*Advances in Brief*

# Induction of Topoisomerase II Activity after ErbB2 Activation Is Associated with a Differential Response to Breast Cancer Chemotherapy

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## Abstract

***ErbB2 (HER-2)* gene amplification and overexpression have been shown to predict a better outcome with doxorubicin-based chemotherapy as opposed to alkylator-based chemotherapy in early stage breast cancer. To understand the mechanism of differential response to these two regimens, we have evaluated the effect of signaling through the ErbB2 receptor on downstream enzymes that may affect drug response, using two different models. The first system employs breast cancer cells that have high levels of endogenous ErbB2 by gene amplification (BT-474 and SKBR3 cells). The second system allows us to isolate the effect of ErbB2 receptor-mediated intracellular signaling using an epidermal growth factor receptor-ErbB2 chimeric receptor activated by epidermal growth factor. Our experiments show that the cytotoxicity of doxorubicin is inhibited in ErbB2+ breast cancer cells by the anti-ErbB2 antibody, Herceptin. This is accompanied by a decrease in topoisomerase (topo) IIα protein and activity, suggesting that this is the mechanism of change in doxorubicin response. In addition, a 10–100-fold (1–2 log) decrease in the $LD_{50}$ of doxorubicin is seen after ErbB2 activation using the chimeric receptor model. Furthermore, we see a 100-fold decrease in the $LD_{50}$ of etoposide, another topo II inhibitor. This increase in doxorubicin sensitivity is associated with a 4.5-fold increase in the amount of topo IIα protein and an increase in topo II activity as measured by DNA decatenating and unknotting activities, as well as cleavable complex formation. In contradistinction to doxorubicin, we have observed an increased resistance to cyclophosphamide chemotherapy after chimeric receptor activation. We propose that the differential benefit seen with doxorubicin- *versus* alkylator-based chemotherapy in ErbB2+ breast cancer is due, in some cases, to ErbB2-mediated topo IIα activation. These data also suggest hypotheses for the optimal sequencing of Herceptin and chemotherapy agents in ErbB2+ breast cancer.**

## Introduction

The use of chemotherapy to treat early stage breast cancer has been proven to extend survival (1). Whereas doxorubicin-containing regimens show a small additional benefit over CMF[3]-based regimens, they are associated with rare but serious side effects [congestive heart failure and acute leukemia (1, 2)]. The choice of regimen often depends on the overall risk of recurrence and comorbidities of the patient but is generally not tumor specific. Better understanding of the predictive value of certain biological markers in the primary tumor should allow us to identify chemotherapy regimens that are most likely to be effective while minimizing toxicity. In addition, appropriate combinations of chemotherapy and new biological agents, such as the anti-ErbB2 antibody Herceptin, are best determined by understanding biological interactions between signaling pathways and treatment effects.

The ErbB2 or HER-2 oncogene is overexpressed in approximately 30% of human breast cancer specimens and is associated with a poor outcome in many studies (3–5). Recent data suggest that ErbB2 amplification and overexpression are associated with improved outcome after doxorubicin-based therapy as compared with alkylator-based therapy [CMF and PF (6, 7)]. This has led to the speculation that ErbB2 confers sensitivity to doxorubicin and resistance to alkylating agents.

In an attempt to understand the mechanism of the differential response to these regimens, we have studied the effect of activating the ErbB2 receptor on downstream enzymes that may affect drug response. Our previously published *in vitro* data have shown that activation of the ErbB2, ErbB3, and ErbB4 receptors using heregulin β-2 is associated with an increase in the DNA-modifying enzyme, topo IIα, which is accompanied by increased sensitivity to doxorubicin but resistance to an alkylator, cisplatin (8). In the current study, we have attempted to dissect the role of ErbB2 in modulating drug response using two ErbB2-dependent *in vitro* models. Data presented here demonstrate that an increase in topo II activity and greater

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[3] The abbreviations used are: CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; EGF, epidermal growth factor; EGFR, EGF receptor; topo, topoisomerase; ECL, enhanced chemiluminescence; FACS, fluorescence-activated cell-sorting; BCS, bovine calf serum; VP-16, etoposide; 4-HC, 4-hydroperoxy-cyclophosphamide.

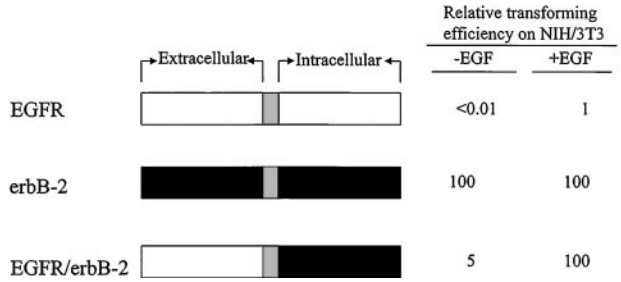


*Fig. 1* EGFR/ErbB2 chimeric receptor. Comparison of structure and biological activity of EGFR, ErbB2, and EGFR/ErbB2 chimeric receptors based on relative transforming efficiency, as measured by colony growth in soft agar of NIH-3T3 fibroblasts in response to EGF (8).

sensitivity to doxorubicin follow ErbB2 receptor signaling. We have also observed an increased resistance to cyclophosphamide after ErbB2 receptor activation and propose that these two observations are linked by changes in topo II activity. Finally, we find that sensitivity to doxorubicin is reversed by Herceptin in ErbB2+ breast cancer cells. Whereas the role of Herceptin in early stage breast cancer has yet to be determined, our study suggests that combining this drug with doxorubicin is unlikely to be the optimal strategy against ErbB2+ tumors.

