

Herceptin Sensitizes ErbB2–Overexpressing Cells to Apoptosis by Reducing Antiapoptotic Mcl-1 Expression

Elizabeth S. Henson, Xiaojie Hu, and Spencer B. Gibson

Abstract **Purpose:** Monoclonal antibodies, such as herceptin and trastuzumab, against the epidermal growth factor receptor ErbB2 (also known as HER2/neu) are an effective therapy for breast cancer patients with overexpression of ErbB2. Herceptin, in combination with standard chemotherapy, such as taxol or etoposide, gives a synergistically apoptotic response in breast tumors. **Experimental Design:** The mechanism underlying this synergy between chemotherapy and herceptin treatment is not well understood. Herein, we have determined that addition of herceptin, sensitized breast cancer cell lines MDA-MB-231 and MCF-7 to etoposide- or taxol-induced apoptosis. **Results:** This treatment resulted in reduced expression of ErbB2 and the antiapoptotic Bcl-2 family member Mcl-1 in MDA-MB-231 cells. Using antisense oligonucleotides against Mcl-1, MDA-MB-231 cells were rendered sensitive to etoposide-induced apoptosis similar to herceptin, but combined treatment of antisense against Mcl-1 and herceptin failed to give a significant increase in apoptosis. In 29 human breast tumors immunostained for ErbB2 and Mcl-1, we found that when ErbB2 was overexpressed, there was a corresponding increase in Mcl-1 expression. **Discussion:** Using murine fibroblasts that express human ErbB2, but no other ErbB family member (NE2), these cells showed resistance to both taxol- and etoposide-induced apoptosis compared with parental cells. In addition, NE2 cells preferentially express the antiapoptotic Bcl-2 family member Mcl-1 compared with parental cells, and treatment with herceptin reduces Mcl-1 expression. Taken together, these results suggest that herceptin sensitizes ErbB2-overexpressing cells to apoptosis by reducing antiapoptotic Mcl-1 protein levels.

The epidermal growth factor (EGF) receptor ErbB2 (also known as HER2/neu) is a 185 kDa transmembrane receptor tyrosine kinase that is amplified or overexpressed in many cancers including breast cancer (1). It has been associated with poor clinical outcome such as shorter survival and short time to relapse (2, 3). This has led to ErbB2 being a target for drug development. Monoclonal antibodies against ErbB2 (herceptin, Trastuzumab) have been developed which reduces tumor growth and induces apoptosis (1, 4, 5). Herceptin is currently used in the treatment of breast tumors expressing high levels of ErbB2 (5). This treatment is most effective when combined with standard chemotherapy such as microtubule toxins (taxol) or genotoxins (etoposide; ref. 6). The mechanism for the synergy between herceptin and standard chemotherapy remains unclear.

Bcl-2 family members consist of both proapoptotic (Bax and Bak) and antiapoptotic (Bcl-2, Bcl-x_L, A1, Mcl-1, and Bcl-w) proteins (7). The association of these proteins with each other dictates whether a cell will survive or undergo apoptosis (8). In cancer, antiapoptotic members are often increased, rendering cancer cells resistant to apoptosis (8). Mcl-1 is one member of the antiapoptotic Bcl-2 family that was initially identified in the human myeloid leukemia cell line, ML-1 (9, 10). The protein inhibits apoptosis, through a direct interaction with proapoptotic members of the Bcl-2 family at the mitochondria. Mcl-1 is essential for hematopoietic stem cells and lymphocyte survival (11). The Mcl-1 protein has a hydrophobic stretch of 20 amino acids at the COOH terminus, which is responsible for localizing both proteins to the outer mitochondrial membrane similar to other Bcl-2 family members. Mcl-1 differs from Bcl-2 at the amino end where Mcl-1 has a unique amino acid segment that contains PEST motifs (sequences rich in proline, glutamate, serine, and threonine; refs. 9, 10). These PEST motifs likely contribute to the fact that the half-life of Mcl-1 is ~1 hour, compared with 10 to 14 hours for Bcl-2. Mcl-1 mRNA is also alternatively spliced into a short form (Mcl-1s; ref. 12). In addition, Mcl-1 mRNA turns over rapidly, and these features explain the ability of Mcl-1 expression to be rapidly increased or down-regulated with cytokines and differentiation factors (13). Mcl-1 thus differs from other members of the Bcl-2 family in acting as an immediate response molecule to protect cells against apoptosis.

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Herein, we will show that ErbB2 expressing cells up-regulate Mcl-1 levels and upon herceptin treatment, Mcl-1 expression is reduced. This corresponds with increased sensitivity in these cells to apoptosis. Using antisense against Mcl-1, MDA-MB-231 breast cancer cells are sensitized to etoposide-induced apoptosis that is not enhanced by further herceptin treatment. This suggests that herceptin antitumor activity could be due to reduced expression of Mcl-1.

Materials and Methods

Materials. Antibodies for Western blot were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): Mcl-1 rabbit polyclonal S-19 (sc-819), Bcl- κ _{S/L} rabbit polyclonal S-18 (sc-634), and Bcl-2 rabbit polyclonal N-19 (sc-492). Actin rabbit polyclonal (A02066), anti-rabbit FITC (F-0382) and Bax mouse monoclonal clone 2D2 (B-8554) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for immunofluorescence were obtained from DAKO Corporation (Carpinteria, CA): Mcl-1 rabbit polyclonal (A3534), c-erbB-2 rabbit polyclonal (A0485), swine anti-rabbit blocking antibody (Z0196). Anti-mouse Rhodamine Red-X (R-6393) was purchased from Molecular Probes (Eugene, OR).

Cell culture. All cell lines (unless otherwise stated) were main-tained in a humidified 5% CO₂ environment, in DMEM supplemented with 100 units of penicillin per mL, 100 μ g of streptomycin (Life Technologies) per mL and in 10% bovine calf serum. Cells were transfected with Mcl-1 cDNA by LipofectAMINE as previously described (14). NIH3T3 cells contain very low levels of expression of EGF receptors and either untransfected or stably transfected with human ErbB2 cDNA in an expression vector as previously described (15). MDA-MB-231 cells were derived from a metastasis in mice (a kind gift from Dr. Peter Watson, Manitoba Institute of Cell Biology and Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada). MCF-7 cells were maintained in α -MEM with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), and 1% penicillin/streptomycin (Invitrogen), HEPES (Invitrogen), and sodium pyruvate (Invitrogen). Cells were visualized using an Olympus CK40 microscope and Spot CCD Camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Measurement of apoptosis. Cells were resuspended in 100 μ L of medium and 2 μ L of acridine orange (100 μ g/mL), and ethidium bromide (100 μ g/mL) in PBS was added. Ten microliters of the solution were applied to a slide, and the cells were viewed on an Olympus BX51 fluorescent microscope using the fluorescein filter set. Cells which showed intense staining of the DNA in the nucleus were defined as apoptotic versus the diffuse staining of the nucleus in healthy cells. At least 200 cells were counted per condition. All statistical analyses for significant differences in apoptotic responses was done using a Student's *t* test method unless otherwise stated.

RNase protection assay. A RiboQuant Multi-Probe RNase Protection Assay system (BD Biosciences PharMingen, Mississauga, Ontario, Canada) was used as per the manufacturer's instructions. MDA-MB-231 cells were treated for 24 hours with 1 or 10 μ g of Herceptin, or left untreated as a control. RNA was harvested using RNA Bee (Tel-Test Inc., Friendswood, TX) as per the manufacturer's instructions and 20 μ g of RNA was hybridized with the hAPO-2b template set (BD Biosciences PharMingen). The housekeeping gene *GAPDH* was used to normalize the samples. Analysis of the signal intensity was done on a Storm Phosphoimager (GE Health Care Biosciences, Little Chalfont, United Kingdom).

