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The members of the type I growth factor receptor family (ErB1-4) appear to have a role in predicting benefit from chemotherapy, particularly ErbB2. During the time of this award we have shown that ErbB2 signaling has an effect on cell cycle distribution of the topo IIa enzyme and its phosphorylation state, leading to increased sensitivity to doxorubicin but resistance to the alkylator cyclophosphamide. We have generated data which suggests that topo IIa is phosphorylated on tyrosine residues as opposed to serine/threonine as previously described. This effect is seen in response to ErbB2-mediated signaling, but not ErbB3 and we believe that this phosphorylation event may be important to determining sensitivity to doxorubicin. In addition, our experiments indicate an increased ability of ErbB2-transfected breast cancer cells to repair double-stranded breaks induced by gamma-irradiation. This supports our hypothesis that ErbB2 increases the cells ability to repair DNA in breast cancer cells which may be the explanation for resistance to alkylating agents seen in both ErbB2 breast cancer cell lines and human tumors. We hope that this work will allow us to tailor breast cancer treatment to the individual patient by understanding the mechanisms behind drug sensitivity and resistance in ErbB2 positive breast cancer.

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Introduction

The members of the type 1 growth factor receptor family (ErbB1-4) appear to have a role in predicting benefit from chemotherapy, particularly ErbB2. The mechanisms by which these growth factors alter cellular pathways to change drug response is poorly understood, however. The objective of this proposal is to identify how this family of growth factors, particularly ErbB2, can be used to predict drug sensitivity in patients with breast cancer.

While it is well established that growth factors and their receptors play a role in the prognosis of breast cancer, recent data suggest they may have a role in predicting response to therapy as well. In general, the EGFR (Epidermal Growth Factor Receptor) and ErbB2 receptors are associated with resistance to some chemotherapeutic agents, especially alkylator-based regimens such as cyclophosphamide, methotrexate and 5-fluorouracil (CMF). Interestingly, benefit from a doxorubicin (Adriamycin®)-containing regimen was limited to ErbB2 overexpressors in three large randomized cohorts of patients, the CALGB 8869/8541, SWOG and NSABP-B14 companion studies. One potential explanation for this finding is our observation of up regulation of the DNA modifying enzyme and target of doxorubicin, topoisomerase II (topo II), in cells in which the ErbB2, ErbB3 and ErbB4 receptors have been activated using the ligand, heregulin. These cells become more sensitive to doxorubicin and etoposide, both topo II inhibitors. We hypothesize that signaling through one or a combination of the ErbB receptors in breast cancer cells increases topoisomerase II which then sensitizes cells to doxorubicin. This predicts that alteration of ErbB signaling by various agents (natural or synthetic) may control response to doxorubicin in a predictable fashion in vitro and in vivo. In this proposal, we are examining the relationship between activation of the ErbB receptors using various ligands (EGF, Heregulin and anti-receptor antibodies) and topoisomerase II regulation.

Technical objectives (specific aims as listed in 1996 proposal)

Aim 1: To determine if one of the ErbB2/3/4 receptors is responsible for the increased topoisomerase II and change in drug sensitivity seen when heregulin is transfected into cancer cells we propose to examine these endpoints in cell lines in which the individual receptors are activated.

Aim 2: To demonstrate whether specific ligands (antibodies, growth factors or compounds which modulate tyrosine kinase phosphorylation) can predictably alter sensitivity of breast cancer cells to doxorubicin and alkylators by topoisomerase II modulation mediated through ErbB receptor phosphorylation.

Aim 3: To determine the mechanism of topoisomerase II accumulation in ErbB receptor-activated cells we will examine three alternative explanations:

3a) Topoisomerase II upregulation is part of a global effect on DNA repair machinery.
3b) Distribution of cell cycle in breast cancer cells is altered by activation of the ErbB2 receptor to produce an accumulation of topoisomerase II.

3c) Accumulation of topoisomerase II in breast cancer cells with activation of the ErbB receptors is due to increase activity of the topo II promoter directly.

RESULTS AND DISCUSSION

Task 1 (0-6 months): Creation of erbB3- and erbB4-EGFR chimera-transfected NIH3T3 cells by transfection with constructs provided by Dr Mattias Kraus and Dr. Careen Tang respectively.

An EGFR/ErbB3 chimeric receptor construct, kindly provided by Dr. Mattias Kraus, was transfected into NIH-3T3 cells using a modified Calcium phosphate precipitation method and selected using G418. Twelve chimera-expressing clones and six vector-transfected clones were chosen for characterization. Six chimeric clones express high levels of EGFR/ErbB3 chimera as compared with vector-transfected cells (see 1997 progress report). Phosphorylation on tyrosine is seen upon receptor activation with EGF (see 1997 progress report).

The ErbB4 chimeric construct was not created, as previously expected, by our collaborator (CT). As creation of such a chimeric construct is beyond the expertise of this investigator, we were not able to pursue the ErbB4 part of the project.