## Materials and Methods

**Cell Lines.** NIH-3T3 cells transfected with an EGFR-ErbB2 chimeric receptor were kindly provided by C. Richter King (Lombardi Cancer Center, Washington, D.C.). The chimeric receptor was constructed by joining the extracellular domain of the EGFR with the transmembrane and intracellular portion of the ErbB2 receptor (Fig. 1; Ref. 9). The receptor is activated by EGF, with subsequent tyrosine phosphorylation of the ErbB2 receptor intracellular tyrosine kinase. Host cells (NIH-3T3 cells) do not express the ErbB2, ErbB3, or ErbB4 receptors. Two clones were available for these experiments, NIH77 and NIH82. Both clones show response to EGF with ErbB2 tyrosine phosphorylation and evidence of tritiated thymidine incorporation after receptor signaling, as confirmed by experiments in this laboratory (data not shown). BT-474 and SKBR3 cells are breast cancer cell lines that overexpress the ErbB2 receptor by amplification and have coexpression of ErbB3 and ErbB4 and EGFR (10). The MCF-7 breast cancer cell line is a transformed, non-ErbB2-amplified cell line that expresses high levels of estrogen receptor and is dependent on estrogen for growth. All breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA).

**Western Blotting of Nuclear Extracts.** For studies of the effect of ErbB2 activation on topo IIα protein levels, nuclear extracts were prepared from either chimeric cells or breast cancer cell lines. Chimeric cells were serum starved for 24 h followed by incubation with or without EGF (10 ng/ml) for 24, 48, 72, and 96 h. Breast cancer cell lines were treated with the mouse monoclonal anti-ErbB2 antibody 4D5 (Genentech, Alameda, CA) at 10 μg/ml, which is the target plasma level in human studies (11). Nuclear extracts were prepared by lysis of cells with a high-salt buffer [100 mM NaCl, 20 mM KCl, 20 mM Tris, and 0.5 mM $Na_2HPO_4$ (pH 7.4)] containing 0.5% Triton X-100 and 1% NP40. Nuclei were pelleted, and the nuclear proteins were solubilized by sonication in 1% SDS. After quantitation, 50 μg of nuclear protein were loaded and separated by 4–20% SDS-PAGE, transferred onto nitrocellulose, and incubated with an antihuman topo IIα rabbit polyclonal antibody (TopoGEN, Inc., Columbus, OH). After secondary antibody incubation, the p170 kDa protein was visualized using ECL (Amersham, Buckinghamshire, United Kingdom).

**Cell Cycle Experiments.** ErbB2-amplified (SKBR3) and ErbB2-nonamplified, ER+ (MCF-7) cells were arrested in $G_1$ using 2 mM hydroxyurea for 24 h, followed by release into serum-containing media. Cells were harvested at 0, 5, 11 and 24 h; nuclear extracts were prepared; and Western blotting for topo IIα was performed as described above. FACS analysis was performed on an aliquot of cells from the same time points to determine their position in the cell cycle. SKBR3 cells were chosen for these experiments to facilitate comparison because their rate of progression through the cell cycle was similar to that of MCF-7 cells. Actin levels were measured as a loading control.

**Cytotoxicity Assays.** Chimeric receptor cells were serum starved for 24 h and plated in 96-well microtiter plates at 1000 cells/well in quadruplicate in IMEM without phenol red containing 2% BCS and EGF (10 ng/ml). Cells were treated with continuous exposure to doxorubicin (0.001–10 μM) or continuous exposure to VP-16 (0.001–100 μM), approximating *in vivo* conditions for drug exposure. Cells treated with 4-HC were exposed to 4-HC (0.001–1.0 μM) for 48 h, washed, and incubated in drug-free media for an additional 5 days. The short exposure period also approximates *in vivo* pharmacokinetics and avoids aerosolization of 4-HC that may contaminate control wells. On day 7, at confluence of the control samples, the cell viability was assessed by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (tetrazolium/formazan) assay (12). Results are expressed as a percentage of the control, where cells not treated with chemotherapy represent control values. The $LD_{50}$ is the amount of drug required to kill 50% of cells. Experiments were repeated a minimum of three times for each drug evaluated.

**Topo II Unknotting and Decatenation Assays.** For the unknotting assay, P4-bacteriophage circular DNA was incubated with increasing dilutions of nuclear extract in a reaction buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 μg/ml BSA, and 1 mM ATP (13). After a 30-min incubation at 30°C, reactions were terminated by SDS-Ficoll stop solution (5:1), and samples were separated by electrophoresis in 0.7% agarose. The fraction of unknotted products in the reaction was determined by densitometry and expressed as a function of the amount of nuclear protein added. All experiments were performed in triplicate.

The decatenation assay uses kinetoplast DNA to measure intermolecular strand passage activity (14). Nuclear extracts prepared as noted above were incubated with kinetoplast DNA at 37°C for 15 min. After gel electrophoresis in 1% agarose, the fraction of decatenated products containing nicked circular or relaxed circular DNA was determined by densitometry and expressed as a function of the amount of nuclear protein loaded. All experiments were performed in triplicate.

