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Award Number: DAMD17-99-1-9111

TITLE: ER/AP-1 Modifications in Tamoxifen-Resistant Human Breast Tumors

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REPORT DATE: September 2002

TYPE OF REPORT: Annual Summary

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

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual Summary (1 Sep 99 - 31 Aug 02)

4. TITLE AND SUBTITLE
ER/AP-1 Modifications in Tamoxifen-Resistant Human Breast Tumors

5. FUNDING NUMBERS
DAMD17-99-1-9111

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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13. ABSTRACT ***(Maximum 200 Words)***
Endocrine therapy in the form of aromatase inhibitors or antiestrogens like tamoxifen is the treatment of choice for most patients with estrogen receptor (ER)-positive breast cancers. However, it remains unclear why up 30-50% of ER-positive tumors demonstrate clinical resistance to these forms of ER-targeted treatments. It is known that ER that is structurally altered such that it cannot bind directly to its ERE, can still affect gene expression via protein-protein interactions with other transcription factors (e.g. AP-1, Sp1, NF-κB, etc.); these protein-protein interactions are mediated by ER domains other than the ER-DBD. The goal of the current study is to identify the molecular changes induced by oxidant stress that result in altered intracellular ER structure, account for its loss of function (DNA-binding), and thereby produce a clinically more aggressive form of tumor behavior that includes loss of responsiveness to ER-targeted endocrine therapy. In order to accurately study these oxidative effects I have been developing a double alkylation, double digestion protocol. The protocol has evolved from its original version (described last year) due to unexpected pitfalls. The revised protocol and its application to the study of oxidant stressed ER isolated from cell lines and tumor samples are described in this report.

14. SUBJECT TERMS
breast cancer, tamoxifen, estrogen receptor, AP-1, Sp1

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The DNA-binding and transactivating functions of many transcription factors, in particular so-called zinc finger proteins, are redox sensitive; and most of these transcription factors appear to be regulated via oxidation of critical Cys residues within their DBDs (1). In particular, transcription factors such as Sp1 and the steroid-binding glucocorticoid receptor (GR) contain redox-sensitive and zinc-coordinated Cys residues supporting the zinc-finger structures necessary for binding the major groove of DNA (1, 2). Interestingly, natural aging causes *in vivo* accumulation of oxyradical tissue damage that results in selective loss of the DNA-binding activity of Sp1 and GR, although no significant decline in the tissue content of either protein is observed (1, 2). Furthermore, *in vivo* studies have shown that this age-associated deficiency is reversible by treatment with dithiothreitol (DTT) (2). The ER-DBD containing two $(Cys)_4$-liganded zinc fingers are homologous to those found in GR, and are similarly redox sensitive. Several lines of evidence support this observation, including the fact that its transcriptional activity and DNA-binding capacity are modulated by the redox effector protein thioredoxin (3). In addition, vigorous thiol reduction often restores the ER's ability to bind DNA both *in vitro* and *in vivo* (4). Furthermore, this functional abnormality can be induced in ER-positive human breast cancer cells by exposure to oxidants *in vitro* (4). Finally, mass spectrometric analysis of recombinant ER-DBD determined that this defect is indeed due to oxidation of the two zinc fingers within the domain and in particular the second zinc finger, which is involved in dimerization, is susceptible to oxidation (5). This defect in DNA binding does not prevent ER from co-activating genes through other DNA-binding transcription factors such as AP-1 and Sp1. ER is known to interact with both these transcription factors when they are DNA-bound and through protein-protein interactions independent of ERE-mediated ER DNA binding. Oxidant stress is associated with increase in AP-1 activity and DNA-binding as well as loss of Sp1 and ER DNA-binding function. In turn, AP-1 regulates a number of genes (collagenase, cathepsin D, and p-glycoprotein multidrug resistance gene) associated with cellular growth resistant deregulation, thus enhamced AP-1 activity may lead to more malignant and invasive phenotype. Having deferred our attempts to develop a cell-free AP-1 transcript forming assay due to methodological difficulties (technical objective 1) and having completed our analysis of 70 ER-positive tumors for age-specific changes in DNA-binding content of AP-1, SP-1 and (P)-Erk5 (technical objective 2), we focused our efforts on technical objective 3. As described in last year's progress report (submitted September 27, 2001) our third objective is to develop a mass spectrometric protocol that will allow us to accurately and quanitatively characterize the oxidant induced molecular lesions within the zinc fingers of full-length ER. We have made modifications to our original two-stage Cys alkylation and two protease in-gel digestion protocol. The new protocol and the reasons for the modifications are described below.