Task 2 (months 6-12): Assessment of response of erbB expressing breast cancer cells and chimera-cells to growth factors EGF, heregulin and amphiregulin with topoisomerase II protein and mRNA activation.

**EGFR/ErbB2 transfectants**

During the first two years of the proposal we determined that ErbB2 signaling led to increased topoisomerase IIα protein and that this effect appeared to be ligand independent (1997 annual report and attached manuscript - Publication #2). We also made the novel discovery that signaling through this receptor is associated with hyper-phosphorylation of topo IIα which appeared to be on tyrosine residues. This discovery, is unique as topo IIα activity was previously thought to be regulated only by serine and threonine phosphorylation. Given the potential importance of this discovery on understanding the fundamental regulation of topoisomerase IIα protein we chose to pursue this area of research more in depth. We further found that the distribution of topo IIα protein and phosphorylation during the cell cycle was different in ErbB2 positive vs negative cells lines (1998 annual report and attached manuscript - Publication #2).

Specifically, ErbB2 positive cells expressed higher levels of topo IIα protein during all phases of the cell cycle and that topo IIα tyrosine phosphorylation was also higher in these cells.

During the third year we focused our efforts on characterizing tyrosine phosphorylation on Topo IIα following ErbB2 signaling. We did this by attempting to dephosphorylate topo IIα using a tyrosine-specific phosphatase. These experiments did
not yield evidence of a band shift, which would be supportive evidence of tyrosine phosphorylation of topo IIα, although tyrosine-specific antibodies did reveal a specific band at the appropriate size for topo IIα. Explanations for this finding are: 1) lack of tyrosine phosphorylation on topo IIα; 2) Lack of mobility shift in topo IIα upon dephosphorylation 3) Endogenous phosphatases present which had dephosphorylated topo IIα during the nuclear extraction procedure, therefore no change in phosphorylation would be seen upon phosphatase treatment.

We also attempted to identify a candidate nuclear tyrosine kinase which might be responsible for phosphorylation of topo IIα. Using a kinase assay with c-abl as the kinase (R. Quackenbush) and purified N-terminal topo IIα as the substrate (provided by Dr. Tao Hsieh), we did not see specific phosphorylation of topo IIα.

We further evaluated the effect of these changes in topo II on its enzymatic activity in response to signaling by ErbB receptors. We found that topo II was upregulated and its ability to cleave DNA, decatenate DNA and damage in response to doxorubicin was increased in response to ErbB signaling (Annual report 1998, Publication #2).

Although this is a departure from the initial SOW we felt that the potential impact of this discovery on understanding the effects of ErbB2 on modulating an important drug target was great and should take precedent over the plans initially stated. Specifically, we chose to pursue the issues of topo II protein phosphorylation and enzymatic activity over and above pursuing mRNA expression in ErbB2/EGFR transfected cell lines. Attempts to further characterize tyrosine phosphorylation on topo IIα are ongoing in the laboratory of our collaborator (T. Hsieh), who is a protein biochemist with an expertise in the topoisomerases. The aforementioned work on topoisomerase II enzymatic activity in response to ErbB2 signaling has recently been accepted for publication to Clinical Cancer Research (publication #2).

**EGFR/ErbB3 transfectants**

EGFR/ErbB3 clones show a similar induction of topoisomerase IIα protein unlike vector-transfected cells which show no difference in response to EGF (see 1998 progress report). In contradistinction to the EGFR/ErbB2 chimeric cells, phosphorylation of topoisomerase IIα (on either tyrosine or serine/threonine) does not appear to be modulated by receptor activation in EGFR/ErbB3 transfectants (see 1998 progress report).

**Breast Cancer Cells**
The ligands chosen are known to phosphorylate either the ErbB2 receptor alone (anti-ErbB2 antibodies), ErbB2 and EGFR concomitantly (EGF) or ErbB2, ErbB3 and ErbB4 (heregulin β-2).

In the first year of this proposal breast cancer cell lines expressing known levels of receptor (see chart in 1997 and 1998 progress reports) were tested with the ligands EGF, heregulin β-2, and 4D5 antibody. Although EGF and heregulin β-2 can slightly increase tyrosine phosphorylation of these receptors after serum starvation, no significant change in topoisomerase II α levels was seen. This may be due to the fact that high levels of topo II α protein are expressed in these cell lines even in the serum starved condition
and further activation of the receptor does not increase these levels. In fact, unlike the NIH 3T3 chimeric receptor model, it is virtually impossible to inactivate ErbB2 receptor kinase activity by serum starvation. When the 4D5 antibody directed against the extracellular domain of the ErbB2 receptor was applied to these cells we showed a direct correlation of receptor activity with topoisomerase II α protein level and phosphorylation (see 1997 Annual Report). We have further confirmed, by immunoprecipitation of nuclear protein that this tyrosine phosphorylation is specific to topo II α and that this activity is downregulated by 4D5 (see 1998 report).