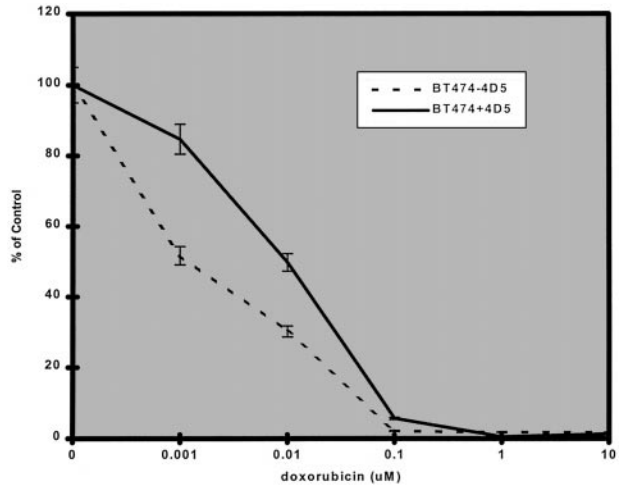


*Fig. 2* Cytotoxicity assay of doxorubicin in BT-474 cells. BT-474 cells were serum starved for 24 h followed by treatment in 10% serum-containing media, with or without 4D5 (10 μg/ml), for 24 h. Cells were then plated at 2000 cells/well in a 96-well microtiter dish on day 0 and treated with increasing concentrations of doxorubicin (0.001–10 μM). Viability was assessed using a tetrazolium-based assay on day 7 (at confluence of control sample) with absorbance read at 490 nm.

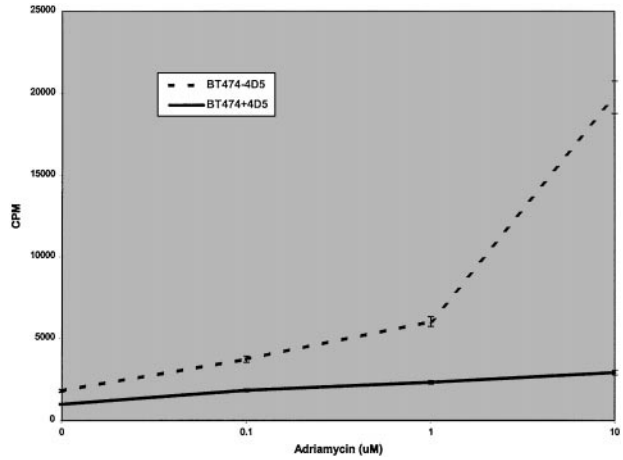


*Fig. 3* Topo II cleavage activity in response to doxorubicin treatment after treatment with anti-ErbB2 antibody in BT-474 cells. BT-474 cells were serum starved for 24 h followed by treatment in serum-containing media, with or without 4D5 (10 μg/ml), for 24 h. After [$^3$H]thymidine incorporation for 12 h, cells were treated with increasing concentrations of doxorubicin (0.1–10 μM) for another 12 h. Cells were washed and lysed, and DNA-topo II protein complexes were precipitated by KCl. The pellet was washed and resuspended in scintillation fluid, and the radioactivity of each sample was determined. Topo II cleavage activity is expressed as cpm *versus* micromolar doxorubicin.

**Topo II Cleavage Assay.** Topo II cleavage assays were performed using the K-SDS assay to measure the formation of protein/DNA covalent complexes after exposure to topo II inhibitor (15). Briefly, cells were serum starved for 24 h, followed by treatment with or without EGF (10 ng/ml) for 24 h. After [$^3$H]thymidine incorporation for 12 h, cells were treated with increasing concentrations of doxorubicin (0.1–10 μM) for a period of 12 h. Cells were washed and lysed with SDS and DNA-topo II protein complexes precipitated by the addition of KCl. The pellet was washed and resuspended in scintillation fluid, and the radioactivity of each sample was determined. Topo II cleavage activity was expressed by plotting the amount of protein-linked DNA *versus* drug concentration (μM doxorubicin). All experiments were performed in triplicate.

## Results

We used two *in vitro* models to evaluate the role of the ErbB2 receptor in response to chemotherapy. The first involved breast cancer cells that overexpress ErbB2 and are well characterized for content of ErbB3, ErbB4, and EGFR receptors that are often coexpressed in ErbB2+ cells (16). This allowed us to evaluate the effects on signaling through ErbB2 in the context of other members of the EGFR superfamily, more closely approaching the *in vivo* situation. ErbB2 is constitutively active in these cells, but it can be inhibited with the anti-ErbB2 antibody, 4D5, the murine form of Herceptin.

Our second model allowed us to isolate the effect of ErbB2-mediated signaling without the presence of heterodimerizing coreceptors. Because ErbB2 has no known ligand, the receptor alone cannot be activated exogenously. Therefore we used an EGFR-ErbB2 chimeric receptor construct transfected into NIH-3T3 cells, which do not express significant levels of the EGFR superfamily members (Fig. 1; Ref. 9). Within 1 h of exposure to EGF (10 ng/ml), the ErbB2 receptor kinase is activated as demonstrated by phosphorylation of intracellular domain tyrosine residues and tritiated thymidine incorporation (data not shown).

