Grant Number DAMD17-96-1-6133

TITLE: ERBB-Receptors and Drug Response in Breast Cancer

PRINCIPAL INVESTIGATOR: Lyndsay N. Harris, M.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, D.C. 20057

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
### 4. TITLE AND SUBTITLE
ERBB-Receptors and Drug Response in Breast Cancer

### 6. AUTHOR(S)
Lyndsay N. Harris, M.D.

### 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University  
Washington, D.C. 20057

### 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Frederick, Maryland 21702-5012

### 11. SUPPLEMENTARY NOTES
19981210 118

### 12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

### 13. ABSTRACT (Maximum 200)
There is a compelling need for better ways to select cytotoxic therapy for a given patient with breast cancer. The role of the members of the type 1 growth factor receptor family (ErbB1-4) and their ligands in predicting response to chemotherapy is still unknown. We hypothesize that signaling through one or a combination of the ErbB receptors in breast cancer cells increases the activity of topoisoasemase IIα which then sensitizes cells to doxorubicin. 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 also made the novel discovery 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. We propose that the biology of the change in topo IIα activity may be understood by global changes in DNA repair enzymes, brought about by signaling through the EGFR superfamily. This may also explain the resistance to alkylators seen in our system and the clinical setting.

### 14. SUBJECT TERMS
Breast Cancer, ErbB2, HER2, ErbB3, Chemotherapy, Cyclophosphamide, Drug Resistance, Doxorubicin

### 15. NUMBER OF PAGES
17

### 16. PRICE CODE
Unclassified

### 17. SECURITY CLASSIFICATION OF REPORT
Unclassified

### 18. SECURITY CLASSIFICATION OF THIS PAGE
Unclassified

### 19. SECURITY CLASSIFICATION OF ABSTRACT
Unclassified

### 20. LIMITATION OF ABSTRACT
Unlimited
FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

[Signature]  9-14-98

FI - Signature          Date
TABLE OF CONTENTS

Introduction .......................................................... 1
Results and Discussion ............................................ 2
Methods ............................................................... 11
Conclusions and Future Direction ......................... 12
References .......................................................... 12
Introduction

There is a compelling need for better ways to select cytotoxic therapy for a given patient with breast cancer. The role of the members of the type 1 growth factor receptor family (ErbB1-4) and their ligands in predicting response to chemotherapy is still unknown. The objective of this proposal is to identify how this family of growth factors, particularly ErbB2, can be used to predict and control 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 two large randomized cohorts of patients, the CALGB 8869/8541 and SWOG companion studies (1)(2). One potential explanation for this finding is our observation of up regulation of the DNA modifying enzyme and target of doxorubicin, topoisomerase II α, in cells in which the ErbB2, ErbB3 and ErbB4 receptors have been activated using the ligand, heregulin (3). These cells also become more sensitive to doxorubicin. We hypothesize that signaling through one or a combination of the ErbB receptors in breast cancer cells increases topoisomerase II α 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. We propose to examine the relationship between activation of the ErbB receptors using various ligands (EGF, Heregulin and anti-receptor antibodies) and topoisomerase II α regulation.

We propose that the biology of the change in topoisomerase II α (topo II α) activity may be understood by global changes in DNA repair enzymes, brought about by signaling through the EGFR superfamily. During the time of this award we have shown that ErbB2 signaling has an effect on cell cycle distribution of the topo II α enzyme and its phosphorylation state, leading to increased sensitivity to doxorubicin but resistance to the alkylator cyclophosphamide (4). We have also made the novel discovery that topo II α is phosphorylated on tyrosine residues as opposed to serine/threonine as previously described (5). 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.

This information will allow the use of biological agents with cytotoxic therapy in combination to produce highest tumor response rates. It will also allow selection of appropriate patients (ie those which over express ErbB2 or other members of the ErbB family, or possibly topo II itself) for a given biological therapy directed at a specific molecular target. Patients whose tumors overexpress a given ErbB receptor, particularly if the receptor can be shown to be activated, may benefit from doxorubicin-containing regimens. By investigating the role of type I growth factors in predicting response to therapy we draw closer to better selecting patients who will benefit from our treatments and avoiding toxicity in those who do not.
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 α 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 α modulation mediated through ErbB receptor phosphorylation.

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

3a) Topoisomerase II α 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 α.