Statistical analysis. Statistical analysis was done using Microsoft Excel, and using Student's *t* test or χ^2 tests as appropriate. *P* < 0.05 was considered statistically significant (16).

Immunoblots. Cells were lysed in Nonidet P-40 lysis buffer [50 mmol/L HEPES (pH 7.25), 150 mmol/L NaCl, 50 μ mol/L ZnCl₂, 50 μ mol/L NaF, 2 mmol/L EDTA, 1.0% Nonidet P-40, 2 mmol/L

phenylmethylsulfonyl fluoride]. Cell debris was removed by centrifugation at 10,000 \times g for 10 minutes and protein concentrations were determined by Bradford assay. Fifty micrograms of lysate were subjected to SDS-polyacrylamide electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in TBS with 0.15% Tween 20 (TBST) and 5% milk. Blots were incubated with a primary antibody overnight, washed thrice in 1 \times TBST and incubated for 1 hour with the appropriate secondary antibody conjugated with alkaline phosphatase. Blots were visualized on X-ray film with enhanced chemiluminescence reagents (Amersham-Biosciences). Breast tumor protein was a kind gift from the Manitoba Breast Tumor Bank. Equal amounts of protein were loaded in each lane as confirmed by Ponceau-S staining.

Transfection of antisense oligonucleotides against Mcl-1. All oligonucleotides (Sigma Genosys) were designed with phosphorothioate nucleotides at each end represented by the lower case letters in the sequence. Antisense (catccCAGCCTCTTgtttA), sense (atttgTTCTCC-GACcctaC) and scramble (tagctTCGTTTCCAcctaT) oligonucleotides were used. The scrambled sequence was run through BLAST search, and no significant homologies existed with other genes. Antisense transfections were done using OligofectAMINE Reagent (Invitrogen) as previously described (14). At this time, 10 μ g/mL of Herceptin was added to the appropriate wells and the cells were incubated for 1 hour before etoposide was added. Cells were harvested 24 hours after etoposide treatment.

Immunofluorescence on breast tumor sections. Paraffin-embedded sections were provided by the Manitoba Breast Tumor Bank. Immunofluorescence was done as described in ref. (17). Briefly, anti-Mcl-1 (DAKO) was diluted 1:250 in blocking buffer [1 \times PBS, 5% goat serum (Life Technologies), 0.2% Triton X-100, 0.02% sodium azide, and 0.1% bovine serum albumin]. All incubations in primary antibody were done overnight, incubations in secondary antibodies were done in 1 hour. Secondary anti-rabbit Rhodamine Red was diluted at 1:500 in blocking buffer. Because both primary antibodies were raised in rabbit, additional blocking was required. Slides were blocked in 100% bovine calf serum for 30 minutes, washed thrice for 5 minutes, and blocked with swine anti-rabbit (Sigma) at a dilution of 1:25 in blocking buffer for 30 minutes. Anti-ErbB2 was diluted 1:350 in blocking buffer. Secondary anti-rabbit FITC was diluted 1:500 in blocking buffer. Nuclei were stained using Hoechst, and the slides were mounted using antifade (Molecular Probes). Fluorescence was visualized and captured on an Olympus BX51 microscope (Olympus) with Scope Pro deconvolution software (Media Cybernetics).

Results

Breast cancer cell lines are sensitized to etoposide- and taxol-induced apoptosis following herceptin treatment. Increased ErbB2 expression has been associated with drug resistance in breast cancer (1, 2). In breast cancer patients with over-expression of ErbB2, herceptin treatment is often used in combination with standard chemotherapy (4, 5). To determine whether herceptin could sensitize breast cancer cells to apoptosis, MDA-MB-231 cells were treated with etoposide (1.5 mmol/L) in the presence or absence of herceptin (0.1 to 10 μ g/mL) for 48 hours. MDA-MB-231 cells are relatively resistant to etoposide-induced apoptosis in which 1.5 mmol/L etoposide treatment induced only 26% apoptosis (Fig. 1A). When 0.5 μ g/mL herceptin was added, the amount of etoposide-induced apoptosis increased to 29% and when 1 or 10 μ g/mL herceptin was added, the amount of apoptosis increased to 36% and 39%, respectively (Fig. 1A). Similar results were found in the breast cancer cell line MCF-7 when 10 μ g/mL herceptin significantly increased the amount of etoposide-induced apoptosis from 18% to 29% apoptosis (Fig. 1B).

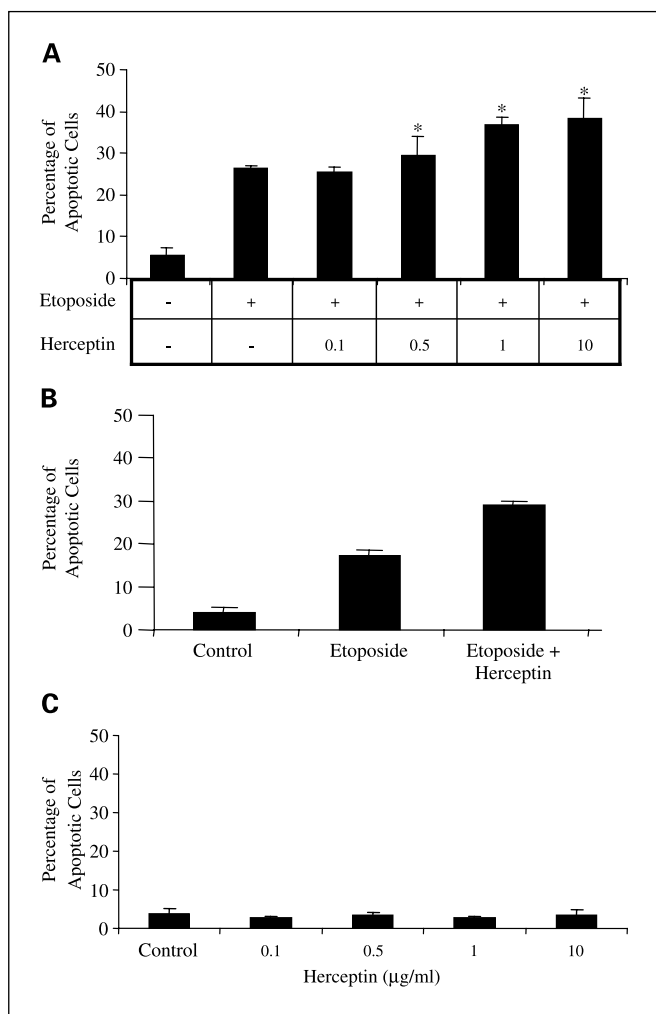


Fig. 1. Breast cancer cell lines are sensitized to apoptosis following herceptin treatment. *A*, MDA-MB-231 cells were left untreated or treated with etoposide (1.5 mmol/L) alone or in combination with herceptin (100 ng/mL to 10 μg/mL). Cells were incubated for 48 hours and apoptosis levels were determined by acridine orange staining. Columns, mean of three independent experiments; bars, SE. Asterisk, significant difference between etoposide-treated cells and cells treated with etoposide and herceptin ($P < 0.05$, Student's t test). *B*, MCF-7 cells were left untreated (control) or treated with 1 mmol/L etoposide alone or in combination with 10 μg/mL herceptin. The cells were then incubated for 48 hours and apoptosis was determined as described in Materials and Methods. *C*, MB-MDA-231 cells were also treated with herceptin alone over a range of concentrations (0.1-10 μg/mL) and the amount of apoptosis was determined as described above.