**Inhibition of ErbB2 Signaling Leads to Increased Resistance to Doxorubicin and Decreased topo II Activity in ErbB2+ Breast Cancer Cells.** We evaluated the effect of modulating ErbB2 signaling on doxorubicin response using 4D5 antibody (Herceptin). We assessed whether inhibition of the ErbB2 kinase was associated with a change in sensitivity to doxorubicin using a tetrazolium-based cytotoxicity assay. In these experiments, we found a reproducible inhibition of doxorubicin cytotoxicity after treatment with 4D5 (10 μg/ml) with an increase in the $LD_{50}$ from 0.001 to 0.01 μM (Fig. 2).

We used the K-SDS assay to evaluate the ability of intracellular topo II to form cleavable complexes after exposure to doxorubicin in the presence or absence of 4D5. Formation of the cleavable complex is the basis of the cytotoxic effect of the topo II poisons (17). In our experiments, topo II cleavage activity was decreased in BT-474 cells treated with 4D5, indicating that modulation of ErbB2 receptor activity is associated with a decreased ability of doxorubicin to induce topo II cleavage (Fig. 3). It is interesting to note that BT-474 cells are most resistant toward antitumor agents targeting either topo I or topo II (18). Even at 10 μM doxorubicin, the cleavage complex formation has not reached the maximal plateau. The addition of 4D5 antibody to these cells can almost eliminate the cleavage complex formation.

To further explore whether topo II activity is altered by changes in ErbB2 signaling, we evaluated ErbB2 kinase activity and levels of topo IIα protein. As reported previously, the 4D5 antibody led to a decrease in ErbB2 receptor tyrosine phosphor-

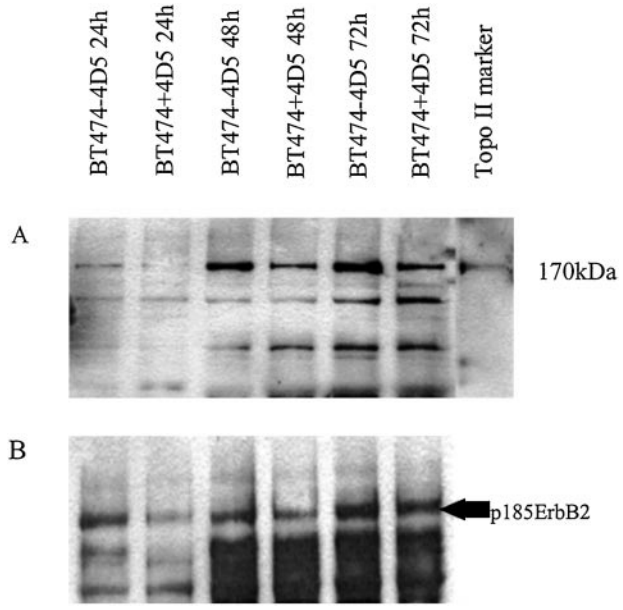


*Fig. 4* Time course of BT-474 breast cancer cells showing down-regulation of both ErbB2 tyrosine phosphorylation and topo IIα after treatment with 4D5. *A,* topo IIα. Nuclear extracts were prepared from BT-474 cells treated with or without 4D5 (10 μg/ml) for 24, 48, and 72 h. After quantitation, 50 μg of nuclear protein were loaded and separated by 4–20% SDS-PAGE, transferred onto nitrocellulose, and incubated with an antihuman topo IIα rabbit polyclonal antibody. After secondary antibody incubation, the p170 kDa protein was visualized using ECL (Amersham, Buckinghamshire, United Kingdom). *B,* ErbB2 tyrosine phosphorylation. BT-474 cells were treated with or without 4D5 (10 μg/ml) for 24, 48, and 72 h. Whole cell extracts were prepared by lysis with 2× sample buffer (Integrated Separation Systems, Natick, MA), and equal volumes were separated using 4–20% SDS-PAGE, followed by transfer to nitrocellulose. ErbB2 tyrosine phosphorylation was detected using 4G10 antiphosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). After secondary antibody incubation, the p180 kDa protein was visualized using ECL.

ylation (Fig. 4*A*). In addition, topo IIα levels decreased during treatment with 4D5, concomitant with the decrease in ErbB2 receptor phosphorylation (Fig. 4*B*). Of note, under the same conditions, topo I protein levels did not change in response to inhibition of ErbB2 activation (data not shown).

**Activation of the EGFR/ErbB2 Chimeric Receptor Leads to Increased Sensitivity to Doxorubicin.** To isolate the effect of ErbB2 receptor signaling, we used a chimeric receptor construct transfected into NIH-3T3 cells, which do not express significant amounts of any of the EGFR superfamily of receptors. We found a reproducible 10–100-fold (1–2 log) decrease in the $LD_{50}$ of doxorubicin after EGF treatment of cells containing the EGFR/ErbB2 chimeric receptor compared with untransfected NIH-3T3 cells treated in a similar fashion. Fig. 5*A* demonstrates this effect. To address possible differences in cell growth rate, chimeric cells growing in serum-containing media were treated with and without EGF and then exposed to increasing doses of doxorubicin. A similar increase in sensitivity to doxorubicin was seen in this experiment (data not shown).