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

During the first year of the proposal we determined that ErbB2 signaling led to increased topoisomerase II α levels. We also made the novel discovery that signaling through this receptor leads to hyperphosphorylation of topoisomerase II α which appeared to be on tyrosine residues. This discovery is unique as it has heretofore been thought that topoisomerase II α activity is regulated by serine/threonine phosphorylation of the protein. During this second year we have further characterized this relationship by evaluating both the cell cycle distribution of topoisomerase II α levels and phosphorylation in ErbB positive vs negative cells lines. We also evaluated the effect of these changes in topoisomerase II α on its enzymatic activity in response to signaling by ErbB receptors.

RESULTS AND DISCUSSION


During the first 6 months of the proposal we created and tested the tools to be used for Aim 1. In order to determine which, if any, of the specific receptors involved are responsible for the phenotype of altered drug resistance we examined activation of both ErbB2 and 3 in
activated artificially by the ligand EGF with subsequent phosphorylation of the ErbB-receptor specific intracellular domain tyrosines (6). This is performed in a cell line (NIH-3T3) which does not express any of the ErbB receptors endogenously, therefore is a relatively clean system in which to look at ErbB2/3/4 receptor activation and signaling (fig 1).

Fig. 1

**EGFR/ErbB2 NIH-3T3 cells**

Two single clone populations of this cell line were kindly provided by Dr. C. Richter King and were characterized. NIH-EGFR/ErbB2-77 and -82 clones demonstrate tyrosine phosphorylation of the chimeric receptor and upon EGF ligand activation using Western blotting techniques (see 1997 annual report).

**EGFR/ErbB3 NIH-3T3 cells**

An EGFR/ErbB3 chimeric receptor construct, kindly provided by Dr. Mattias Kraus, was stably transfected into NIH-3T3 cells using a modified Calcium phosphate precipitation method suggested and selected using G418. Six chimera-expressing clones and five vector-transfected clones were chosen for characterization (see 1997 annual report). Growth rates EGFR/ErbB3 chimeric cells do not seem to reflect amount of receptor expressed in preliminary experiments. Phosphorylation on tyrosine was seen upon receptor activation with EGF 10 ng/ml (see 1997 annual report).

**EGFR/ErbB4 NIH-3T3 cells**

Data not yet available.

**Topoisomerase II levels, phosphorylation and activity in EGFR/ErbB Clones**

**EGFR/ErbB3 Clones:**

In the first year of our proposal we showed that activation of ErbB2 in the chimeric receptor model leads to increased topoisomerase II α protein and phosphorylation on tyrosine but not serine and threonine (see 1997 report). Due to technical difficulties, data is only available on one EGFR/ErbB3 clone and this shows a similar induction of topoisomerase II α protein upon activation with EGF, unlike the vector transfected control cell lines which show no difference in topo II α levels (Fig 2). Interestingly, the phosphorylation status of topoisomerase II α does not appear to change upon ErbB3 signaling in preliminary experiments (Fig 3).
**EGFR/ErbB2 Clones:**

To further characterize the effect of increased topoisomerase II α protein and phosphorylation on tyrosine we conducted several experiments designed to test topo II α function. In both the decatenation assay and the unknotting assay, methods used to test ability of the enzyme to unwind DNA and cause DS breaks (see methods) we show an increase of topo IIα activity upon ErbB2 receptor activation (Fig. 4).

---

**Decatenation Activity of NIH-EGFR/ErbB2 Chimera**
**Cells Treated +/- EGF**

![Graph](image)

---

**Topoisomerase II regulation in Breast Cancer Cell Lines**

**Breast cancer cell lines**

In order to demonstrate the relationship of receptor activation to topoisomerase II α modulation in the context of human breast cancer we chose to examine several breast cancer cell lines which are well characterized with respect to ErbB-family receptors. The following cell lines were obtained from American Tissue Culture Center (ATCC), Rockville, MD:

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EGFR(erbB1)</th>
<th>erbB2</th>
<th>erbB3</th>
<th>erbB4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKBR3</td>
<td>++</td>
<td>++++</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>T47D</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>BT-474</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SKOV3</td>
<td>-</td>
<td>++++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MCF-7</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>
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 above) were tested with the ligands EGF, heregulin β-2 (10ng/ml; kindly supplied by Dr. Dajun Yang), and 4D5 antibody (10 ug/ml; kindly provided by Genentech, Alameda, CA). 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 (data not shown). 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 (Herceptin®) 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(see methods) that this tyrosine phosphorylation is specific to topo II α and that this activity is downregulated by 4D5 (Fig. 5).