Herceptin treatment alone failed to increase apoptosis in the cell lines tested (Fig. 1C). Taken together, herceptin has the ability to increase etoposide-induced apoptosis in MCF-7 and MDA-MB-231 cells.

Herceptin treatment reduces ErbB2 and Mcl-1 expression in MDA-MB-231 cells. The mechanism for ErbB2-mediated cell survival is unclear. Expression of Bcl-2 family members regulate apoptosis (14, 18). MDA-MB-231 cells were treated with 1 or 10 μg/mL herceptin and mRNA levels of Bcl-2 family members were determined. We found that Bcl-x_L levels increased to 1.8-fold following 10 μg/mL herceptin treatment, whereas the proapoptotic family member Bax levels remained the same, and Mcl-1 mRNA levels decreased (Fig. 2A). Besides mRNA levels, Mcl-1, Bax, and Bcl-x_L protein levels were determined. Following treatment with 1 and 10 μg/mL herceptin, the

amount of Mcl-1 was significantly reduced, but levels of Bax and Bcl-x_L seemed slightly increased (Fig. 2B). In addition, ErbB2 expression decreased following herceptin treatment (Fig. 2B), which indicates inactivation of ErbB2 signal transduction (19). This suggests that herceptin treatment reduces Mcl-1 protein expression compared with other Bcl-2 family members by inhibiting ErbB2 signaling.

Antisense against Mcl-1 renders MDA-MB-231 cells sensitive to etoposide-induced apoptosis. Antisense oligonucleotides against Bcl-2 have been shown to be effective at sensitizing cancer cells to apoptosis (20, 21). To determine whether reduced Mcl-1 expression will sensitize cells to etoposide-induced apoptosis, antisense oligonucleotides against Mcl-1 were transfected into MDA-MB-231 cells and the amount of etoposide-induced apoptosis was determined. Previously, we and other have shown that antisense oligonucleotides against Mcl-1 were specific for Mcl-1 and not other Bcl-2 family members (14, 22, 23). Etoposide treatment alone induced 18% apoptosis, whereas antisense, sense, and scrambled oligonucleotides against Mcl-1 failed to induce apoptosis. When etoposide was treated in combination with antisense against Mcl-1, the amount of apoptosis increased. In contrast, the combination of etoposide and sense or scrambled oligonucleotides failed to increase the amount of apoptosis (Fig. 3A). In addition, the antisense against Mcl-1 was transfected into MDA-MB-231 cells in combination

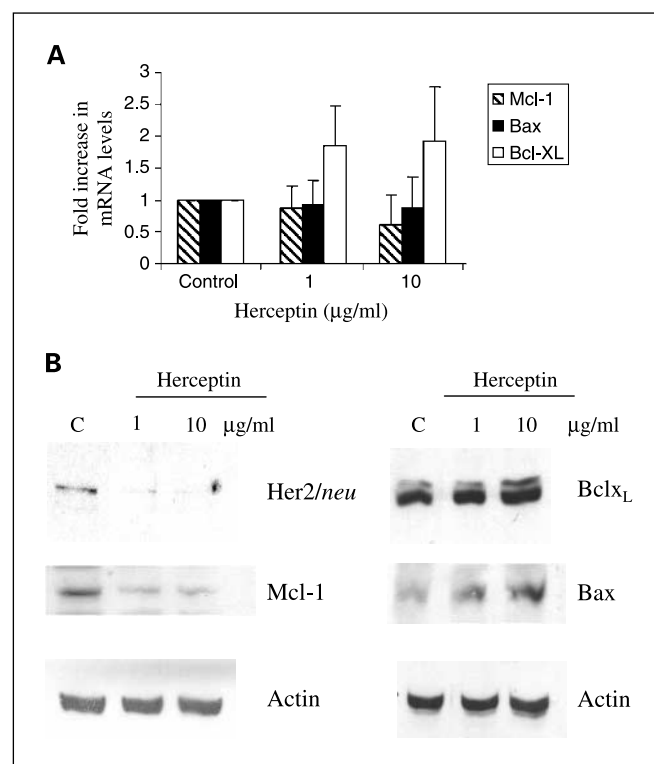


Fig. 2. Herceptin treatment lowers the level of Mcl-1 expression. *A*, mRNA for Bcl-2 family members was determined by RNase protection assay. MDA-MB-231 cells were left untreated (control) or treated with 1 or 10 μg/mL herceptin. Total RNA was isolated after 24 hours, and 20 μg of RNA was hybridized with hAPO-2b template set as described in Materials and Methods. Analysis of the signal intensity was done on a Storm Phosphorimager (Amersham-Biosciences). Fold change in mRNA levels were normalized to L32 mRNA. Columns, mean of three independent experiments; bars, SE. *B*, MDA-MB-231 cells were treated with 1 and 10 μg/mL herceptin or left untreated (*C*). Cells were lysed 24 hours posttreatment and Western blotted for ErbB2, Mcl-1, Bcl-x_L, Bax, and actin.

with herceptin (1 µg/mL) and etoposide (1.5 mmol/L). The amount of apoptosis increased to 38% but did not give an additive effect (predicted additive effect would be 45%; Fig. 3B). To ensure that antisense against Mcl-1 was reducing Mcl-1 protein levels, cells were lysed and Western blotted for Mcl-1 in the presence or absence of antisense oligonucleotides. This indicated that antisense oligonucleotides decreased Mcl-1 expression (Fig. 3C). Taken together, this suggests that herceptin and antisense against Mcl-1 target similar antiapoptotic pathways.