To determine whether these cells were sensitive to other topo II inhibitors that are not DNA intercalators, we tested the cytotoxicity of VP-16 in this system. Chimeric cells were 100-fold more sensitive to VP-16 as compared with control NIH-3T3 cells that do not express the chimeric receptor (Fig. 5*B*). The similarity of the effect of ErbB2 activation on doxorubicin and VP-16 dose-response curves suggests that the doxorubicin sensitivity noted in our system is mediated by topo II.

**Topo II Activity and Protein Levels Are Increased in Chimeric Cells after ErbB2 Receptor Activation.** We again used the K-SDS assay to evaluate the intracellular topo activity in chimeric cells after activation of ErbB2. We observed a marked increase in DNA cleavage complexes in the chimeric cells after treatment with EGF as compared the same cells without EGF treatment (Fig. 6*B*). At a concentration of 1 μM doxorubicin, approximately six times more protein-linked DNA cleavage can be detected in the EGF-treated cells. At an even higher concentration, there is a decrease in the cleavage complex, a phenomenon common to the intercalative topo II inhibitors (17).

To further explore the functional consequence of ErbB2 receptor signaling on topo II activity, we performed two different assays: (*a*) unknotting of knotted P4 DNA; and (*b*) decatenating of catenated kinetoplast DNA rings. Both assays measure the ability of topo II to catalyze strand passage between two double-stranded DNA segments; however, the decatenation assay evaluates intermolecular strand passage activity, whereas the unknotting assay evaluates intramolecular activity. On activation with EGF, we found an increase in topo II enzymatic activity in chimeric cells by both measures. Data shown are for the decatenation assay, although similar results were seen in the unknotting assay (Fig. 6*A*). After ErbB2 activation, there is an enhancement of 2–4-fold strand passage activity in these assays. Taken together, these data suggest that the increase in topo II enzymatic activity on ErbB2 receptor signaling leads directly to an increase in sensitivity of these cells to topo II poisons like doxorubicin and VP-16.

Using our EGFR-ErbB2 chimeric receptor model in a time course experiment, we observed that activation of ErbB2 is associated with an increase in topo IIα protein from 48 to 72 h after receptor activation for both clones. This corresponds to a 4.5-fold increase in topo IIα protein, based on band densitometry (Fig. 7*A*). Equal loading is seen for actin (Fig. 7*B*).

**Increased Activity of Topo IIα Is Seen throughout the Cell Cycle in ErbB2-overexpressing Cells but not in ER+ Cells that Do Not Overexpress ErbB2.** The effect of ErbB2 signaling on topo IIα may be specific to the effects of this oncogene on downstream targets or may be a nonspecific effect of mitogenesis. To address this question, we performed cell cycle experiments on ErbB2-overexpressing (SKBR3) and non-ErbB2-overexpressing (MCF-7) breast cancer cells. MCF-7 cells are known to be dependent on estrogen receptor signaling for their growth. Both cell lines were blocked in early S-phase using hydroxyurea treatment and released into serum-containing media. A time course was performed to demonstrate topo IIα activity at different phases of the cell cycle. The results show that ErbB2-overexpressing cells have higher levels of topo IIα protein throughout different phases of the cell cycle compared with non-ErbB2-overexpressing MCF-7 cells (Fig. 8), although the pattern of expression is similar for both cell lines. The two cell lines had a similar

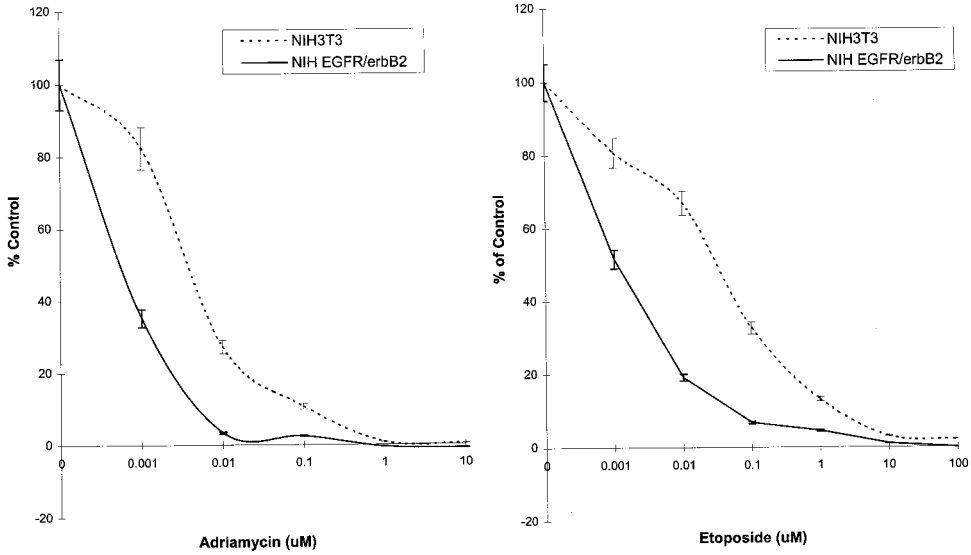


*Fig. 5* Cytotoxicity assays with topo II inhibitors doxorubicin and VP-16 in NIH-EGFR/ErbB2 cells. *A,* NIH-EGFR/ErbB2 chimera-transfected cells and NIH-3T3 wild-type cells were starved in serum-free media for 24 h and then incubated in media supplemented with 2% BCS and 10 ng/ml EGF. Cells were then plated at 1000 cells/well in a 96-well microtiter dish on day 0 and treated with increasing concentrations of doxorubicin (0.001–10 μM). Viability was assessed using a tetrazolium-based assay on day 7 (at confluence of control sample) with absorbance read at 490 nm. *B,* cells were treated as described above, except that the topo II inhibitor used for this experiment was VP-16 (0.001–100 μM).

