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| <b>13. ABSTRACT (Maximum 200 Words)</b><br>The factors driving resistance to antiestrogens are unknown. Comparing the transcriptomes of antiestrogen responsive and resistant MCF-7 variants by serial analysis of gene expression, we have implicated several genes, including the human X-box binding protein-1 (XBP-1). XBP-1 is a cAMP response element (CRE) binding protein associated with estrogen receptor (ER) expression in gene expression profiles of human breast cancers. <b>We hypothesize</b> that overexpression of XBP-1 and/or activation of CRE contribute functionally to the ability of responsive cells to survive the metabolic stresses induced by exposure to antiestrogens. <b>We also hypothesize</b> that measuring expression of the XBP-1 protein will assist in better identifying antiestrogen resistant and/or responsive tumors. <b>Aim 1:</b> We will further study the likely functional role of XBP-1/CRE by overexpression through transfection into responsive cells, and inhibiting expression in resistant cells using novel CRE oligonucleotide decoys, antisense and/or ribozymes. Effects of these molecular manipulations on responsiveness to antiestrogens will be studied <i>in vitro</i> and <i>in vivo</i> . <b>Aim 2:</b> We will explore the prognostic and predictive significance of XBP-1 expression in a unique series of human breast cancer biopsies. Thus, we will assess the extent to which XBP-1 is an independent prognostic factor, and whether it is associated with response to antiestrogens. Where possible, we will explore whether XBP-1 expression data allow us to build better predictive/prognostic models. |   |  |  |                                  |
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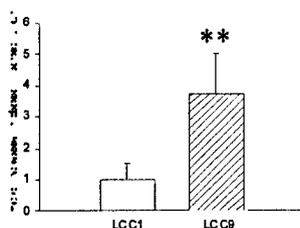
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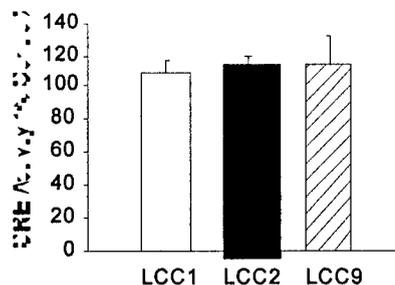
## INTRODUCTION

Antiestrogens are effective in premenopausal and postmenopausal patients, and in the chemopreventive, adjuvant and metastatic settings (1), probably through the induction of growth arrest/apoptosis (1). The triphenylethylene TAM, a partial agonist, is the most widely used antiestrogen. Long term TAM use reduces the incidence of contralateral breast cancer (antagonist) and primary breast cancer in high risk women (antagonist), maintains bone density (agonist) and increases the risk of endometrial carcinomas (agonist) (2). Newer antiestrogens include the "pure antagonist" ICI 182,780 (no agonist activity). In patients that had previously shown a response to TAM but recurred, ICI 182,780 produces a response rate significantly higher than the response rate for crossover to another triphenylethylene (Toremifene) following TAM failure (3).

**Antiestrogen Resistance.** Most breast tumors that initially respond to TAM recur and require other endocrine or cytotoxic therapies (4). Despite over 10 million patient years of experience with TAM, the precise mechanisms that confer acquired resistance are unknown (1). Absence of ER expression is clearly important for *de novo* resistance (1). ER expression is *not* lost in most breast tumors that acquire antiestrogen resistance (5). Currently, there is little compelling evidence that expression of ER splice variants and mutant ER contribute significantly to antiestrogen resistance in patients (1,6). While the importance of wild type ER $\alpha$  is established as a mediator/predictor of antiestrogen responsiveness, that of ER $\beta$  remains unclear. ER $\alpha$  may be the predominant species in most ER+ breast tumors (7,8), and is associated with a better prognosis (9). ER $\beta$  is associated with a poorer prognosis, absence of PgR, and lymph node involvement (8,10). One small study reported higher ER $\beta$  mRNA levels in resistant tumors (11). However, this association could not be separated from that between ER and a more aggressive phenotype (8,10). Some studies report activities independent of ER function, which may initiate events that are necessary but not sufficient for antiestrogen-induced effects (1). Our research team has recently reviewed in detail the potential mechanisms of antiestrogen resistance in ER+ tumors (12).