![Graph showing TOPOII quan](image)

**Fig. 5**

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 (7)(8). 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 (data not shown). Interestingly, SKBR3 cells are still growth inhibited by 4D5 as are all ErbB2 overexpressing cell lines (6). 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 (7). 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 general, no difference in ability to decatenate or unknot DNA was seen when BT-474 cells were treated with 4D5 (Fig 6). This further supports the work of Pegram et al where no synergy between 4D5 and doxorubicin was seen in BT-474 cells and is in contrast to experiments in nude mice where synergy between 4D5 and doxorubicin was seen (9)(10). The fact that BT-474 cells have more ErbB3 than other breast cancer cells may be relevant here, in that topo II phosphorylation was not seen in our ErbB3 chimera cells either. It is possible that increased tyrosine phosphorylation is a critical event in increasing sensitivity to doxorubicin.

![Decatenation Activity of BT474 treated +/- 4D5](image)

To determine if there was a difference in the cell cycle regulation of topo II α in ErbB2 positive vs negative cell lines we tested the expression of the protein and its level of phosphorylation in SKBR3 as well as MCF-7 cell lines. While topo II α was clearly regulated through the cell cycle in both, the amount of topo II α protein was reproducibly higher per in the ErbB2 cell line, SKBR3 (Fig. 7). In addition, the amount of tyrosine phosphorylation was clearly higher per molecule of topo IIα in the SKBR3 cells (Fig. 8).
Fig. 7a Cell cycle distribution of Topo IIa protein and Phosphotyrosine in SKBR3 cells

Fig. 7b Cell cycle distribution of Topo IIa protein and Phosphotyrosine in MCF-7 cells

Fig. 8 Tyrosine phosphorylation of Topoisomerase II during cell cycle
This suggests that ErbB2 signaling may lead to constitutively higher levels of tyrosine phosphorlylation on topo II α which may be responsible for the differences in drug resistance seen in the clinical setting.

**Task 4:** Months 18-24: Cytotoxicity assays (anchorage dependent) using doxorubicin and 4-OH 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 (Fig. 9). 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.

![Cytotoxicity of NIH3T3 and NIH EGFR/erbB2 Cells Treated with EGF in 4HC](image)

**Fig 9.**

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 II α activity (Fig. 10). Interestingly, this did not relate to increased growth rate as one might expect - the EGFR/ErbB3 clone demonstrating topo II α induction had the slowest growth rate. Two other clones tested showed little modulation of topo II α although there were technical difficulties and these experiments need to be repeated.
Cytotoxicity assay of NIH3T3-vector and EGFR/erbB3 chimera cells treated with EGF

Fig 10.

However, resistance to 4-HC was seen in all the EGFR/ErbB3 cells tested, despite a lack of topo II upregulation (Fig. 11).

Cytotoxicity assay of NIH3T3-vector and EGFR/erbB3 chimera cells treated with EGF and 4HC

Fig. 11
These data suggest that both ErbB2 and ErbB3 receptor activation are associated with resistance to the alkylator cyclophosphamide, however, not all ErbB3 clones showed increased sensitivity to doxorubicin. As ErbB3 is overexpressed in 22% of breast cancer it is possible that this receptor may also be used as a marker of resistance to alkylators but that ErbB2 may be necessary for sensitivity to doxorubicin (11).

METHODS

Western Blotting of Nuclear Extracts for Topoisomerase α, anti-Pyr, anti-Phosphoserine, anti-Phosphothreonine

These experiments were performed by preparation of nuclear extracts from chimeric cells which were serum starved X 24 hours and subsequently incubated + or - EGF 10 ng/ml for 24, 48 and 72 hours respectively. Nuclear extracts were prepared by lysis of cells with high salt buffer with Triton X100 and NP40, pelleting of nuclei, washing and disruption of nuclear envelope using a buffer containing SDS and sonication. Nuclear protein was separated using 4-20% SDS-PAGE, transferred onto nitrocellulose and incubated with a anti-human Topo II α rabbit polyclonal antibody (1U/ml, TopoGEN, Inc, Columbus, Ohio), anti-Pyr (Upstate Biotechnology, Lake Placid, NY), anti-Pser or Pthr (Sigma, St Louis, MO). After secondary Ab incubation, the p170 kDa protein was visualized using Enhanced Chemo-luminescence (ECL-Amersham, Buckinghamshire, England).