Increased receptor phosphorylation is not the rule, however with Ab treatment in cells which overexpress ErbB2. SKBR3 cells, in our hands as well as others, respond to the Ab with an increase in phosphorylation of the receptor as well as activation of the ras-MAP kinase pathway\(^1\). Our experiments demonstrate that this corresponds to an increase in tyrosine phosphorylation on topo II α as one might expect if the effect on topo II α was related to receptor activation. Interestingly, SKBR3 cells are still growth inhibited by 4D5 as are all ErbB2 overexpressing cell lines. These observations suggest that the relationship between topo II α and receptor activation is not simply a result of cell growth and division because we still see topo II α upregulation in response to receptor activation in cells which are growth-inhibited by 4D5 Ab. The reasons for these differences between cell lines which express ErbB2 at a high level are unclear but may lie in the fact that they express different levels of the other ErbB receptors. BT-474 cells express high levels of both ErbB3 and 4 and it has been suggested that it is interference with receptor heterodimerization rather than ligand binding that is responsible for the inhibition of phosphorylation by anti-ErbB2 antibodies. SKBR3, on the other hand, has very low levels of either ErbB3 and 4 but has significantly more EGFR which may allow for activation of certain signal transduction pathways but not others. Evaluation of topo II α activity in breast cancer cell lines by the decatenation and unknotting assays was not as clear cut. In BT474 cells we saw a reproducible inhibition of decatenation and unknotting activities after treatment with 4D5 antibody (see 1998 progress report and Publication #2). However, this effect was not seen in other breast cancer cell lines (SKBR3, MDA 453).

Due to the conflicting results in cell lines we felt it was important to assess the potential relevance of our in vitro findings in human tissues. In order to do this we collaborated with Drs Stuart Schnitt and Timothy Jacobs to further explore activity of topo II in human tissues. We evaluated a cohort of 100 primary breast cancer cases by immunohistochemistry for both topo IIα and ErbB2 (HER-2). For each case, topo II expression was quantified as the proportion of positively stained nuclei among 1000 nuclei counted. The proportion of tumor cell nuclei showing topo IIα staining was significantly higher for the 44 HER2 positive cases (median 48.2%, mean 45.8%) than for 46 HER2 negative cases (median 8.9%, mean 23.2%). In addition, the % topo IIα -positive nuclei showed a progressive increase with increasing tumor grade (grade I: median 5.2%, mean 5.2%; grade II:median 18.7%, mean 18.8%; grade III: median 52.4%; mean 51.8%). Double immunostaining on a subset of HER2-positive cases with various levels of topo II staining was performed and in all cases, HER2 and topo II were co-expressed in the same cells. This data, although preliminary, suggests that co-expression of ErbB2 and topo IIα occurs in breast cancer cases and that topo IIα expression is higher in ErbB2 positive breast tumors\(^7\). We have recently received
approval to perform topoisomerase immunostaining and FISH analysis on patient samples from a large (1000 patient) clinical trial where patients were treated with doxorubicin-containing chemotherapy. This, we hope, will definitively answer the question as to whether ErbB2 signaling leads to increased topoisomerase II activity and increased sensitivity to doxorubicin in vivo.


Given the novel findings listed above we chose to focus this work on Topo IIα protein expression. Therefore our efforts were centered more on understanding the impact of ErbB2 signaling on Topo IIα as it relates directly to drug resistance. In addition, we focused effort on designing correlative clinical studies and generating preliminary data from clinical specimens to help understand the relevance of these findings in human subjects (see above). The promotor experiments, while important in understanding the mechanism behind upregulation of Topo IIα, are less clinically relevant therefore we chose to focus on the issues of topo II protein phosphorylation and enzymatic activity over and above pursuing topoisomerase promotor activity in ErbB+/EGFR and breast cancer cell lines.

To further explore regulation of topo II by these receptors we did a number of experiments to evaluate cell cycle regulation of this protein in response to ErbB-signaling. As the effect of ErbB2 signaling on topo IIα may be specific to effects of this oncogene on downstream targets or may be a non-specific effect of mitogenesis we performed cell cycle experiments on ErbB2 overexpressing (SKBR3) and non-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-overexpressing MCF-7 cells (Publication #2), although the pattern of expression is similar for both cell lines. The two cell lines had a similar rate of progression through the cell cycle after release into serum containing media as measured by FACS analysis.

Task 4: Months 18-24:Cytotoxicity assays (anchorage dependent) using doxorubicin and 4-OHH Cyclophosphamide to evaluate drug sensitivity/resistance in erbB expressing breast cancer cells and chimera cells.