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BODY

Progress Overview:

Progress since year 01 according to the technical objectives as originally proposed and subsequently amended (11/30/00).

Technical Objective 1: Develop a new assay to measure AP-1 transcriptional activity as a correlate of increased AP-1 DNA-binding and JNK activity in human tumor samples.

Unchange since original Progress Report and with reprioritization and addition of new objective (Technical Objective 3, see annual report submitted on September 27, 2001).

Technical Objective 2: Compare zinc finger (ER, Sp1) DNA-binding with ER content, AP-1 DNA-binding, and associated mitogen activated protein kinase (Jun N-terminal kinase (JNK) or Erk5) activity from primary breast tumors.

Completed Objective (see annual report submitted on September 27, 2001)

Technical Objective 3: Extend recently developed mass spectrometric approach to identify differentially oxidized cysteine (Cys) residues within the ER DBD zinc-fingers and characterize the oxidative damage detected in purified full-length human breast tumor ER.

Mass spectrometric studies on oxidant-stressed isolated recombinant ER-DBD showed that its two zinc fingers readily oxidize, particularly the second zinc finger which engages in dimerization. The goal of our third research objective is take these studies a step further and examine the structural changes induced by oxidative stress on the ER-DBD within the context of the full length protein purified from cell lines and breast tumor samples. Our initial studies focused on full-length recombinant protein (purchased from PanVera) and involved an in-solution digestion of non-treated and oxidant treated full-length recombinant ER protein followed by liquid chromatography and mass spectrometry analysis. We realized immediately that reproducing the studies on recombinant ER-DBD was going to be challenging for a couple of reasons: 1) the resulting ER-DBD peptides spontaneously oxidized during the analysis and 2) there was a significant loss of absolute signal for each of the ER-DBD peptides after oxidant treatment of the full-length recombinant ER. Thus, in an effort to circumvent these problems, we proposed a two-stage, two-protease in-gel digestion protocol (Fig.1). Since our last progress report (submitted September 27, 2001) we have modified our initial

protocol at different steps in order to ultimately produce a protocol that will allow us to accurately and quantitatively access the degree of oxidation of ER purified from cell lines and breast tumor samples. The following paragraphs will summarize our findings and conclusions concerning the best approach to use in the study of our particular protein.

We have tried four different procedures in an attempt to derive the best protocol for our studies (Fig. 2). The first procedure which involves digesting the protein and analyzing it by LC/MS is not adequate because reduced recombinant ER spontaneous oxidizes during the digestion and LC/MS procedure. The second procedure involves the initial selective carboxymethylation of non-oxidized Cys residues in ER with doubly labeled ^{13}C bromoacetic acid, an in-gel reduction and a second alkylation step with iodoacetic acid to identify Cys residues that were originally oxidized, followed by in-gel proteolytic digestion and peptide analysis by LC/MS. Endoproteinase Lys-C and Asp-N enzymes have been selected giving peptides that each contain two Cys residues, showing mass differences of 2 or 4 Da for single or double incorporation of ^{13}C labeled carboxymethyl groups. The procedure was designed to circumvent the problems mentioned above and to more precisely quantitate the amount of reduced versus oxidized zinc finger peptides by using chemically identical but isotopically tagged alkylating agents of different molecular weights to selectively label free cysteines with one agent and previously oxidized cysteines with the other. However, we found a problem with this protocol. Both the first and the second alkylation steps were inefficient. Particularly when it came to the alkylation of zinc finger 1. We tested various temperature conditions for varying amount of alkylation time and found that we could not completely alkylate zinc finger 1 from a reduced form ER either during the first or second alkylation steps. Complete alkylation of reduced protein is key in our analysis in order to accurately determine the oxidation state ER.

Our inability to efficiently alkylate ER prompted modifications to our protocol. We reasoned that if we digested the protein in the presence of the alkylating agent, then any structural obstructions as a result of the tertiary structure of the protein would be destroyed during the digestion and thus the alkylating agent would have access to alkylate the resulting Cys containing petides (Fig.2 procedure #3). Procedure #3 differs from procedure #2 in two ways. First the digestion is performed immediately after the first alkylation (without removal of BAA*). Secondly, the gel purification step is ommited and thus the alkylation reaction is quenched with excess L-Cysteine (Cys). The resulting peptides are reduced and any previously oxidized Cys are alkylated by the second alkylating agent (IAA) and analyzed by LC/MS. Our logic was correct, for allowing the alkylating agent to work in the digestion solution yielded complete alkylation of the various ER-DBD fragments.