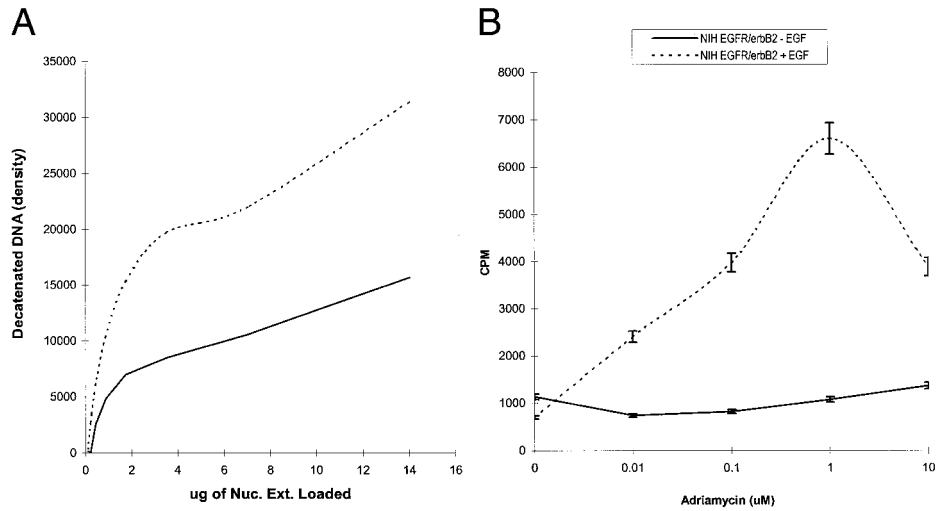


*Fig. 6* Topo II cleavage activity in response to doxorubicin after ErbB2 receptor signaling in NIH-EGFR/ErbB2 cells. *A,* decatenation assay. Chimeric cells were starved in serum-free media for 24 h and then treated with or without EGF (10 ng/ml) for 48 h. Nuclear extracts were incubated with kinetoplast DNA at 37°C for 15 min. After gel electrophoresis in 1% agarose, the decatenated products containing nicked circular and relaxed circular DNA were determined by densitometry and expressed as a function of the amount of nuclear protein loaded. *B,* cleavage assay. Chimeric cells were serum starved for 24 h, followed by treatment with or without EGF (10 ng/ml) for 24 h. After [$^3$H]thymidine incorporation for 12 h, cells were treated with increasing concentrations of doxorubicin (0.1–10 μM) for an additional 12-h period. Cells were washed and lysed, and DNA-topo II protein complexes were precipitated by KCl. The pellet was washed and resuspended in scintillation fluid, and the radioactivity of each sample was determined. topo II cleavage activity was expressed as cpm *versus* micromolar doxorubicin.

rate of progression through the cell cycle after release into serum-containing media, as measured by FACS analysis (data not shown).

**Signaling through ErbB2 Is Associated with Resistance to 4-HC.** Cyclophosphamide is the main component of the regimen CMF that has been used to treat breast cancer. Several studies have suggested a correlation of resistance to CMF-based regimens in patients whose tumors overexpress ErbB2 (19, 20). Using the active metabolite, 4-HC, we assessed the effect of ErbB2 receptor signaling on the cytotoxicity of cyclophosphamide in our *in vitro* system. Cells containing the chimeric receptor were stimulated by ligand and then treated with 4-HC. In contrast to the increased sensitivity seen with doxorubicin, we saw a 10–100-fold (1–2 log) increase in the $LD_{50}$ of 4-HC in cells with activated ErbB2 receptor, demonstrating increased resistance to the alkylator. Fig. 9 demonstrates this effect from one of three such experiments. Therefore, the activity of the ErbB2 receptor may influence resistance to cytotoxic agents other than those that specifically target topo II.

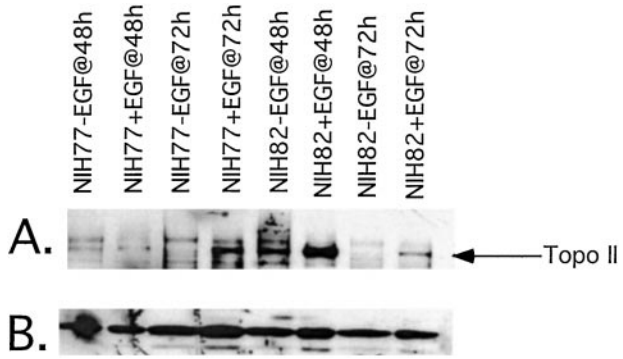


*Fig. 7* Time course of NIH-3T3 EGFR/ErbB2 chimera cells showing induction of topo IIα on receptor activation. *A*, NIH-EGFR/ErbB2 chimera-transfected cells (clones 77 and 82) and NIH-3T3 wild-type cells were starved in serum-free media for 24 h and then treated with 10 ng/ml EGF for 48 and 72 h. Nuclear protein extracts were prepared and separated using 4–20% SDS-PAGE and transferred to nitrocellulose. After quantitation, 50 μg of nuclear protein were loaded and separated by 4–20% SDS-PAGE, transferred onto nitrocellulose, and incubated with an antihuman topo IIα rabbit polyclonal antibody. After secondary antibody incubation, the p170 kDa was visualized using ECL. *B*, actin loading control visualized with mouse monoclonal antibody (Sigma).