**Fig 1:** Transcriptional activation of CRE is increased 4-fold in MCF7/LCC9 cells. **\*\*** $p < 0.02$ ,  $n = 6$ .



**Fig 2:** ICI 182,780 does not regulate CRE activity in either MCF7/LCC1 or MCF7/LCC9 cells. Not significantly different from controls (activity in untreated cells for each variant).

### Implicating XBP-1 in Antiestrogen Resistance.

**SAGE.** Initially, we explored differences in the transcriptomes of the MCF7/LCC1 (antiestrogen sensitive) and MCF7/LCC9 cells (antiestrogen resistant – resistant to both TAM and ICI 182,780) by serial analysis of gene expression (SAGE) as previously described (13), using the "SAGE" software (Dr. Kinzler, Johns Hopkins University). Most genes identified are not differentially expressed between MCF7/LCC1 and MCF7/LCC9 cells. Differentially

expressed genes were selected by (a) the Tags compared represent  $\leq 2$  genes, (b) a Tag found in either the MCF7/LCC1 or MCF7/LCC9 SAGE library must represent 0.10% of the database, and (c) fold difference  $\sim 2$ -fold. Evidence that a gene is expressed in breast cancers also was considered. No single criterion was considered an absolute requirement for selection. Among the genes we identified were cathepsin D, nucleophosmin (NPM) and XBP-1.

***Altered expression of XBP-1 protein and transcriptional activation (CRE).***

To confirm the altered expression of XBP-1, we first performed Western analysis on proteins from MCF7/LCC1 and MCF7/LCC9 cells. We initially detected a  $\sim 5$ -fold induction of XBP-1 protein in MCF7/LCC9 cells, comparable with the 4-fold induction in mRNA levels (14). Measuring protein levels and/or protein bound to responsive elements can be poor indicators of the functional

activation of transcription factors. Since XBP-1 activates CREs, we measured directly CRE transcriptional activation using a CRE promoter-firefly luciferase reporter assay (PathDetect *in vivo* signal transduction pathway *cis*-reporting system; Stratagene). Cells were transiently transfected with the appropriate plasmids using Qiagen's Superfect reagent. Normalization of transfection efficiency was made to a *Renilla* luciferase reporter driven by the constitutive cytomegalovirus promoter (Promega's Dual-luciferase reporter assay). The data in Fig. 1 show that basal CRE activity is significantly increased in MCF7/LCC9 cells compared with MCF7/LCC1 cells (4-fold;  $p < 0.02$ ).

***Regulation of CRE (XBP-1) activity by ICI 182,780.*** The upregulation of CRE activation would be of limited use to cells if it could be inhibited by ICI 182,780-occupied ERs. Thus, we assessed the ability of ICI 182,780 to affect CRE activation using the promoter-reporter assay. ICI 182,780 treatments (10 nM) were administered for 48 hrs post-transfection. ICI 182,780 treatment does not alter the transcriptional regulatory activities of the CRE promoter in either responsive MCF7/LCC1 or resistant MCF7/LCC9 cells (Fig 2). These data further imply a functional role for XBP-1 in acquired resistance to ICI 182,780. In responsive cells, the inability to induce CRE in the presence of ICI 182,780 allows for the dominance of growth inhibitory signals leading to growth arrest/apoptosis. Resistant cells may survive growth inhibition/apoptosis by upregulating signaling through CREs. ***Since CRE-activation is required for MCF-7 cell proliferation (15), some breast cancer cells may survive antiestrogen treatment by upregulating factors that are not affected by ER-mediated signaling, e.g., XBP-1/CRE.***

XBP-1  
M.W = 28kDa



Fig. 3. Western blot of two MCF-7/XBP-1 clones expressing the XBP-1 protein.

## BODY OF REPORT

### KEY RESEARCH ACCOMPLISHMENTS

We have continued to make good progress and have almost completed Task 1 in the MCF-7 cell model. We had (last year) noted that we had worked out of sequence and presented data on Task 4 (expression in tissue samples). Thus, we are on target overall.