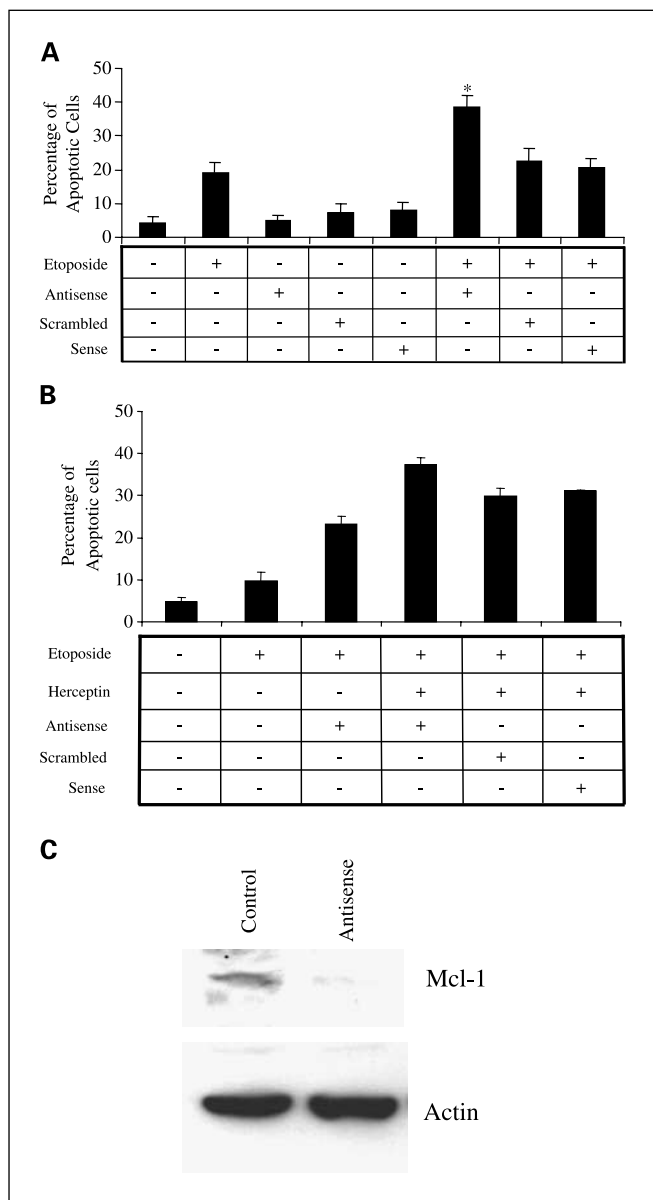


Fig. 3. Antisense oligonucleotides against Mcl-1 sensitizes cells to etoposide-induced apoptosis, and treatment with herceptin does not increase this sensitivity. MDA-MB-231 cells were transfected with antisense, sense, and scrambled oligonucleotides against Mcl-1 as described in Materials and Methods. *A*, cells were then treated with 1.5 mmol/L etoposide or (*B*) herceptin (10 µg/mL) in combination with etoposide (1.5 mmol/L). Twenty four hours posttreatment, apoptosis levels were determined using acridine orange staining. Columns, mean of three independent experiments; bars, SE. Asterisk, significant difference ($P < 0.05$, Student's test) between cells treated with etoposide alone and cells treated with etoposide with antisense against Mcl-1. *C*, 24 hours posttransfection with antisense against Mcl-1, the cells were lysed and Western blotted for Mcl-1 and stripped and reprobed with actin as a loading control.

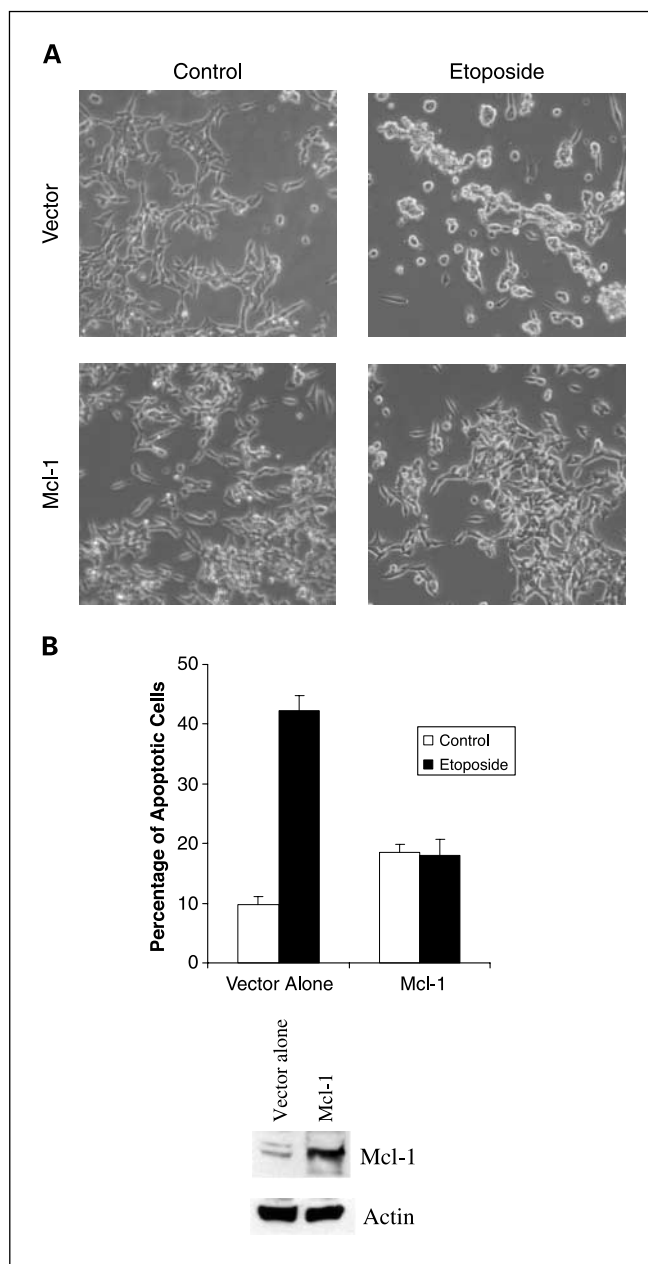


Fig. 4. Mcl-1 overexpression is sufficient for protection against etoposide-induced apoptosis. *A*, HEK293 cells were transiently transfected with Mcl-1 cDNA in an expression vector (Mcl-1) or vector alone (Vector). The cells were then left untreated (control) or treated with 100 µmol/L etoposide. Twenty four hours posttransfection, cells were visualized using an Olympus CK40 and Spot camera. *B*, the amount of apoptosis was determined by acridine orange staining. (Original magnification, $\times 400$). Cells were also lysed and then Western blotted for Mcl-1 and actin (loading control).

Overexpression of Mcl-1 blocks etoposide-induced apoptosis. Decreased levels of Mcl-1 expression could explain the increase in the amount of etoposide-induced apoptosis following herceptin treatment. To determine whether overexpression of Mcl-1 blocks etoposide-induced apoptosis, we overexpressed Mcl-1 in the epithelial derived human embryonic kidney (HEK) 293 cells and treated the cells with etoposide. In cells overexpressing Mcl-1, the amount of etoposide-induced apoptosis was reduced compared to cells transfected with vector

alone as indicated by changes in cellular morphology (Fig. 4A) and by chromatin condensation (Fig. 4B). Overexpression of Mcl-1, however, increased the amount of apoptosis in untreated cells (Fig. 4B). Cells were lysed and Western blotted for Mcl-1 to indicate overexpression of Mcl-1.

ErbB2 and Mcl-1 expression are associated in estrogen receptor-negative breast cancers. ErbB2 expression is often increased in cancer, especially in breast cancer (1). We obtained 29 estrogen receptor-negative tumors from the Manitoba Breast Tumor Bank and immunofluorescently stained for ErbB2 and Mcl-1. Scoring of ErbB2-positive tumors was conducted according to standard pathology practices (24, 25). We found that tumors that were positive for ErbB2 were all positive for Mcl-1 expression (Fig. 5A; Table 1). In tumors that were positive for Mcl-1 expression, only one showed low staining for ErbB2 (Table 1). These results were statistically significant using a χ^2 test ($P < 0.00001$). Furthermore, using Western blotting for ErbB2 and Mcl-1, tumors with high ErbB2 expression showed high Mcl-1 protein levels whereas tumors with low ErbB2 expression could express low or high Mcl-1 protein levels (Fig. 5B).

Exclusive ErbB2 expression in cells confers protection against taxol- and etoposide-induced apoptosis that is inhibited by herceptin treatment. To evaluate the role ErbB2 plays in inhibiting apoptosis in the absence of other EGF receptor family members, we used NIH3T3 cells that have been selected for low expression of EGF receptor family members (a kind gift from Dr. Hynes, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland, ref. 15). These cells were either parental cells or parental cells stably transfected with human ErbB2 cDNA (NE2). The cells were then treated with a

microtubule toxin taxol or etoposide. Untreated parental cells showed higher levels of apoptosis compared with NE2 cells (Fig. 6). On treatment of taxol over a dose range of 2.5 to 12.5 $\mu\text{mol/L}$, the amount of apoptotic cells increased to a maximum of 38% in parental cells after 48 hours. In contrast, the amount of apoptosis in NE2 cells following taxol treatment increased to only 15% after 48 hours (Fig. 6A). When etoposide (100 $\mu\text{mol/L}$) was added to parental cells, the amount of apoptosis increased to 40% at 48 hours. Similar to taxol, NE2 cells exposed to etoposide for 48 hours showed only 27% apoptosis (Fig. 6B). This indicates that expression of human ErbB2 decreases the amount of apoptosis in these cells following apoptotic stimuli.