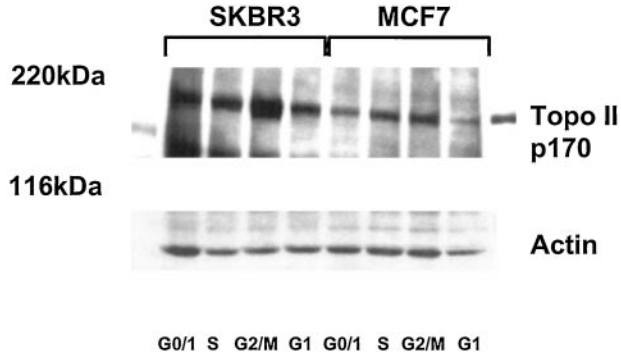


*Fig. 8* Cell cycle distribution of topo IIα protein and phosphotyrosine in ErbB2+ and ErbB2− breast cancer cell lines. SKBR3 and MCF-7 cells were arrested in serum-free media containing 2 mM hydroxyurea for 24 h, followed by release into serum-containing media. Cells were harvested at 0, 5, 11, and 24 h, and nuclear extracts were prepared, followed by Western blotting for topo IIα protein. FACS analysis was performed in parallel to determine position in the cell cycle. Actin levels are depicted as a loading control.

## Discussion

Our previous work has shown that the ErbB family of receptors can be activated in MCF-7 breast cancer cells transfected with the ligand heregulin. This is associated with up-regulation of the nuclear enzyme topo IIα and a change in sensitivity to chemotherapy agents used in breast cancer (8). In the current study, we have pursued the relationship between chemotherapy, ErbB2, and topo II. We have examined the effect of ErbB2 activation in cells that highly overexpress the receptor by activation of a chimeric receptor in heterologous cells and by inhibition of receptor signaling by the 4D5 monoclonal antibody. In each of these model systems, we were able to modulate the dose-response relationship to doxorubicin and observed consistent and concomitant changes in topo IIα levels. Furthermore the topo II enzyme activity is also affected by changes in ErbB2 receptor activity.

Our data suggest a direct relationship between ErbB2 receptor signaling and topo II modulation. We have observed increases in both topo IIα protein and enzymatic activity after receptor activation. These changes are accompanied by increase in sensitivity to the enzyme-specific inhibitors doxorubicin (Adriamycin) and VP-16. In addition, the anti-ErbB2 antibody, Herceptin, can reverse this effect. Furthermore, we see the opposite effect on response to cyclophosphamide, with increased resistance to the latter. We hypothesize that the increase in topo II activity observed after ErbB2 receptor signaling is responsible for the differential sensitivity of these cells to doxorubicin- *versus* alkylator-based therapy seen in the *in vivo* setting.

Topo IIα is a DNA-modifying enzyme that can pass a segment of DNA duplex through a reversible, enzyme-mediated double-strand break (21). Drugs that target topo II include the anthracyclines (doxorubicin and daunorubicin), VP-16, teniposide, and amascarine (17). These agents appear to act by binding to the enzyme-DNA complex and inducing lethal cellular damage by the inhibition of the religation step during the transient DNA cleavage reaction. Increase in topo IIα expression is associated with sensitivity to these agents in both cell lines and tumors, presumably due to increased target on which the drug may act (22, 23).

A study evaluating 230 breast cancer tumor specimens demonstrated that increased expression of topo IIα is associated with ErbB2 overexpression, even when adjusted for proliferative index (24). In a subset of 49 of these tumors, coamplification of topo IIα and ErbB2 genes occurred as these genes colocalize to chromosome 17 (25). This study shows that topo IIα may also increase by nongenetic mechanisms through temporary increases in ErbB2 receptor activation. We see increased topo II levels and activity in cell lines where ErbB2 is not amplified but is activated by a ligand. In addition, NIH-3T3 cells that contain a normal gene dosage of topo IIα up-regulate this protein after activation of a chimeric receptor that has been transfected into these cells. Therefore, we conclude that increased levels of topo IIα may be due either to gene amplification or to increased activity of the ErbB2 receptor. In addition, we have shown that topo IIα levels appear to be higher in some ErbB2-amplified breast cancer cells, compared with transformed breast cancer cells that are not dependent on ErbB2 for growth. In this setting, both ErbB2 and topo IIα activity can be down-regulated using the anti-ErbB2 antibody, Herceptin.