### Bulleted List of Research Accomplishments (this report)

- Completed characterization of XBP-1 overexpressing MCF-7 cells
- Shown that XBP-1 can confer estrogen-independence
- Estrogen-independence conferred by XBP-1 is associated with reduced cell cycle arrest in the absence of estradiol
- XBP-1 reduces sensitivity to both 4-hydroxyTamoxifen (4OH-TAM) and ICI 182,780 (Fulvestrant; Faslodex)
- Reduced antiestrogen sensitivity by XBP-1 is partly a result of a reduced ability of these drugs to induce cell cycle arrest
- Reduced antiestrogen sensitivity conferred by XBP-1 also is partly the result of an inability of antiestrogens to induce apoptosis

### TASK 1: Overexpress XBP-1 in antiestrogen sensitive cells

- a. Transfect and select transfectants (months 1-2)
- b. Characterize transfectants for XBP-1 protein expression and CRE transcriptional activity (months 3-4)
- c. Determine response of transfectants and controls to E2 and antiestrogens *in vitro* (months 4-8)
- d. Determine response of transfectants and controls to E2 and antiestrogens *in vivo* (months 8-12)

**Transfection of MCF-7 cells with XBP-1 (reported in the previous report).** We first introduced the XBP-1 cDNA into MCF7 cells (MCF7/XBP-1), which are antiestrogen sensitive and estrogen dependent for growth *in vitro* and *in vivo*. Cells were transfected with a pcDNA 3.1 expression vector (Invitrogen) containing the XBP-1 cDNA. The empty vector (same construct but without the XBP-1 cDNA) also was transfected into MCF-7 cells to generate control cell populations. We had some difficulty getting XBP-1 overexpressed, the reasons for which are not yet apparent. Nonetheless, after several attempts, we successfully obtained clones resistant to G418 (selectable marker). We presented the initial characterization of these cells last year (e.g., increased XBP-1 protein expression and CRE activity) and additional data showing the ability of XBP-1 to bind to the estrogen receptor protein.

We have since also had problems with the T47D cell model (we started to generate the T47D model at the end of year 1 and into year 2), in that isolating stable transfectants also proved difficult (we noted some initial problems with the MCF-7 cells in last year's report). We

plan to revisit selecting T47D cells to broaden the models in which we can work. However, we believe that we can adequately address our hypotheses without the T47D cells but would prefer to have two cell lines if possible.