To confirm that ErbB2 is conveying this resistance to apoptosis, parental and NE2 cells were exposed to etoposide alone, or in combination with increasing concentrations of herceptin (0.1-10 $\mu\text{g/mL}$). As the concentration of herceptin increased, the amount of apoptosis in NE2 cells increased following etoposide treatment until the level of apoptosis was similar to parental cells treated with etoposide (Fig. 6C). Herceptin treatment did not alter the amount of etoposide-induced apoptosis in parental cells due to the lack of ErbB2 expression. Similar results were found after taxol treatment with the addition of 1 $\mu\text{g/mL}$ herceptin increasing taxol-induced apoptosis in NE2 cells from 15% to 24% (Fig. 6D). Herceptin alone failed to induce apoptosis in NE2 cells over a range of concentrations (Fig. 6E).

The expression levels of antiapoptotic Bcl-2 family members were also determined in parental and NE2 cells. We found that parental cells expressed detectable levels of Bcl-x_L and Bcl-2 but failed to express detectable levels of Mcl-1. In contrast, NE2

Fig. 5. Patients that overexpress ErbB2 show an up-regulation of Mcl-1. **A**, breast tumor sections were selected from the Manitoba Breast Tumor bank based on high grade and negative ER status. Paraffin-embedded tumor sections were deparaffinized, rehydrated, and processed for immunofluorescence as described in Materials and Methods. ErbB2 immunostaining (green), Mcl-1 immunostaining (red). DNA was stained using 4',6'-diamidino-2-phenylindole (blue). Image was captured using the 40x objective lens, 400 \times magnification. **B**, protein from tumors was selected from the Manitoba Breast Tumor bank based on high tumor grade and negative ER status. The protein lysate was Western blotted for ErbB2 and Mcl-1. Equal amounts of protein were loaded in each lane and confirmed by Ponceau S staining.

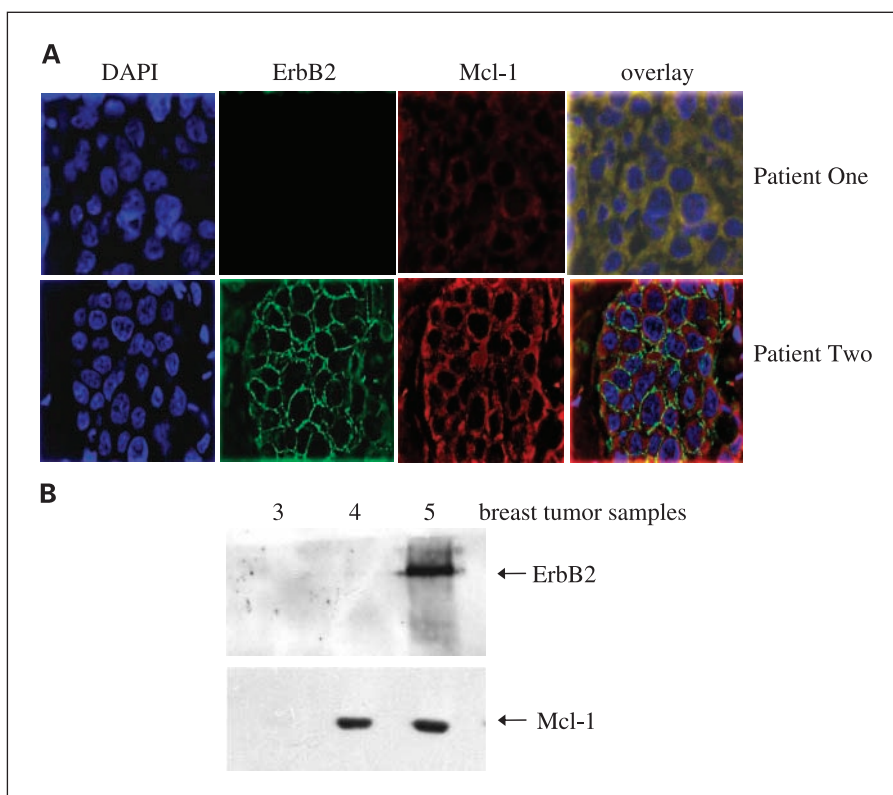


Table 1. Correlation of ErbB2 status with Mcl-1 expression levels

ErbB2 status	Mcl-1 status			Total
	Low	Medium	High	
0-1+	17 (58.6%)	1 (3.4%)	1 (3.4%)	19
2+	0 (0%)	3 (10%)	4 (13.8)	7
3+	0 (0%)	2 (6.9%)	1 (3.4%)	3
				29
χ^2				0.00001

NOTE: Expression of ErbB2 was determined using the HerceptTest. Tumors in which the entire membrane was stained for ErbB2 in >10% of the tumor cells were considered positive; those that did not show membrane staining, or only partial membrane staining, were considered negative. Mcl-1 levels were graded based on intensity of cytoplasmic staining from distinct punctuate staining in all tumor cells (low) to intense staining that fills the entire cytoplasm of the tumor cells (high). χ^2 analysis was done. Expected observations were the equal distribution within each level of ErbB2 expression across all levels of Mcl-1 expression. Analysis showed a χ^2 of 0.00001, tumors that were positive for ErbB2 were positive for Mcl-1.

cells expressed all three Bcl-2 family members (Fig. 7A). To confirm that NE2 cells express ErbB2 and parental cells express low levels of ErbB2, the cells were lysed and Western blotted

for ErbB2. As expected, NE2 cells expressed higher levels of ErbB2 than parental cells (Fig. 7A). Because herceptin increases etoposide-induced apoptosis, we determined whether herceptin treatment changes expression of Mcl-1 protein levels in NE2 cells. Using 10 $\mu\text{g}/\text{mL}$ of herceptin, the level of Mcl-1 was significantly reduced at 24 hours, whereas 1 $\mu\text{g}/\text{mL}$ herceptin did not reduce Mcl-1 protein levels (Fig. 7B). To determine whether lower concentrations of herceptin reduces Mcl-1 protein levels, NE2 cells were treated with 1 $\mu\text{g}/\text{mL}$ of herceptin over a 72-hour time course. This resulted in decreased levels of Mcl-1 after 48 hours of herceptin treatment (Fig. 7C). This further illustrates that herceptin treatment reduces Mcl-1 expression and sensitizes cells to apoptosis when ErbB2 is expressed.

Discussion

In many tumors, ErbB2 expression contributes to tumor progression and is a target for therapy (1, 2, 4, 5). ErbB2 expression has been correlated with increased drug resistance and aggressive disease (1). Inactivation of EGF signaling pathways using antibodies against ErbB2 or EGF receptors has been effective at increasing chemotherapy-induced apoptosis (5). Herceptin that targets ErbB2 is routinely used to treat breast cancer patients with increased ErbB2 expression (2). Herceptin is most effective when used in combination with standard