The initial step in the study of ER from cell lines and tumor samples involves the purification by immunoprecipatation of the protein from cell lysates. However, given that the ER-DBD fragments are very susceptible to spontaneous oxidation, the first step in any protocol addressing ER oxidant sensitivity should involved the complete alkylation of non-oxidized Cys residues. Our studies so far show that complete alkylation of ER only occurs under digested (ie. denaturing) conditions and thus it would not be possible to immunoprecipate a digested protein. Therefore, while procedure # 3

would allow us to accurately study the oxidant sensitivity of the various Cys residues within the two zinc fingers of purified recombinant ER protein, we had to again modify the procedure to allow us to study protein isolated from cell lines and tumor samples. So procedure # 4 (Fig. 2) allows for the alkylation in the presence of a denaturing agent (2M guanidine hydrochloride) rather than in the presence of the proteases. It also differs from procedure #3 in that the double digestion is the last step in the procedure and precedes only the LC/MS analysis. We found that employing this procedure also yields complete alkylation of the various ER-DBD fragments. Additionally, if we allowed the second alkylating agent (the in-gel alkylation) to work in the digestion solution, these reactions also yielded complete alkylations of the fragments.

Conclusions:

Thus through a series of adjustments we have developed a protocol (Fig. 3) to study oxidant stress in ER purified from cell lines and breast tumor samples. The procedure involves denaturing ER in cell lysates with GnHCl in the presence of the alkylating agent, followed by dilution of the denaturing agent to a concentration where it does not denature the antibody so that immunoprecipatation of ER can take place. Then the protein is further gel purified and an in-gel reduction and alkylation is performed (in the presence of proteases) and the ER-DBD fragments are LC/MS analyzed.

We are currently in the process of optimizing the isolation of ER from cell lines and tumor samples. We have tested a variety commercially available ER antibodies (Upstate biotechnology, Santa Cruz Biotechnology, Calbiochem, etc) under a variety of reaction conditions and determine that we can successfully immunoprecipitate recombiant ER with greater than 90% efficiency. Maximizing both the efficiency with which ER is immunoprecipitated and recovered from the immunocomplex is particularly important to enable future purification of ER from breast tumor samples of ~100mg (wet weight) where cellular quantity and ER content are more limiting.

Finally, we have recently examined the efficiency of various extraction protocols for recovering ER from MCF-7 cells (i.e. nuclear extraction, high salt extraction, detergent extraction protocols). Our results show that the nuclear extraction method yields the greatest amounts of ER from MCF-7 cells. Thus, once optimized the procedure described above (Fig. 3) will be applied to ER nuclear extracts. Because treatment of these cells with oxidizing agents (i.e. hydrogen peroxide, diamide) has been shown to eliminate ER DNA-binding without reducing intracellular ER content, the proposed experiments will be performed under similar non-lethal cell culture treatment conditions. If, as expected, many of the observed *in vivo* ER defects correspond to those defects observed following *in vitro* ER treatment or culture stressed ER-positive breast cancer cell lines, these results will support the proposed mechanistic basis for loss of ER DNA-binding and provide further clues as to the *in vivo* mechanisms leading to this oxidative damage and thus possible therapeutic strategies for preventing or reversing these ER defects associated with altered ER function and breast cancer clinical behavior.

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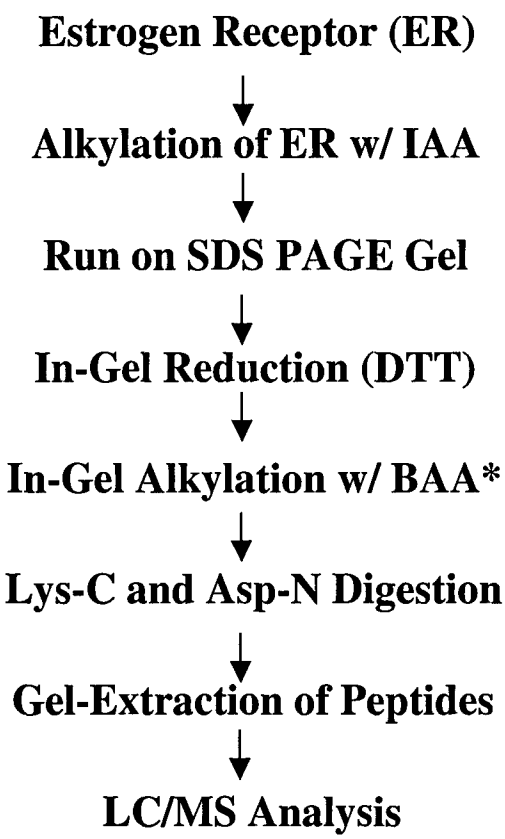