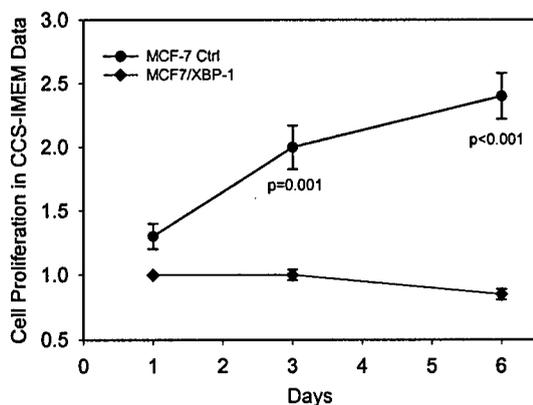


Fig 4: Growth of cells in CCS-IMEM

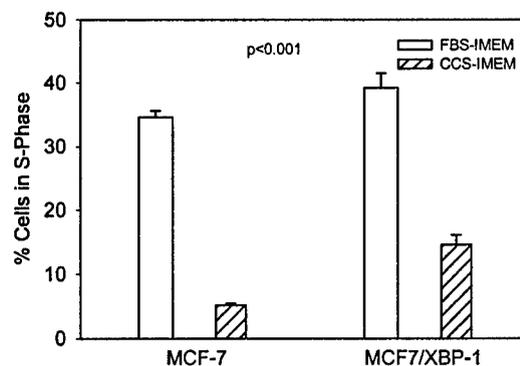


Fig 5: S-phase distribution in FBS-IMEM and CCS-IMEM

#### **MCF7/XBP-1 cells are estrogen independent *in vitro***

We assessed whether overexpression of XBP-1 could confer on MCF-7 cells the ability to grow *in vitro* in the absence of estrogenic supplementation. Cells (we used MCF7/XBP-1 and the MCF-7 vector control in all studies described in this report) were grown in cell culture medium without phenol red and supplemented with 5% DCC (dextran coated charcoal) stripped calf serum (CCS-IMEM) for three days, with one medium change per day. Monolayers were washed in fresh CCS-IMEM, plated into 12-well culture dishes in this medium, and the cells counted on the following days 1 (24 hrs later), 3, and 6. Cells were counted following trypsinization and dilution in saline using a Coulter Counter. The data in Fig 4 (replicate data of three or more replicates/experiment combined from three experiments) show that XBP-1 enabled the cells to survive and proliferate in CCS-IMEM, whereas the control cells did not proliferate and began to die between days 3 and 6.

We also compared cell cycle distribution of the control and MCF7/XBP-1 cells. In these experiments we included additional controls (cells growing in the regular MCF-7 medium). In CCS-IMEM, we found approximately 6% of MCF-7 cells to be in S-phase, compared with almost 20% of the MCF7/XBP-1 cells (Fig 5; data combined from three or more experiments). These data are consistent with the differences in Fig 4. Cell cycle analysis was done by Flow Cytometry using standard methods (16).

#### **MCF7/XBP-1 cells are less sensitive to TAM and ICI 182,780 resistant than controls**

Since XBP-1 overexpression conferred estrogen-independence, we then assessed its ability to affect responsiveness to 4-hydroxytamoxifen (4OH-TAM) and ICI 182,780 (each 1 nM). First, we determined the antiproliferative activity of these drugs, using simple cell counts on days 1, 4 and 6 following initiation of continuous exposure to the drugs. The greater final cell density of the untreated MCF7/XBP-1 cells tends to obscure the significant growth of these cells in the presence of drug relative to the MCF-7 vector controls (the controls did not grow or died). The final cell numbers for the control populations reflect no net increase in response to ICI 182,780

and only a 20% increase in the presence of 4OH-TAM. This compares with 3-fold and 4-fold respective increases in the MCF7/XBP-1 cells.

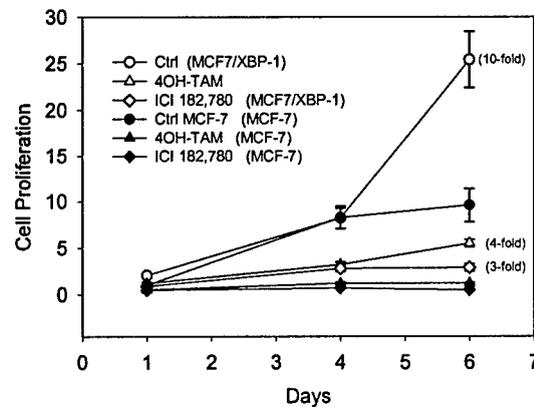


Fig 6: Cell proliferation in response to antiestrogen treatment. Fold differences are shown relative to cell number on Day 1. Cell proliferation values are cell number  $\times 10^3$ . Data represent the combined results of three experiments, with three or more replicates in each experiment. MCF-7 = MCF-7 vector control (transfected with the empty expression vector)

#### **MCF7/XBP-1 cells are less sensitive to the cell cycle arrest effects, and are resistant to the proapoptotic effects, of 4OH-TAM and ICI 182,780**