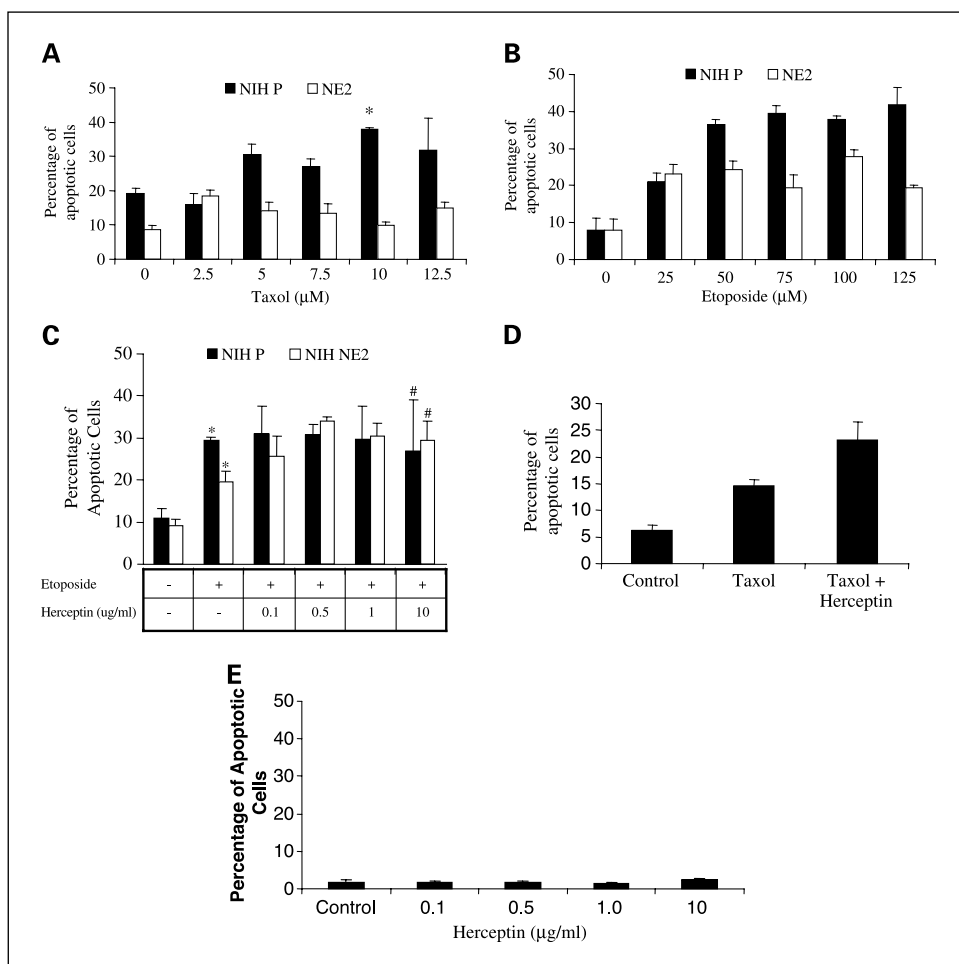


Fig. 6. NIH3T3 cells that express only ErbB2 are resistant to chemotherapeutic drug-induced apoptosis that is reversed by herceptin treatment. Cells were treated with increasing amounts of taxol (A) or etoposide (B) as indicated. Apoptotic cells were counted at 48 hours as determined by detection of condensed chromatin using acridine orange staining. Bars, SE. Asterisk, taxol induced a statistically significant amount of apoptosis ($P < 0.05$) in NIH parental cells. C, NIH3T3 cells were left untreated or treated with 100 ng/mL, 500 ng/mL, 1 $\mu\text{g}/\text{mL}$, or 10 $\mu\text{g}/\text{mL}$ of Herceptin and incubated for 1 hour. The cells were then treated with 75 $\mu\text{g}/\text{mL}$ of etoposide for 48 hours. The level of apoptosis was determined by acridine orange staining. Bars, SE; *, parental versus NE2 cells with etoposide alone ($P < 0.05$), indicating statistically significant differences. #, parental versus NE2 with etoposide plus 10 $\mu\text{g}/\text{mL}$ Herceptin ($P > 0.2$), which represents no statistical differences as determined by Student's t test. D, NE2 cells were left untreated (control) or treated with taxol alone (10 $\mu\text{g}/\text{mL}$) or in combination with 1 $\mu\text{g}/\text{mL}$ herceptin. The amount of apoptosis was determined as described above. E, NE2 cells were also treated with herceptin alone over a range of concentrations (0.1-10 $\mu\text{g}/\text{mL}$) and the amount of apoptosis determined as mentioned above at 48 hours following treatment. Bars, SE.

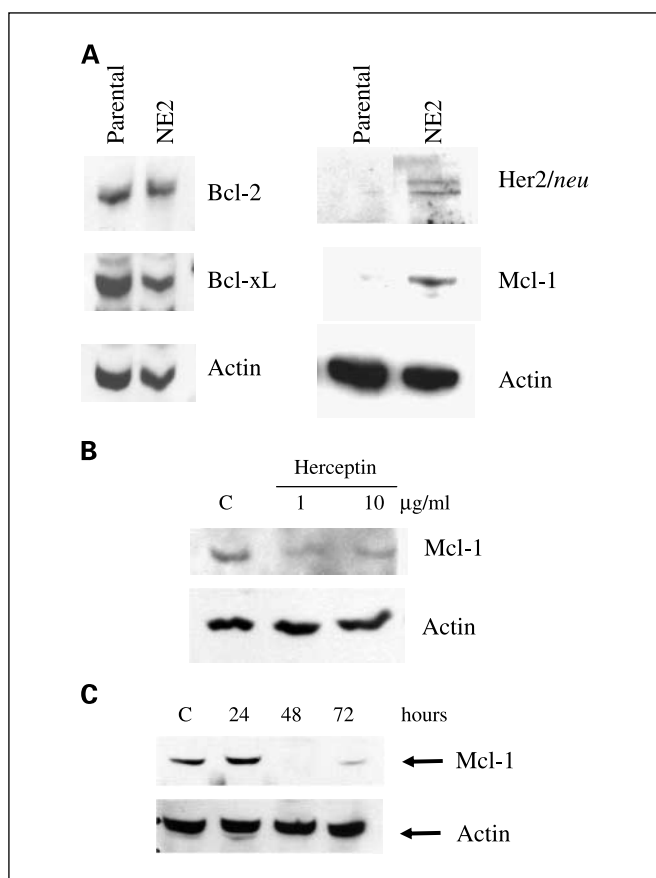


Fig. 7. NIH3T3 cells that express ErbB2 have higher levels of Mcl-1 but not Bcl-2 or Bcl-xL. **A**, NIH3T3 parental and NE2 cells were grown to confluence and then the cells were lysed and Western blotted for Bcl-2, Bcl-xL, ErbB2, Mcl-1, or actin as described in Materials and Methods. **B**, NE2 cells were left untreated (C) or treated with herceptin (1 or 10 $\mu\text{g}/\text{mL}$). After 24 hours, the level of Mcl-1 protein or actin (loading control) was determined. **C**, NE2 cells were either left untreated (C) or treated with 1 $\mu\text{g}/\text{mL}$ herceptin over a 72 hour time course. Cells were then lysed and level of Mcl-1 expression determined. Blots were stripped and reprobed with actin as a loading control.

chemotherapy (26). The increase in apoptosis associated with combined herceptin and chemotherapy has been attributed to inhibition of cell survival signaling pathways such as AKT signaling pathways (26, 27). Indeed, breast tumors with reduced PTEN expression are resistant to herceptin treatment due to increased AKT activation (27). This, however, only attributes the signaling pathways that block apoptosis but fails to account for herceptin-mediated activation of apoptotic pathways. For the first time, we have shown that herceptin reduces the expression of the antiapoptotic protein Mcl-1, and that elimination of Mcl-1 expression renders cells sensitive to chemotherapeutic drugs similar to herceptin treatment whereas overexpression of Mcl-1 inhibits apoptosis.

Members of the Bcl-2 family are one of the most important regulators of apoptotic potential (7, 8). Alterations in Bcl-2 family members are often found in cancers (8). Mcl-1 is overexpressed in many cancers (13, 28). In lymphoma, Mcl-1 is increased and contributes to tumor progression (29). In ovarian cancer, Mcl-1 expression correlates with poor prognosis (30). In chronic lymphocytic leukemia, Mcl-1 levels correlate with drug resistance and mutations in the Mcl-1 promoter seems to increase Mcl-1 expression (31–33). In

invasive breast tumors, Mcl-1 expression levels failed to correlate with disease outcome (25), but we have now shown that Mcl-1 expression is increased by overexpression of ErbB2 in estrogen-negative tumors. Taken together, Mcl-1 seems to promote cell survival and its expression is elevated in cancer cells.