Drug Sensitivity/Resistance in EGFR/ErbB Clones

We have previously shown that EGFR/ErbB2 chimeric cells have increased sensitivity to doxorubicin (see 1997 report). We now show that these cells also become more resistant to the alkylator 4-OH cyclophosphamide (4-HC) which is the active metabolite of cyclophosphamide used in the CMF regimen (1998 report). This result is consistent with the clinical association of improved outcome seen with doxorubicin-containing regimens in ErbB2-positive breast cancer while resistance is seen to CMF-type regimens.
EGFR/ErbB3 clones, on the other hand, did not show consistent sensitivity to doxorubicin. In fact, only one clone was more sensitive to doxorubicin and this is the clone which was shown to have induction of topoisomerase IIα activity (1998 report). Interestingly, this did not relate to increased growth rate as one might expect - the EGFR/ErbB3 clone demonstrating topo IIα induction had the slowest growth rate. Interestingly, these data are not dissimilar to a recent report in a parallel system recently published by Stacey et al. (“Influence of Cell Cycle and Oncogene Activity upon Topoisomerase IIα Expression and Drug Toxicity”, Mol. Cell. Biol., 20(24): 9127-37). Their data suggest that oncogenic ras increases both levels and activity of topoisomerase IIα as well as activity of topo II poisons in a subset of slowly dividing cells. They postulate that their system may be useful for studying tumor cell biology as the ‘slowly dividing cells’ seen in their experiments are similar to malignant human tumor cells.

Alkylator (cyclophosphamide) resistance seemed to be common to activation of either receptor, however. We postulated that the tyrosine phosphorylation of topo II (seen on only upon ErbB2 activation) was responsible for the doxorubicin sensitivity whereas alkylator resistance might be cause by changes in topo II levels alone.

Drug Sensitivity/Resistance in Breast Cancer Cells

We evaluated the effect of modulating ErbB2 signaling using 4D5 antibody on doxorubicin response. We assessed whether inhibition of the ErbB2 kinase was associated with 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 with an increase in the LD50 from .001 to .01 uM (Publication #2). We further evaluated the repair response of ErbB2 transfected MCF7 cells after exposure to doxorubicin – see task 6).

Task 5: Months 18-24: Cytotoxicity assays (anchorage independent growth in spheroid cultures) using doxorubicin and 4-OHH cyclophosphamide to evaluate drug sensitivity/resistance in erbB expressing breast cancer cells and chimera cells.

We made extensive efforts to grow both EGFR/erbB2 and –erbB3 chimeric receptor cells in a spheroid model system to evaluate anchorage-independent drug resistance. We were not able to induce spheroid formation in any of the transfected clones or the wild-type cells, despite multiple attempts. We did not use matrigel, as suggested by some reviewers, as this contains EGF ligand that would invalidate the results of these experiments. Interestingly, all breast cancer cell lines tested grew in spheroid culture - a property that may be particular to malignant cells.

In an attempt to create a ‘breast cancer’ model where we could more easily modulate the function of ErbB receptors we attempted to create an inducible system for ErbB2 using the Tet-off® system. We were able to create stable transfectants in MCF-7 cells with the pTet-off regulator plasmid and demonstrate, using a lac-z that the system could be induced. However, after co-transfection with pBI-SUPER-erbB2 and selection of dozens of clones, we were unable to demonstrate clones which were inducible for ErbB2 protein. We concluded that our difficulty was peculiar to our construct.

Sensitivity to doxorubicin and resistance to 4HC was seen in a spheroid model of breast cancer cells (BT474’s) after treatment with 4D5. These data need to be repeated and experiments are ongoing.

In the fourth year of the grant we shifted focus to the functionality of increase in topo II activity seen after ErbB2 signaling. We believe that the increased sensitivity to topo II is part of a global effect on DNA repair activity, as has been suggested by other authors (Arteaga, Pietras). We have assessed the repair activity of ErbB2 chimeric cells compared with wild type cells after exposure to gamma radiation using pulse-field gel electrophoresis4. Our preliminary experiments suggested that ErbB2 activation led to increased DNA repair, however, despite over 14 experiments with optimization of conditions we were unable to reproduce this result consistently. Potential explanations for our lack of proof of our hypothesis are: 1) This method is unable to accurately reflect changes in DNA repair, 2) There is no difference in the ability of ErbB2 signaling to induce repair of double-stranded breaks compared to wild-type cells or 3) Response to gamma irradiation is not the same as response to drugs (which is the basis for this hypothesis), 4) The fibroblast-transfected chimeric receptor model does not adequately represent the effects in breast cancer cells.

We attempted to look at another model system that became available to us in the last year of the grant – MCF-7 breast cancer cells which were stable transfectants for ErbB2 and vector control cells. We evaluated the ability of these cells to repair dsDNA breaks after exposure to gamma irradiation, again using pulse field gel electrophoresis. Results consistently showed that ErbB2 transfectants were better able to repair dsDNA breaks as evidenced by decreased release of fragmented DNA into the gel after exposure to gamma irradiation (Figure 1).

![DNA repair MCF-7/Erb-2 vs MCF-7/Vector cells after radiation](image)

FIGURE 1.
To characterize the specificity of the effect of ErbB2 signaling on topo II, at the suggestion of a reviewer, we assessed changes of another DNA modifying enzyme, topoisomerase I, after inhibition of ErbB2. No change was seen in topo I levels in experiments where ErbB2 was inhibited in overexpressing breast cancer cell lines (Figure 2).

FIGURE 2.