Large clinical trials suggest a benefit from doxorubicin in ErbB2+ breast cancer that is not as great as that with alkylator-based therapy. In the cooperative group study performed by the National Surgical Adjuvant Breast and Bowel Project (NSABP-B11), an improved outcome with a doxorubicin-containing regimen was seen only in patients whose tumors overexpressed ErbB2 (7). In another large study, Cancer and Leukemia Group B (CALGB) 8869, patients whose tumors overexpressed ErbB2 had a better survival if they were treated with higher doses of doxorubicin-containing chemotherapy, in contrast to their ErbB2− counterparts (26). Furthermore, we have also

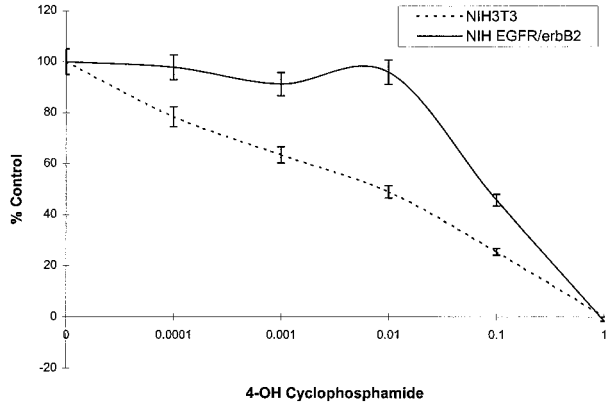


*Fig. 9* Cytotoxicity assays with 4-HC in NIH-EGFR/ErbB2 cells. NIH-EGFR/ErbB2 chimera-transfected cells and NIH-3T3 wild-type cells were harvested in serum-free media for 24 h and then incubated in 2% BCS + 10 ng/ml EGF. Cells were then plated at 1000 cells/well in a 96-well microtiter dish on day 0 and treated with increasing concentrations of 4-HC (0.001–1.0 μM). Viability was assessed using a tetrazolium-based assay on day 7 (at confluence of control sample) with absorbance read at 490 nm.

shown that stage IV breast cancer patients who exhibit higher levels of circulating ErbB2-extracellular domain, are 6 times more likely to respond to doxorubicin-containing therapy than to CMF regimens (27). These clinical studies suggest that response to doxorubicin is influenced by ErbB2. *In vitro* studies presented in the current report corroborate clinical studies and suggest that topo IIα is involved in the mechanism behind response to chemotherapy in ErbB2+ cells.

The relationship between ErbB2 overexpression in human breast cancer and poor outcome after CMF treatment suggests resistance to alkylating agents (20, 21). Although other agents (methotrexate and 5-fluorouracil) are part of this regimen, it is generally accepted that cyclophosphamide is the most effective agent in this combination. In two separate studies, patients whose breast tumors overexpressed ErbB2 did not appear to achieve as much benefit from postoperative CMF chemotherapy as did their non-ErbB2-overexpressing counterparts. Thus, our *in vitro* observations are consistent with the clinical picture, where overexpression of ErbB2 is associated with relative resistance to cyclophosphamide.

Previous studies have demonstrated that resistance to alkylators such as cyclophosphamide is multifactorial (28, 29). The most commonly described mechanisms are alterations in drug transport, modulation of glutathione levels, and enhanced repair of DNA adducts. It has been observed that some cell lines selected for resistance to alkylators have elevated levels of topo IIα, and it has been suggested that topo II is involved in DNA repair through its modulation of chromatin structure (30, 31). Although cause and effect have not been proven, similar resistance to 4-HC was associated with increased topo II activity in the *in vitro* system used for our studies. It has been shown that inhibition of ErbB2 can reduce the rate of unscheduled DNA repair, increase intrastrand adduct formation, and delay the rate of adduct decay in ErbB2+ cell lines (32, 33). We speculate that topo II activity may be, in part, responsible for this modulation of repair activity. It is possible that other replication and repair-associated proteins are involved in a response to ErbB2 signaling and that components of this response confer sensitivity to topo II inhibitors, whereas other components confer resistance to alkylators. Topo II may be a member of this multifactorial response, perhaps as part of a multienzyme complex important in repair of DNA damage.

Our experiments demonstrate that ErbB2 signaling leads to alterations in both the level and enzymatic activity of topo II. This, in turn, is associated with increased sensitivity to doxorubicin and resistance to cyclophosphamide. Although a direct connection has not been proven, we suggest that alteration in topo II activity, brought on by ErbB2 receptor signaling, may be one mechanism by which differential sensitivity of ErbB2+ tumors to doxorubicin- *versus* cyclophosphamide-based regimens occurs in the clinical setting.

Recent development of the humanized monoclonal antibody, Herceptin, has provided useful therapy for HER-2+ patients, particularly in combination with chemotherapy. However, many questions remain about the ideal way to give Herceptin, its duration of use, and how best to monitor patients on therapy. Our study further suggests that the ideal way to combine Herceptin with chemotherapy involves combinations with cyclophosphamide where inhibition of topo II may lead to reversal of drug resistance. This hypothesis is supported by observational data from other *in vitro* work (34). Moreover, our experiments suggest that combinations of Herceptin with doxorubicin are not ideal because this does not allow us to take advantage of increased topo II activity in ErbB2+ cells. Further understanding of the relationship between a given molecular lesion and response to chemotherapy will help us recommend our treatments in a more patient-specific manner.

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