Besides cancer cells, Mcl-1 plays a critical role in hematopoietic stem cell survival. Mcl-1 is expressed at high levels in hematopoietic stem cells, B cells, and T cells (11, 34). Targeted deletion of Mcl-1 in these cell types leads to a loss in ear bone marrow progenitor cells, mature B lymphocytes, and T lymphocytes. Moreover, growth factors such as stem cell growth factor, interleukin 7, and interleukin 2 increases and/or maintains Mcl-1 expression levels in hematopoietic cells similar to cancer cells (11, 34). The importance of Mcl-1 in development was also shown in mice lacking Mcl-1 expression, which fail to develop past the blastocyst stage and fail to implant *in utero*, giving an embryonic-lethal phenotype (35). Mcl-1 seems to provide a survival response that is tightly regulated by growth factors in several different cell types.

Mcl-1 could be cleaved into a proapoptotic protein similar to Bcl-2 when cells undergo apoptosis or when Mcl-1 is overexpressed (12). Indeed, we have shown that overexpression of Mcl-1 increases the amount of apoptosis in untreated cells. However, we failed to detect cleavage of Mcl-1 in cells expressing endogenous Mcl-1 and in cells treated with herceptin. Furthermore, etoposide failed to increase the amount of apoptosis in Mcl-1-overexpressing cells. This indicates that Mcl-1 is acting as an antiapoptotic protein in cancer cells.

Growth factors and their corresponding receptors have been shown to promote cell survival in cancer cells (36). Activation of EGF receptor family members leads to up-regulation of Mcl-1 (14, 37). This up-regulation has been associated with protection against apoptosis following apoptotic stimulation. Indeed, chemotherapeutic drug-induced apoptosis is effectively blocked by overexpression of EGF receptor family members and Mcl-1 (14, 37–39). In chronic lymphocytic leukemia, Mcl-1 protein levels decreased following treatment with genotoxic agents (33). We have previously shown that EGF binding to its receptors leads to increased Mcl-1 expression preventing death receptor-induced apoptosis (14). ErbB2 overexpression is also effective at blocking both death receptor- as well as chemotherapeutic drug-induced apoptosis (19). This supports our results that herceptin treatment reduces Mcl-1 levels and renders cells sensitive to chemotherapy.

Regulation of Mcl-1 proteins levels could occur in several ways. We and others have shown that EGF activates the phosphoinositide-3-kinase/AKT signaling pathway, leading to activation of the transcription factor nuclear factor κB and up-regulation of Mcl-1 (14, 40). In ErbB2-positive breast tumors, nuclear factor κB is preferentially activated (41), corresponding with our findings that Mcl-1 expression is associated with ErbB2-positive tumors. Furthermore, the stability of Mcl-1 mRNA and proteins is shorter than other Bcl-2 family members (13). This implies that subtle alterations in signaling pathways such as ErbB2 overexpression could change the expression levels for Mcl-1 by altering the stability of either mRNA or proteins without affecting other Bcl-2 family members. In NE2 cells, Bcl-2 and Bcl-xL protein levels did not change compared with parental cells but Mcl-1 expression increased. In MDA-MB-231 cells, it seems that Mcl-1 mRNA levels decrease, suggesting alterations in mRNA stability or transcription. It is still possible

that ErbB2 signaling could be stabilizing the protein because stabilization of p21^{CIP1} by EGF receptors has been shown (42). In chronic lymphocytic leukemia, Mcl-1 proteins levels decreased following treatment with genotoxic agents, but its mRNA levels remain unchanged (33). In addition, Mcl-1 is unique in the Bcl-2 family in having a role in cell cycle control, and by binding to proliferating cell nuclear antigens, could prevent the passage of cells through S phase (26). This is similar to herceptin being implicated in retarding tumor growth (43). Our results suggest that sensitization of breast cancer cells to apoptosis following herceptin treatment is due to decreased Mcl-1 protein expression. The regulation of Mcl-1-decreased expression of Mcl-1 by ErbB2 will be the focus for future investigations.

Targeting Bcl-2 family members with therapeutics could be a good strategy to sensitize cancer cells to undergo apoptosis (21). Antisense oligonucleotides against Bcl-2 have been shown to be effective at inducing apoptosis in several leukemia cells that have high Bcl-2 expression (21). This drug is currently in clinical trials for several leukemias and has shown some efficacy (44). The development of small molecule inhibitors of Bcl-2 or Bcl-x_L interactions with proapoptotic Bcl-2 family members such as Bax has been successfully shown to induce apoptosis in a variety of cancer cell lines (45–47). These

molecules bind to the BH3 cleft found in Bcl-2 and Bcl-x_L, preventing binding of Bax or Bak. It has been shown that these BH3-like molecules could also interfere with Mcl-1 binding to proapoptotic Bcl-2 family members (48–50). Similarly, antisense oligonucleotides against Mcl-1 have been effective at inducing apoptosis in several cell lines and, in combination with chemotherapy, enhances apoptotic responses in cell lines and in xenografted tumors (20, 22, 23). Using small interfering RNA to target Mcl-1 expression was also effective at inducing and enhancing apoptosis in cancer cells (51). Our data suggests that treatment with antisense oligonucleotides against Mcl-1 might render cells sensitive to chemotherapy and reduce drug resistance but addition of herceptin will not enhance this apoptotic response. Thus, overexpression of ErbB2 could increase drug resistance and cell survival by inducing Mcl-1 expression. Targeting Mcl-1 expression could be an effective treatment in herceptin-resistant ErbB2-overexpressing breast tumors.