Figure 1: Schematic diagram of the two-stage alkylation and two-protease in gel-digestion and extraction procedure originally used in our studies. The alkylation steps circumvent the spontaneous oxidation of Cys residues that occurs during the analysis of ER. Alkylation with iodoacetic acid (IAA) adds 58 Da to the molecular weight per reactive cysteine, while doubly ^{13}C-labeled bromoacetic acid (BAA*) adds 60 Da.

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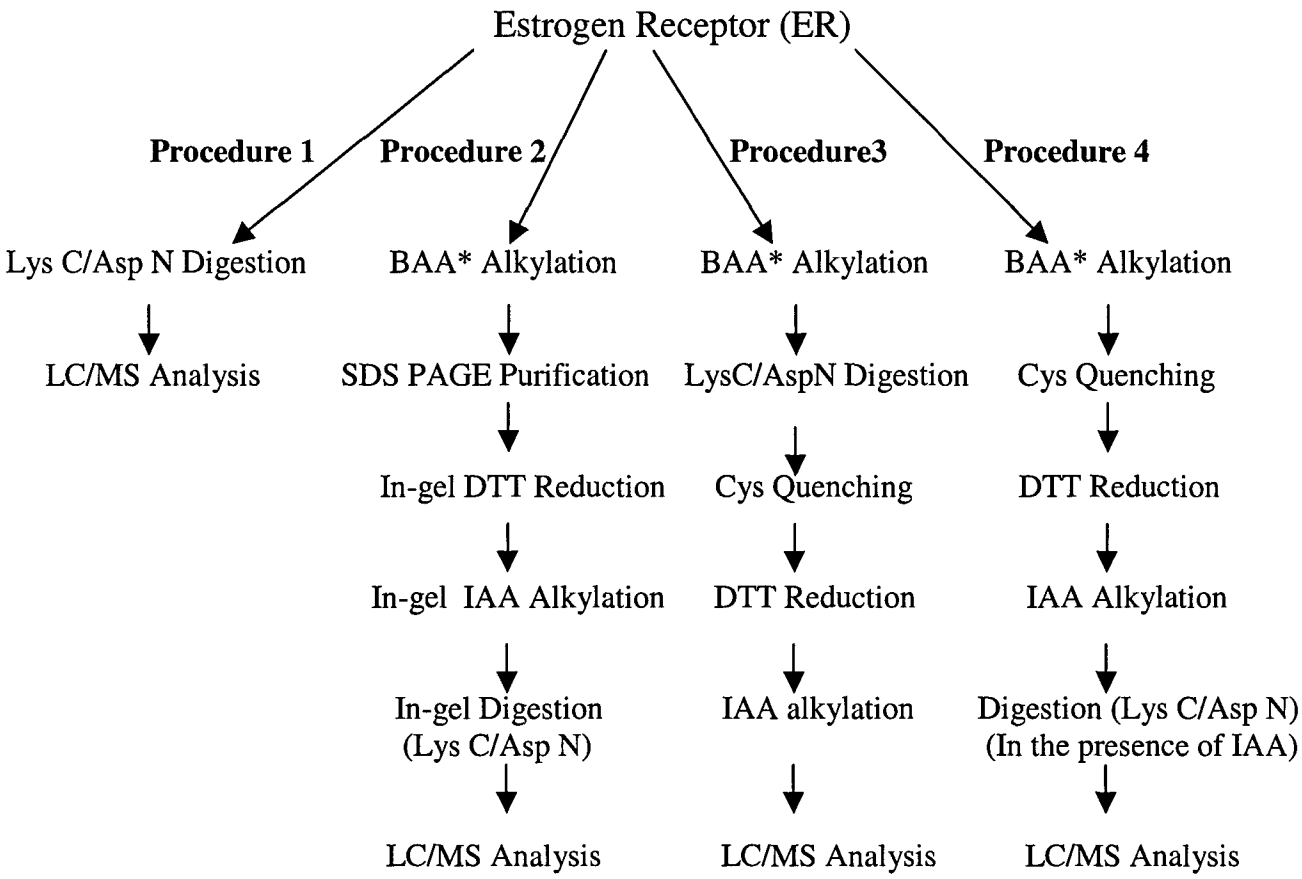

Figure 2: Schematic diagrams of the four different approaches attempted in an effort to accurately and quanitatively access the effect of oxidative stress on the ER-DBD in the context of the full-length esrtrogen receptor. See text for details.