Since the cell proliferation assay data could reflect changes in cell cycle and/or apoptosis, to better understand the nature of the altered responsiveness to antiestrogens, we evaluated the effects of these antiestrogens on cell cycle progression and apoptosis. Cell cycle analysis was done by Flow Cytometry using standard methods (16); apoptosis was measured using the FITC Annexin V/propidium iodide method as previously described (17). In sensitive cells, antiestrogens induce a G<sub>0</sub>/G<sub>1</sub> cell cycle arrest that is most readily detected as a reduction in the proportion of cells in S-phase. Cell growth arrest is also accompanied by increased cell death occurring through an induction of apoptosis (1,12,18). The data in Fig 7 show that significantly more MCF7/XBP-1 cells remain in S-phase compared with controls. Nonetheless, MCF7/XBP-1 cells still show evidence of growth arrest in the presence of 4OH-TAM and ICI 182,780. In marked contrast, MCF7/XBP-1 cells appear fully resistant to the proapoptotic effects of both ICI 182,780 and 4OH-TAM (Fig 8). These observations have significant implications - cells that are growth arrested may recover and proliferate (*i.e.*, acquire a resistant phenotype - in a patient the tumor could recur). Apoptosis clearly occurs in the parental cells, the extent reflecting the relative potency of the two drugs, and is consistent with the known survival benefit seen in some patients (4,19). Apoptotic cell death is essentially eliminated in MCF7/XBP-1 cells; if this occurred in a patient, the tumor would be expected to slow (some cell growth arrest still occurs) but would likely recur (no cell death occurs and some cells still proliferate).

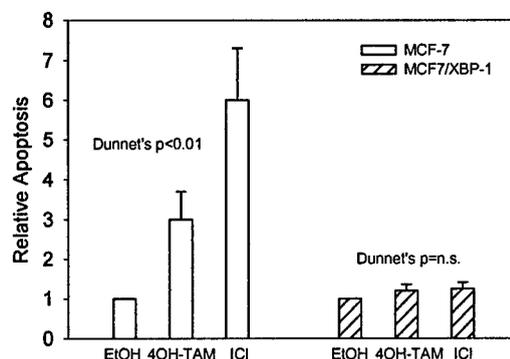
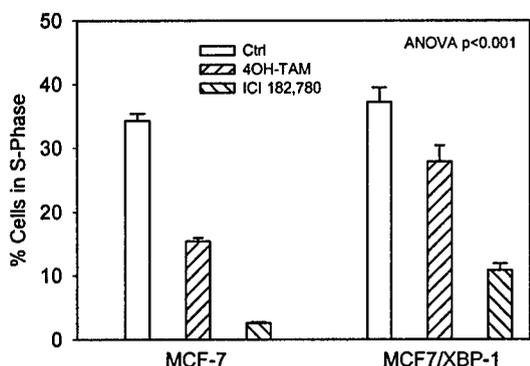


Fig 7: Cell cycle effects of antiestrogens

Fig 8: Proapoptotic effects of antiestrogens

For both Figs 7 and 8, data represent the combined results of three experiments, with  $\geq 3$  replicates/experiment

## TASK 2: Inhibit XBP-1 expression in antiestrogen resistant cells

- Determine ability of CRE decoys to affect CRE activity and response to E2 and antiestrogens *in vitro* (months 13-14)
- Determine ability of antisense oligos to affect XBP-1 expression, CRE activity, and response to E2 and antiestrogens *in vitro* (months 14-16)
- Select active ribozyme from ribozyme library and transfect into MCF7/LCC9 cells (months 17-19)
- Determine ability of ribozyme transfectants to affect XBP-1 expression, CRE activity, and response to E2 and antiestrogens *in vitro* (months 19-21)
- Determine the ability of decoys, antisense or oligos to affect response to E2 and antiestrogens *in vivo* (months 21-24)

We have had some delays in getting these experiments initiated and some technical problems with personnel changes and medical leave. The technical difficulties in obtaining and working with some of the decoys (which we only recently explored) should be resolved in the coming months. We also have now obtained good experience in working with siRNA and will likely choose to use this as an alternative to the ribozyme approach (we will clarify this in our next report). This should also allow us to make up some time, since we will not need to screen the ribozyme library and can more rapidly design and test some siRNA constructs.