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References

- Roskoski R, Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem Biophys Res Commun* 2004;319:1–11.
- Menard S, Casalini P, Campiglio M, Pupa SM, Tagliabue E. Role of HER2/neu in tumor progression and therapy. *Cell Mol Life Sci* 2004;61:2965–78.
- Tagliabue E, Agresti R, Carcangiu ML, et al. Role of HER2 in wound-induced breast carcinoma proliferation. *Lancet* 2003;362:527–33.
- Ferrone M, Motl SE. Trastuzumab for the treatment of non-small-cell lung cancer. *Ann Pharmacother* 2003;37:1904–8.
- Kaklamani V, O'Regan RM. New targeted therapies in breast cancer. *Semin Oncol* 2004;31:20–5.
- Pegram MD, Lopez A, Konecny G, Slamon DJ. Trastuzumab and chemotherapeutics: drug interactions and synergies. *Semin Oncol* 2000;27:21–5; discussion 92–100.
- Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004;305:626–9.
- Heiser D, Labi V, Erlacher M, Villunger A. The Bcl-2 protein family and its role in the development of neoplastic disease. *Exp Gerontol* 2004;39:1125–35.
- Yang T, Buchan HL, Townsend KJ, Craig RW. MCL-1, a member of the BCL-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. *J Cell Physiol* 1996;166:523–36.
- Reynolds JE, Yang T, Qian L, et al. Mcl-1, a member of the Bcl-2 family, delays apoptosis induced by c-Myc overexpression in Chinese hamster ovary cells. *Cancer Res* 1994;54:6348–52.
- Opferman JT, Iwasaki H, Ong CC, et al. Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* 2005;307:1101–4.
- Weng C, Li Y, Xu D, Shi Y, Tang H. Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. *J Biol Chem* 2005;280:10491–500.
- Michels J, Johnson PW, Packham G. Mcl-1. *Int J Biochem Cell Biol* 2005;37:267–71.
- Henson ES, Gibson EM, Villanueva J, Bristow NA, Haney N, Gibson SB. Increased expression of Mcl-1 is responsible for the blockage of TRAIL-induced apoptosis mediated by EGF/ErbB1 signaling pathway. *J Cell Biochem* 2003;89:1177–92.
- Olayioye MA, Graus-Porta D, Beerli RR, Rohrer J, Gay B, Hynes NE. ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol Cell Biol* 1998;18:5042–51.
- Emberley ED, Alowami S, Snell L, Murphy LC, Watson PH. S100A7 (psoriasin) expression is associated with aggressive features and alteration of Jab1 in ductal carcinoma *in situ* of the breast. *Breast Cancer Res* 2004;6:R308–15.
- de Melo J, Qiu X, Du G, Cristante L, Eisenstat DD. Dlx1, Dlx2, Pax6, Brn3b, and Chx10 homeobox gene expression defines the retinal ganglion and inner nuclear layers of the developing and adult mouse retina. *J Comp Neurol* 2003;461:187–204.
- Leu CM, Chang C, Hu C. Epidermal growth factor (EGF) suppresses staurosporine-induced apoptosis by inducing mcl-1 via the mitogen-activated protein kinase pathway. *Oncogene* 2000;19:1665–75.
- Cuello M, Ettenberg SA, Clark AS, et al. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. *Cancer Res* 2001;61:4892–900.
- Derenne S, Monia B, Dean NM, et al. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. *Blood* 2002;100:194–9.
- Kim R, Emi M, Tanabe K, Toge T. Therapeutic potential of antisense Bcl-2 as a chemosensitizer for cancer therapy. *Cancer* 2004;101:2491–502.
- Thallinger C, Wolschek MF, Maierhofer H, et al. Mcl-1 is a novel therapeutic target for human sarcoma: synergistic inhibition of human sarcoma xenotransplants by a combination of mcl-1 antisense oligonucleotides with low-dose cyclophosphamide. *Clin Cancer Res* 2004;10:4185–91.
- Thallinger C, Wolschek MF, Wacheck V, et al. Mcl-1 antisense therapy chemosensitizes human melanoma in a SCID mouse xenotransplantation model. *J Invest Dermatol* 2003;120:1081–6.
- Lottner C, Schwarz S, Diermeier S, et al. Simultaneous detection of HER2/neu gene amplification and protein overexpression in paraffin-embedded breast cancer. *J Pathol* 2005;205:577–84.
- O'Driscoll L, Cronin D, Kennedy SM, et al. Expression and prognostic relevance of Mcl-1 in breast cancer. *Anticancer Res* 2004;24:473–82.
- Kataoka A, Ishida M, Murakami S, Ohno S. Sensitization of chemotherapy by anti-HER. *Breast Cancer* 2004;11:105–15.
- Nagata Y, Lan KH, Zhou X, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* 2004;6:117–27.
- Kaufmann SH, Karp JE, Svingen PA, et al. Elevated expression of the apoptotic regulator Mcl-1 at the time of leukemic relapse. *Blood* 1998;91:991–1000.
- Michels J, O'Neill JW, Dallman CL, et al. Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. *Oncogene* 2004;23:4818–27.
- Shigemasa K, Katoh O, Shiroyama Y, et al. Increased MCL-1 expression is associated with poor prognosis in ovarian carcinomas. *Jpn J Cancer Res* 2002;93:542–50.
- Moshynska O, Sankaran K, Pahwa P, Saxena A. Prognostic significance of a short sequence insertion in the MCL-1 promoter in chronic lymphocytic leukemia. *J Natl Cancer Inst* 2004;96:673–82.
- Saxena A, Viswanathan S, Moshynska O, Tandon P, Sankaran K, Sheridan DP. Mcl-1 and Bcl-2/Bax ratio are associated with treatment response but not with Rai stage in B-cell chronic lymphocytic leukemia. *Am J Hematol* 2004;75:22–33.
- Johnston JB, Paul JT, Neufeld NJ, et al. Role of myeloid cell factor-1 (Mcl-1) in chronic lymphocytic leukemia. *Leuk Lymphoma* 2004;45:2017–27.
- Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature* 2003;426:671–6.

35. Rinckenberger JL, Horning S, Klocke B, Roth K, Kormsmeier SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev* 2000;14:23–7.
36. Kabore AF, Johnston JB, Gibson SB. Changes in the apoptotic and survival signaling in cancer cells and their potential therapeutic implications. *Curr Cancer Drug Targets* 2004;4:147–63.
37. Song L, Coppola D, Livingston S, Cress D, Haura EB. Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. *Cancer Biol Ther* 2005;4:267–76.
38. Andersson Y, Juell S, Fodstad O. Downregulation of the antiapoptotic MCL-1 protein and apoptosis in MA-11 breast cancer cells induced by an anti-epidermal growth factor receptor—Pseudomonas exotoxin an immunotoxin. *Int J Cancer* 2004;112:475–83.
39. Le Gouill S, Podar K, Amiot M, et al. VEGF induces Mcl-1 up-regulation and protects multiple myeloma cells against apoptosis. *Blood* 2004;104:2886–92.
40. Francois S, El Benna J, Dang PM, Pedruzzi E, Gougerot-Pocidal MA, Elbim C. Inhibition of neutrophil apoptosis by TLR agonists in whole blood: involvement of the phosphoinositide 3-kinase/Akt and NF- κ B signaling pathways, leading to increased levels of Mcl-1, A1, and phosphorylated bad. *J Immunol* 2005;174:3633–42.
41. Biswas DK, Shi Q, Baily S, et al. NF- κ B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc Natl Acad Sci U S A* 2004;101:10137–42.
42. Johannessen LE, Knardal SL, Madshus IH. Epidermal growth factor increases the level of the cyclin-dependent kinase (CDK) inhibitor p21/CIP1 (CDK-interacting protein 1) in A431 cells by increasing the half-lives of the p21/CIP1 transcript and the p21/CIP1 protein. *Biochem J* 1999;337:599–606.
43. Marches R, Uhr JW. Enhancement of the p27Kip1-mediated antiproliferative effect of trastuzumab (Herceptin) on HER2-overexpressing tumor cells. *Int J Cancer* 2004;112:492–501.
44. Chanan-Khan A. Bcl-2 antisense therapy in B-cell malignancies. *Blood Rev* 2005;19:213–21.
45. Real PJ, Cao Y, Wang R, et al. Breast cancer cells can evade apoptosis-mediated selective killing by a novel small molecule inhibitor of Bcl-2. *Cancer Res* 2004;64:7947–53.
46. Pinto M, Perez JJ, Rubio-Martinez J. Molecular dynamics study of peptide segments of the BH3 domain of the proapoptotic proteins Bak, Bax, Bid and Hrk bound to the Bcl-xL and Bcl-2 proteins. *J Comput Aided Mol Des* 2004;18:13–22.
47. Wang S, Yang D, Lippman ME. Targeting Bcl-2 and Bcl-XL with nonpeptidic small-molecule antagonists. *Semin Oncol* 2003;30:133–42.
48. Kuwana T, Bouchier-Hayes L, Chipuk JE, et al. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005;17:525–35.
49. Han J, Goldstein LA, Gastman BR, Froelich CJ, Yin XM, Rabinowich H. Degradation of Mcl-1 by granzyme B: implications for Bim-mediated mitochondrial apoptotic events. *J Biol Chem* 2004;279:22020–9.
50. Chen L, Willis SN, Wei A, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005;17:393–403.
51. Nencioni A, Hua F, Dillon CP, et al. Evidence for a protective role of Mcl-1 in proteasome inhibitor-induced apoptosis. *Blood* 2005;105:3255–62.