Immunoprecipitation and Western Blot Analysis for Topo II α Protein:

Nuclear protein extracts will be prepared from EGFR-ErbB-chimeric NIH-3T3 cells before and after treatment with EGF 10 ng/ml. Extracts were prepared from a non-SDS containing buffer using mechanical disruption of nuclei. After preclearing the extracts with protein A sepharose, samples were immunoprecipitated with an anti-p170 human Topo II α anti-rabbit polyclonal antibody (1U/ml, TopoGEN, Inc, Columbus, Ohio). After incubation on ice, the antigen-antibody complex will be precipitated with 10% protein A-agarose, washed and separated using 4-20% SDS-PAGE. Purified p170 kDa Topo II α will be used as a marker. Protein samples were transferred onto nitrocellulose using Western blot technique. After incubation with a Topo-II α anti-mouse monoclonal antibody (1U/ml), blots are visualized using Enhanced Chemo-luminescence (ECL-Amersham, Buckinghamshire, England).

Cytotoxicity Assays

Growth assays were performed in a standard fashion by exposing cells to increasing doses of doxorubicin (.001-10 uM) and counting viable cells at 5-7 days post-exposure. Cytotoxicity was expressed as % control of untreated cell number.

Topo II α decatenating assays

This assay is based upon decatenation of kinetoplast DNA (KDNA) and is specific for topoisomerase type II reactivity. The assay is performed by extracting nuclei from either chimeric cells treated with EGF 10 ng/ml or breast cancer cells treated with 4D5 10 ug/ml. The nuclear extracts are incubated with KDNA at 37°C X 15 minutes. The reaction is terminated with stop buffer and loaded directly onto a 1% agarose gel containing ethidium bromide. After
electrophoresis the gel is destained X 30 minutes and photographed. The density of decatenated products containing nicked circular or relaxed circular DNA are determined and expressed as a function of nuclear protein concentration loaded.

CONCLUSIONS AND FUTURE DIRECTIONS

Taken together, these data suggest that activation of the ErbB2 and ErbB3 receptors leads to increase in topoisomerase II α protein and increase in sensitivity to doxorubicin. Increased tyrosine phosphorylation is seen on topoisomerase II α post-receptor activation and this effect seems to be limited to ErbB2 receptor signaling, to date. Tyrosine phosphorylation has not been described on the topo II α protein but has recently been observed for other nuclear proteins involved in cell cycle and transcriptional machinery(12). These changes may lead to specific response to doxorubicin and cyclophosphamide which may be useful clinically.

In our future experiments we will attempt to continue the Aims outlined in this proposal and will further explore these new findings in the following experiments:

1. We will further characterize the signaling pathway from ErbB2 to topoisomerase II α phosphorylation by evaluating the ability of tyrosine kinases to phosphorylate this protein in in vitro kinase assays. We plan to evaluate c-abl, WEE-1 and src, yes and other non-receptor tyrosine kinases in collaboration with Dr. Robert Quackenbush from Dr. Ann-Marie Pendergast’s lab.

2. We will further characterize NIH-3T3 EGFR/ErbB3 chimera cells as to topo II α activity (decatenation and unknotting) after receptor stimulation with EGF to determine if the difference in protein level and phosphorylation translates into different activity of the protein. We are fortunate that we have 2 effects: topo II α protein upregulation upon activation of both ErbB2 and ErbB3, but topo II α phosphorylation only with ErbB2, as this allows us to distinguish the functional effect of changes in protein concentration from tyrosine phosphorylation of the enzyme.

3. We will transfet the NIH-3T3 EGFR/ErbB4 construct and characterize these cell lines to determine the role of ErbB4 in topo II α regulation.

4. We will transfet an erbB2 construct (kindly provided by Dr. Jeffrey Marks) into MCF-7 breast cancer cells under the control of a tet-inducible promoter to observe the effect of growth factor signaling in the context of an estrogen-dependent cell line. This will allow us to evaluate the effect of addition of ErbB2 receptor to a system where we have already characterized cell cycle regulation of topo II α. In addition, it will help us understand whether the higher degree of topo II α protein and tyrosine phosphorylation seen in SKBR3 cells is due to ErbB2 specifically.

5. We will pursue changes in transcription and translation of topo II α where protein levels are seen to be modulated using methods outlined in the original award.

REFERENCES