## TASK 3: Timing of acquired increase in XBP-1 expression and CRE activation

- Thaw and expand four passages from selection of MCF7/LCC1 to MCF7/LCC9 cells (month 25)
- Study expression of XBP-1 protein by Western (month 26)
- Study CRE activation by promoter-reporter assay (month 27)
- Study XBP-1 expression and CRE activation in other resistance models as appropriate (months 28-32)

We did not propose to start these studies in year 2. However, we have thawed several early passage cultures (those that were generated during LCC9 selection and that exhibited varying levels of drug resistance) and we have begun to recharacterize their resistance phenotypes in preparation for the studies proposed above. This should also enable us to initiate these studies more rapidly in year 3.

#### TASK 4: Explore XBP-1 expression in clinical samples

- a. Complete predictive study (months 24-30)
- b. Complete prognostic study (months 30-36)

We had moved up our initial studies on clinical samples to more rapidly assess whether XBP-1 protein is detectable in breast cancers and to begin exploring its potential as a predictive (improve prediction of endocrine responsiveness) and/or prognostic factor. We first established the optimum design for tissue microarrays and then measured expression of XBP-1 and several other proteins we have implicated in acquired antiestrogen resistance (14). These data were presented last year (the Table is included below merely as a reminder of the data for reviewers).

We have not addressed this task in year 2 beyond working to obtain the key tissue arrays of samples with clinical outcomes data for TAM responsiveness. We now expect to obtain these samples within the next two months and will immediately screen these with the XBP-1 immunohistochemistry methods worked out and used in year 1 (and reported in last year's report). The preliminary data on tissue arrays from last year's report has now been written as a manuscript and will be submitted within the next 2-4 weeks. We hope to include a reprint or preprint in the final report.

**Table 1 (reported last year):** *Correlation of IRF-1, XBP-1, and NFκB expression from tissue microarrays. \*Numbers are p-values. (-) = inverse correlation, (+) = direct correlation. IRF-1c = cytoplasmic staining; IRF-1n = nuclear staining; NS=not significant.*

|        | ERα       | PgR       | IRF-1c    | IRF-1n    | NFκB      |
|--------|-----------|-----------|-----------|-----------|-----------|
| PgR    | 0.001 (+) | -         | -         | -         | -         |
| ErbB2  | NS        | 0.005 (+) | -         | -         | -         |
| IRF-1c | 0.079 (+) | NS        | -         | -         | -         |
| IRF-1n | NS        | 0.014 (+) | 0.088 (-) | -         | -         |
| NFκB   | NS        | NS        | 0.002 (+) | 0.034 (-) | -         |
| XBP-1  | NS        | NS        | 0.001 (+) | 0.082 (-) | 0.018 (+) |

**REPORTABLE OUTCOMES**

We have reported our new preliminary data in two abstracts and have prepared a manuscript for submission.

1. Gomez, B.P., Riggins, R., Zhu, Y., Wart A. & Clarke, R. "Human X-box binding protein in antiestrogen resistance." *Proc 95<sup>th</sup> Am Assoc Cancer Res*; Abstr 1213, 2004.
2. Clarke, R., Riggins, R.B., Bouker, K.B., Nehra, R., Gomez, B. & Zwart, A. "Molecular mechanisms of endocrine resistance in breast cancer." *Anticancer Res*, 24: 3455-3456, 2004.

The manuscript to be shortly submitted is:

Zhu, Y., Singh, B., Hewitt, S., Liu, A., Gomez, B., Wang, A. & **Clarke, R.** "Expression Patterns Among Interferon Regulatory Factor-1 (IRF-1), Human X-Box Binding Protein-1 (hXBP-1), Nuclear Factor Kappa B (NFκB), Nucleophosmin (NPM), Estrogen receptor-alpha (ERα), and Progesterone Receptor Proteins in Breast Cancer Tissue Microarrays." We will include a copy of any accepted manuscript in our next report.

**CONCLUSIONS**

Our data are consistent with a potentially important role for XBP-1 in breast cancer. We have successfully overexpressed XBP-1 in MCF-7 cells, shown (last year) that XBP-1 binds to ERα, and now show its ability to induce a degree of estrogen-independence and antiestrogen resistance. We have previously (last year) optimized the use of tissue microarrays and demonstrated the detectable presence of XBP-1 protein in breast tumors.

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