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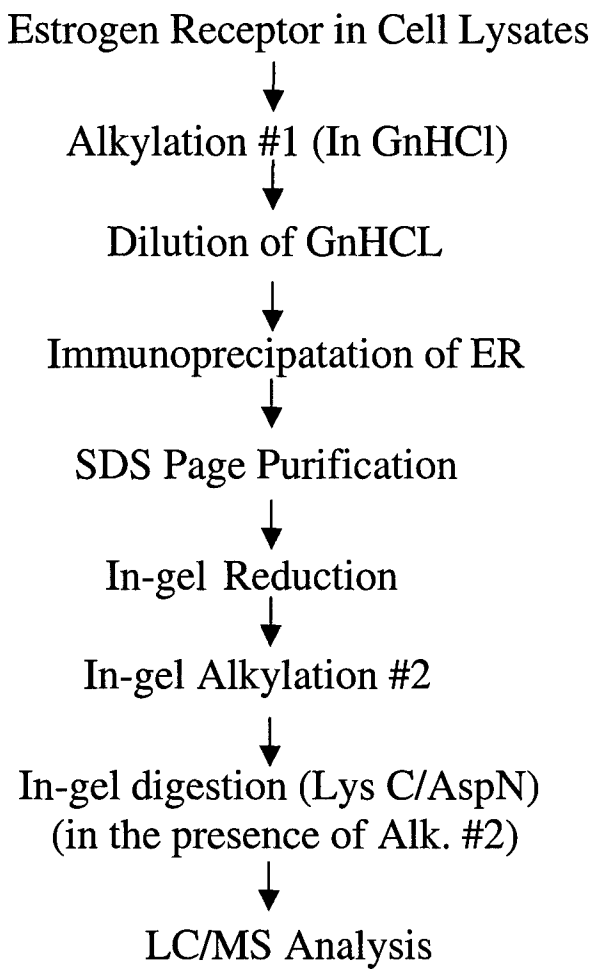

Figure 3: The optimized procedure to be used in the analysis of oxidant treated ER from cell lines and breast tumor samples. See text for more details.

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APPENDIX

Key Research Accomplishments (according to funding year):

1. (year 1) Two different 4AP1/Luc constructs were made during the first year for feasibility assessment of prototype in vitro transcription assay and collaborations with the UCB Tjian lab (and others) were put into place for assistance in reagent production and assay development. Project technical objective 1 was later amended to enable full scale development of this complicated in vitro transcription assay by collaborating labs with specialized expertise, reagents, and interest in this assay.
2. (years 1-2) Completed analysis of ER content, AP-1 DNA-binding, Sp1 DNA-binding, Sp1 protein content, and Erk5 activation in 70 outcome-linked and biomarker-characterized breast tumors. Significant statistical associations with patient age at diagnosis were discovered, potentially explaining the increasing incidence of ER-positive/PR-negative breast cancers occurring with age >50 years, as confirmed by review of SEER database information on >80,000 US breast cancer cases diagnosed between 1992-1997.
3. (year 2) Developed a two-step Cys alkylation and two-protease in-gel digestion and extraction protocol that has enabled us to successfully isolate and identify fragments comprising the ER-DBD from full-length recombinant ER by mass spectrometry. Our preliminary data suggest that this method will allow us to circumvent the unforeseen problems described above (i.e. spontaneous oxidation of Cys residues and loss of peptide signals) and enable us to efficiently study the effect of oxidative stress on the ER-DBD within full-length recombinant ER and on ER purified from cell lines and tumor samples.
4. (year 3) Tested four different procedures in order to derive a protocol that will allow us to best study oxidative stress of ER in cell lines and breast tumor samples. The new protocol in particular allows us to efficiently alkylate all the ER-DBD peptides, which was issue when employing our original two-step Cys alkylation and two-protease in-gel digestion and extraction protocol.

Reportable Outcomes:

1. Quong, J.N.; Eppenberger-Castori, S.; Birrer, M.; Eppenberger, U., and Benz, C.C. Redox-sensitive kinase (Erk5) activation and altered transcription factors (Ap-1, SP1) DNA binding in untreated primary breast tumors. American Association of Cancer Research 2000 Meeting, April 1-5, San Francisco, CA.
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DEPARTMENT OF THE ARMY
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ATTENTION OF:

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FOR THE COMMANDER:

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