CONCLUSIONS/KEY RESEARCH ACCOMPLISHMENTS

During the time of this award we have shown that ErbB2 signaling has an effect on cell cycle distribution of the topo IIα enzyme and its phosphorylation state, leading to increased sensitivity to doxorubicin but resistance to the alkylator cyclophosphamide. We have generated data which suggests that topo IIα is phosphorylated on tyrosine residues as opposed to serine/threonine as previously described. This effect is seen in response to ErbB2-mediated signaling, but not ErbB3 and we believe that this phosphorylation event may be important to determining sensitivity to doxorubicin. Attempts to further characterize tyrosine phosphorylation on topo IIα are ongoing in the laboratory of our collaborator (T. Hsieh), who is a protein biochemist.

We have begun to explore the observation that ErbB2 positive cells are more resistant to DNA damaging agents. Our initial experiments do not indicate an increased ability of these cells to repair double-stranded breaks induced by gamma-irradiation in the Chimeric receptor (fibroblast) model, however we do see increased ability of ErbB2 transfected cells to repair DNA in the breast cancer model. The latter data supports our hypothesis that ErbB2 increases the cells ability to repair DNA in breast cancer cells which may be the explanation for resistance to alkylating agents seen in both ErbB2
breast cancer cell lines and human tumors. Our ongoing experiments are exploring the ability of these cells to repair damage induced by the alkylator, cyclophosphamide.

In order to understand if our in vitro observations of increased topo II activity after ErbB2 signaling are relevant in patients we have begun to explore expression of topo IIα in human breast cancer specimens. In 100 cases tested by immunohistochemistry, topo IIα was more highly expressed in ErbB2 positive cells and was found to be co-expressed in the same cells which overexpressed ErbB2.

The work performed during the time of this grant suggests that topo IIα modulation by ErbB2 signaling may be responsible for the clinical observation of greater benefit from doxorubicin in ErbB2 positive breast cancer. Therefore a final goal of this project and this laboratory are to characterize topo IIα and ErbB2 co-expression in a large cohort of patients treated with doxorubicin-containing chemotherapy. Based on the work performed during the time of this grant, we have received approval from the cooperative group CALGB to proceed with such a project by measuring ErbB2 and topoisomerase IIα in tumor specimens from patients enrolled on a doxorubicin chemotherapy study, CALGB 8541. In addition, we are part of a larger effort to understand the role of ErbB2 in predicting response to doxorubicin-containing chemotherapy followed by taxanes in CALGB 9841, in collaboration with Dr. Daniel Hayes (Georgetown University).

We hope that this work will allow us to tailor breast cancer treatment to the individual patient by understanding the mechanisms behind drug sensitivity and resistance in ErbB2 positive breast cancer.

REPORTABLE OUTCOMES:

Publications:


Patents and Licenses:
None

Degrees Obtained:
None

Development of Cell Lines, Tissue or Serum Repositories:
ErbB3-EGFR Cell lines: Clones 1H, 2H, 3H (high expressers ErbB3); 1-7M (moderate expressers); 4-5 and 8-10L (low expressers).

Informatics:
None

Funding Applied for:

- DOD Research Award, 1997 - not awarded
- R01 - CA86774-02, “ErbB2/p53 and Taxane Chemotherapy for Breast Cancer” Principal Investigator Lyndsay Harris, MD – awarded August 1999
- SPORE award 1 P50 CA89293-01 - Developmental Project “Detection of HER-2 in patients receiving Herceptin and chemotherapy” – Principal Investigator Lyndsay Harris, MD – awarded October 2000
- RO1 - CA 092461-01, “ErbB2/p53 in 9344 – doxorubicin dose escalation with or without Taxol” - Principal Investigator Daniel Hayes, MD - pending
- CALGB Development Foundation Award– “Study Of HER-2 And Topoisomerase IIalpha And Outcome After Adriamycin-Based Chemotherapy For Early Stage Breast Cancer” - Principal Investigator Lyndsay Harris, MD – awarded March 2001

Employment or Research Opportunities:
Duke University – Assistant Professor with laboratory space.
Harvard University – Assistant Professor with laboratory space.

REFERENCES


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 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 employs breast cancer cells that have high levels of endogenous ErbB2 by gene amplification, (BT-474, SKBR3). The second system allows us to isolate the effect of ErbB2 receptor-mediated intracellular signaling using an EGFR-ErbB2 chimeric receptor, activated by Epidermal Growth Factor (EGF).

