the increased translation of XIAP through its IRES element present in its 5′UTR (8–10). These unique secondary structures can be used as noncanonical translation start sites during times of cellular stress when traditional protein translation is shut down (7), identifying XIAP as a stress-related target for therapeutic intervention and establishing the feasibility of targeting XIAP in combination with lapatinib to enhance tumor apoptosis in IBC therapy.

**Figure 6.** Effect of embelin, GW583340 alone, and in combination on cell death. A, effect of embelin on procaspase-9 (left, immunoblot analysis); right, caspase-9 activity and nucleosome enrichment. GAPDH was used as a loading control for the immunoblots. Numbers represent densitometric analysis of procaspase-9 normalized to GAPDH. Columns, mean of triplicate values (n = 2); bars, SEM. B, effect of GW583340 and embelin treatments as shown by + and − on total cell death in SUM149 and rSUM149 (growing in GW583340; left) and SUM190 and rSUM190 (growing in GW583340; right) cells. Columns, mean SEM of triplicate values; bars, SEM. Resistant cells treated with GW583340 versus embelin, P < 0.05; n = 2–3.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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XIAP and ErbB1/2 Tyrosine Kinase Inhibitor Resistance

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References


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