Our experiments show the cytotoxicity of doxorubicin is inhibited in ErbB2 positive breast cancer cells by the anti-ErbB2 antibody, Herceptin®. This is accompanied by decrease in topoisomerase IIα (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 lethal dose – 50 (LD50) of doxorubicin is seen following ErbB2 activation using the chimeric receptor model. Furthermore, we see a 100-fold decrease in the LD50 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 increase in topo II activity measured by DNA decatenating and unknotted 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 positive 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 positive breast cancer.
Introduction

The use of chemotherapy to treat early stage breast cancer has been proven to extend survival\(^1\). While doxorubicin-containing regimens show a small additional benefit over CMF (cyclophosphamide, methotrexate, 5-fluorouracil)-based regimens they are associated with rare but serious side effects (congestive heart failure, acute leukemia)\(^1\)\(^2\). The choice of regimen often depends upon the overall risk of recurrence and comorbidities of the patient but are 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\(^3\)\(^4\)\(^5\). Recent data suggests that ErbB2 amplification and overexpression is associated with improved outcome after doxorubicin-based therapy as compared with alkylator-based therapy(CMF,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 \textit{in vitro} data has shown that activation of the ErbB2, 3 and 4 receptors using Herregulin β-2 is associated with an increase in the DNA modifying enzyme, topoisomerase IIα (topo II), 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 \textit{in vitro} models. Data presented here demonstrate that increase in topo II activity and greater sensitivity to doxorubicin follow ErbB2 receptor signaling. We have also observed an increased resistance to cyclophosphamide following 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 positive breast cancer cells. While the role of Herceptin® in early stage breast cancer is yet to be determined, our study suggests that combining this drug with doxorubicin is unlikely to be the optimal strategy against ErbB2 positive tumors.

Materials and Methods

Cell Lines

NIH3T3 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 (epidermal growth factor receptor) with the transmembrane and intracellular portion of the ErbB2 receptor (Figure 1)⁹. The receptor is activated by epidermal growth factor (EGF) with subsequent tyrosine phosphorylation of the ErbB2 receptor intracellular tyrosine kinase. Host cells (NIH-3T3) do not express the ErbB2, 3 or 4 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 following receptor signaling, confirmed by experiments in this laboratory (data not shown). BT474 and SKBR3 cells are breast cancer cell lines which overexpress the ErbB2 receptor by amplification and have co-expression of ErbB3 and 4 and EGFR¹⁰. The MCF-7 breast cancer cell line is a transformed, non-ErbB2 amplified cell line which expresses high levels of estrogen receptor and is dependent on estrogen for growth. All breast cancer cell lines were obtained from the American Tissue Culture Corporation (Rockville, MD).

Western Blotting of Nuclear Extracts

For studies of the effect of ErbB2 activation on topoisomerase IIα protein levels, nuclear extracts were prepared from either chimeric cells or breast cancer cell lines. Chimeric cells were serum starved for 24 hours followed by incubation with or without EGF (10 ng/ml) for 24, 48, 72 and 96 hours. 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\textsuperscript{11}. Nuclear extracts were prepared by lysis of cells with high salt buffer (100 mM NaCl, 20 mM KCl, 20 mM Tris, 0.5 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4) containing Triton X-100 (0.5%) and NP-40 (1%). Nuclei were pelleted, and the nuclear proteins were solubilized by sonication in 1% SDS. After quantitation, 50 \( \mu \)g of nuclear protein was loaded and separated by 4-20% SDS-PAGE, transferred onto nitrocellulose and incubated with an anti-human topo II\( \alpha \) rabbit polyclonal antibody (TopoGEN, Inc, Columbus, Ohio). Following secondary antibody incubation, the p170 kDa protein was visualized using Enhanced Chemi-luminescence (ECL-Amersham, Buckinghamshire, England).

**Cell Cycle Experiments**

ErbB2-amplified (SKBR3) and ErbB2 non-amplified, ER positive (MCF7) cells at were arrested in G1 using hydroxyurea (2 mM) for 24 hours followed by release into serum-containing media. Cells were harvested at 0,5,11 and 24 hours, nuclear extracts prepared and Western Blotting for topoisomerase II\( \alpha \) performed as described above. FACS (fluorescence activated cell sorting) 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 due to their similar rate of progression through the cell cycle compared with MCF-7 cells, to facilitate comparison. Actin levels were measured as a loading control.

**Cytotoxicity Assays**

Chimeric receptor cells were serum starved for 24 hours and plated in 96 well microtiter plates at 1000 cells/well in quadruplicate in IMEM without phenol red containing 2% BCS (bovine calf serum) and EGF (10 ng/ml). Cells were treated with continuous exposure of doxorubicin (0.001\( \mu \)M-10\( \mu \)M) or continuous exposure VP-16 (0.001\( \mu \)M – 100\( \mu \)M), approximating \textit{in vivo} conditions for drug exposure. Cells treated with 4-HC were exposed to 4-hydroperoxy-cyclophosphamide (4-HC, 0.001\( \mu \)M-1.0\( \mu \)M) for 48 hours, washed and incubated in drug-free media for an additional five days. The short exposure period also approximates \textit{in vivo} pharmacokinetics and avoids
aerosolization of 4HC that may contaminate control wells. On day 7, at confluence of control samples, the cell viability was assessed by XTT (tetrazolium/formazan) assay\textsuperscript{12}. Results are expressed as percent control, where cells not treated with chemotherapy represent control values. The LD50 (lethal dose 50) is the amount of drug required to kill 50% of cells. Experiments were repeated a minimum of three times for each drug evaluated.

**Topoisomerase 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 50mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM MgCl\textsubscript{2}, 0.5 mM DTT, 0.5 mM EDTA, 30 \mu g/ml bovine serum albumin and 1 mM ATP\textsuperscript{13}. After 30-minute incubation at 30\degree C, reactions were terminated by 5:1 of SDS-Ficoll stop solution and samples were separated by electrophoresis in 0.7\% agarose. 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\textsuperscript{14}. Nuclear extracts prepared as noted above were incubated with kinetoplast DNA at 37\degree C for 15 minutes. 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 amount of nuclear protein loaded. All experiments were performed in triplicate.

**Topoisomerase II Cleavage Assay**

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

Results

We employed 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 co-expressed in ErbB2 positive 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 co-receptors. As ErbB2 has no known ligand, the receptor by itself cannot be activated exogenously. Therefore we employed an EGFR-ErbB2 chimeric receptor construct transfected into NIH-3T3 cells which do not express significant levels of the EGFR superfamily members (Figure 1) 9. Within one hour 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 Topoisomerase II Activity in ErbB2 positive Breast Cancer Cells**

We evaluated the effect of modulating ErbB2 signaling using 4D5 antibody (Herceptin®) on doxorubicin response. We assessed whether inhibition of the ErbB2 kinase was associated with 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 LD50 from .001 to .01 uM (Figure 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\(^\text{17}\). 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 (Figure 3). It is interesting to note that BT-474 cells are most resistant toward anti-tumor agents targeting either topo I or topo II\(^\text{18}\). Even at 10 \(\mu\)M 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 II\(\alpha\) protein. As previously reported, the 4D5 antibody led to decrease in ErbB2 receptor tyrosine phosphorylation (Figure 4A). In addition, topo II\(\alpha\) levels decreased during treatment with 4D5, concomitant with the decrease in ErbB2 receptor phosphorylation (Figure 4B). 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 employed a chimeric receptor construct transfected into NIH-3T3 cells that do not express significant amounts of any of the EGFR superfamily of receptors. We found a reproducible 10 to 100-fold (1-2 log) decrease in the LD50 of doxorubicin after EGF treatment of cells containing the EGFR/ErbB2 chimeric receptor compared with untransfected NIH-3T3 cells treated in a similar fashion. Figure 5A demonstrates this effect. To address possible differences in cell growth rate, chimeric cells growing in serum-containing media were treated with and without EGF then exposed to increasing doses of doxorubicin. A similar increase in sensitivity to doxorubicin was seen in this experiment. (data not shown).

To determine if these cells were sensitive to other topo II inhibitors that are not
DNA intercalators, we tested the cytotoxicity of VP-16 (etoposide) 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 (Figure 5B). The similarity of effect of ErbB2 activation on doxorubicin and etoposide dose-response curves suggests that the doxorubicin sensitivity noted in our system is mediated by topo II.

*Topoisomerase II activity and protein levels are increased in chimeric cells following 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 (Figure 6B). At a concentration of 1µM doxorubicin, approximately six times more protein-linked DNA cleavage can be detected in the EGF-treated cells. At even higher concentration, there is a decrease in the cleavage complex, a phenomenon common to the intercalative topo II inhibitors

To further explore the functional consequence of ErbB2 receptor signaling on topo II activity, we performed two different assays: unknotting of knotted P4 DNA and 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. Upon 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 (Figure 6A). After ErbB2 activation, there is an enhancement of two to four-fold strand passage activity in these assays. Taken together, these data suggest that the increase in topo II enzymatic activity upon ErbB2 receptor signaling leads directly to an increase in sensitivity of these cells to topo II poisons like doxorubicin and etoposide.

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

**Increased activity of topo IIα is seen throughout the cell cycle in ErbB2 overexpressing cells but not ER+, ErbB2 non-overexpressing cells**

The effect of ErbB2 signaling on topo IIα may be specific to effects of this oncogene on downstream targets or may be a non-specific effect of mitogenesis. To address this question, we performed cell cycle experiments on ErbB2 overexpressing (SKBR3) and non-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-overexpressing MCF-7 cells (Figure 8), although the pattern of expression is similar for both cell lines. The two cell lines had a similar 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-hydroperoxy-cyclophosphamide**

Cyclophosphamide is the main component of the regimen 'CMF' which 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. Using the active metabolite, 4-hydroperoxy-cyclophosphamide (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 to 100-fold (1-2-log) increase in the LD50 of 4-HC in cells with activated ErbB2 receptor, demonstrating increased resistance to the alkylator. Figure 9 demonstrates this effect
from one of three such experiments. Therefore, activity of the ErbB2 receptor may influence resistance to cytotoxic agents other than those that specifically target topo II.

**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 topoisomerase IIα and change in sensitivity to chemotherapy agents used in breast cancer. In the current study, we have pursued the relationship between chemotherapy, ErbB2 and topoisomerase 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 topoisomerase IIα levels. Furthermore the topoisomerase 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 both increases in topo IIα protein and enzymatic activity following 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 topoisomerase 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. Drugs that target topo II include the anthracyclines (doxorubicin, daunorubicin), etoposide, teniposide and amascarine. These agents appear to act by binding to the enzyme/DNA complex and inducing lethal cellular damage by the inhibition of the re-ligation step during the transient DNA cleavage reaction. Increase in topo IIα expression is associated with
sensitivity to these agents, both in cell lines and tumors, presumably due to increased 
target on which the drug may act\textsuperscript{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\textsuperscript{24}. In a subset of these tumors (49) this group has shown 
that co-amplification of topoisomerase IIα and ErbB2 genes occurs as these genes co-
localize to chromosome 17\textsuperscript{25}. This study shows that topo IIα may also increase by non-
genetic mechanisms through temporary increases in ErbB2 receptor activation. We see 
increased topoisomerase II levels and activity in cell lines where ErbB2 is not amplified, 
but is activated by a ligand. In addition, NIH-3T3 cells which contain a normal gene 
doseage of topo IIα upregulate this protein after activation of a chimeric receptor which 
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 topoisomerase IIα 
activity can be downregulated using the anti-ErbB2 antibody, Herceptin.

Large clinical trials suggest a benefit from doxorubicin in ErbB2 positive breast cancer 
that is not seen with alkylator-based therapy. In the cooperative group study performed by the 
NSABP (B11), an improved outcome with a doxorubicin-containing regimen was seen only in 
patients whose tumors overexpress ErbB2\textsuperscript{7}. In another large study, CALGB 8869, patients 
whose tumors overexpressed ErbB2 have a better survival if they were treated with higher doses 
of doxorubicin-containing chemotherapy, in contrast to their ErbB2 negative counterparts\textsuperscript{26}. 
Furthermore, we have also shown that Stage IV patients who exhibit higher levels of circulating 
ErbB2-extracellular domain (ECD), a surrogate marker for ErbB2 expression in this population, 
are 6 times more likely to respond to doxorubicin-containing therapy than to CMF regimens\textsuperscript{27}. 
These clinical studies suggest that response to doxorubicin is influenced by ErbB2. \textit{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 positive cells.

The relationship between ErbB2 overexpression in human breast cancer and poor
outcome after CMF treatment suggests resistance to alkylating agents\textsuperscript{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 tumours overexpressed ErbB2 did not appear to achieve as much benefit from postoperative CMF chemotherapy as did their non-overexpressing counterparts. Thus, our \textit{in vitro} observations are consistent with the clinical picture where overexpression of ErbB2 is associated with resistance to cyclophosphamide.

Previous studies have demonstrated that resistance to alkylators such as cyclophosphamide is multifactorial\textsuperscript{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 II\textalpha{} and it has been suggested that topo II is involved in DNA repair through its modulation of chromatin structure\textsuperscript{30,31}. Although cause and effect has not been proven, similar resistance to 4HC was associated with increased topoisomerase II activity in the \textit{in vitro} system used for our studies. It has been shown that inhibiting ErbB2 can reduce the rate of unscheduled DNA repair, increase intrastrand adduct formation and delay the rate of adduct decay in ErbB2 positive cell lines\textsuperscript{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 topoisomerase II inhibitors whereas other components confer resistance to alkylators. Topoisomerase II may be a member of this multifactorial response, perhaps as part of a multienzyme complex important in repair from DNA-damaging agents.

Our experiments demonstrate that ErbB2 signaling leads to alterations in both the level and enzymatic activity of topoisomerase 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 topoisomerase II activity, brought on by ErbB2 receptor signaling, may be one mechanism by which differential sensitivity of ErbB2 positive tumors to doxorubicin- versus cyclophosphamide-based regimens occurs in the clinical setting.

Recent development of the humanized monoclonal antibody, Herceptin\textregistered{}, has
provided useful therapy for HER-2 positive 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 idea 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 by other in vitro work. Moreover, our experiments suggest that combinations of Herceptin® with doxorubicin are not ideal as this does not allow us to take advantage of increased topo II activity in ErbB2 positive 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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REFERENCES


31 Pu QQ, and Bezwoda WR. Induction of alkylator (Melphalan) resistance in HL60 cells is accompanied by increased levels of topoisomerase II expression and function. Molecular Endocrinology, 56(1): 147-153, 1999.


Figure 1

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Figure 3

TOPO II CLEAVAGE ASSAY OF BT474 CELLS TREATED WITH 4D5
Figure 5

![Graphs showing the effects of Adriamycin and Etoposide on NIH3T3 and NIH EGFR/erbB2 cell lines.](image)
Figure 6

Decatenated DNA (density)

ug of Nuc. Ext. Loaded

NIH EGFR+shB2 - EG
NIH EGFR+shB2 + EC

CPM

doxorubicin (μM)
Figure 6 Continued
Figure 7

A

B

170kDa

p185ErbB2
Figure 8

Cell cycle distribution of TOPO II α Protein/Ptyr in erbB2 positive and negative breast cancer cell lines

220kDa

116kDa

Topo II p170

Actin

G0/1  S  G2/M  G1  G0/1  S  G2/M  G1