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14. ABSTRACT The HER-2 receptor tyrosine kinase (erbB-2) and the estrogen receptor (ER) participate in the establishment and progression of breast cancer. While the anti-estrogen, tamoxifen, is used as a therapeutic, resistance is a clinical problem. Overexpression of HER-2 confers resistance to the antiestrogen, tamoxifen. Purpose. The objective was to evaluate the effects of Herstatin, a naturally occurring inhibitor of the HER-2 receptor, on hormonal responsiveness of breast cancer cells. Scope. The effects of Herstatin were tested on MCF-7 and MCF-7/HER-2 breast carcinoma cells by stable transfection or treatment with Herstatin, in the presence or absence of estrogen. Results. There was no effect of Herstatin on hormonal response of MCF-7 cells with low levels of HER-2 in vivo or in vitro. In MCF-7/HER-2 cells, Herstatin enhanced response to estrogen and depressed levels of HER-3. Significance. These studies suggest that Herstatin has no effect on hormonal response of MCF7 cells with low levels of HER-2, but may restore hormonal response to breast cancer cells that overexpress HER-2.					
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INTRODUCTION:	4
BODY:	4
KEY RESEARCH ACCOMPLISHMENTS:	7
REPORTABLE OUTCOMES:	8
CONCLUSIONS:	8
REFERENCES:	8
APPENDICES:	9
SUPPORTING DATA:	9

INTRODUCTION

Subject: The establishment and progression of breast cancer is controlled by receptors for estrogens (ER) and peptide growth factors (1, 2, 3). Several lines of evidence suggest that estrogen responsiveness and resistance to anti-estrogens may be influenced by cross-talk between ER and erbB receptor pathways. Overexpression of HER-2 (erbB2) and signaling triggered by the erbB growth factor, Heregulin (HRG), has been found to confer resistance to the antiestrogen, tamoxifen (4-7). The involvement of erbB receptors has led to suggestions that receptor targeted inhibitors may enhance the therapeutic efficacy of tamoxifen. We recently discovered an alternative HER-2 product called Herstatin, which binds to HER-2 and the EGF receptor (EGFR) and blocks their activation (8-11). Preliminary studies indicate that Herstatin blocks both EGF and HRG signaling in estrogen responsive MCF7 cells (11) and therefore may enhance tamoxifen sensitivity in breast cancer cells. **Purpose:** The objective of this proposal is to thoroughly evaluate the effects of Herstatin on hormonal responsiveness of ER positive breast cancer cells. **Scope:** The proposed research will evaluate the potential therapeutic efficacy of Herstatin combined with tamoxifen in the treatment of breast cancer.

BODY

STATEMENT OF WORK

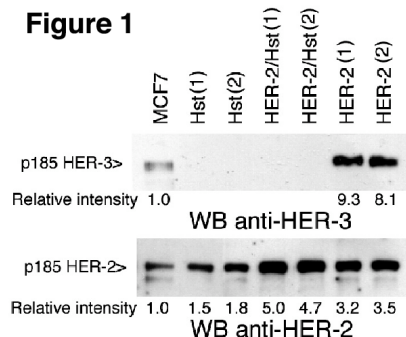
The following outlines the statement of work conducted and the progress made.

Task. Compare the tumorigenic growth of MCF7/HER-2 with MCF7/HER-2/Hst.

This task included establishing the transfected cells lines, characterizing these cells for receptor content and signaling, and examining the growth of the cells lines *in vitro* and *in vivo*.

Two separate clonal cells lines doubly transfected with HER-2 and then Herstatin (MCF7HER-2Hst[1] and MCF7HER-2Hst[2]) were selected and found to have about 3 fold enhanced levels of HER-2 compared to the parental MCF-7

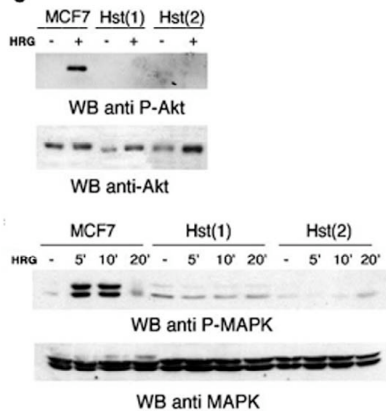
cells demonstrated by Western blot analysis (Figure 1 lower panel [WB anti-HER-2]). Further, in the Herstatin transfected cells, HER-2 overexpression was maintained when Herstatin was overexpressed. An interesting feature of these cells was that the HER-3 receptor was amplified several fold concomitant with HER-2 overexpression (Figure 1 upper panel [WB anti-HER-3]). An important finding of this study was that Herstatin expression eliminated amplification of HER-3 in the context of HER-2 overexpression (Figure 1 lower panel). Moreover, this effect was observed



in two separate clonal cell lines indicating that the effect was not an artifactual property of a clonal isolate.

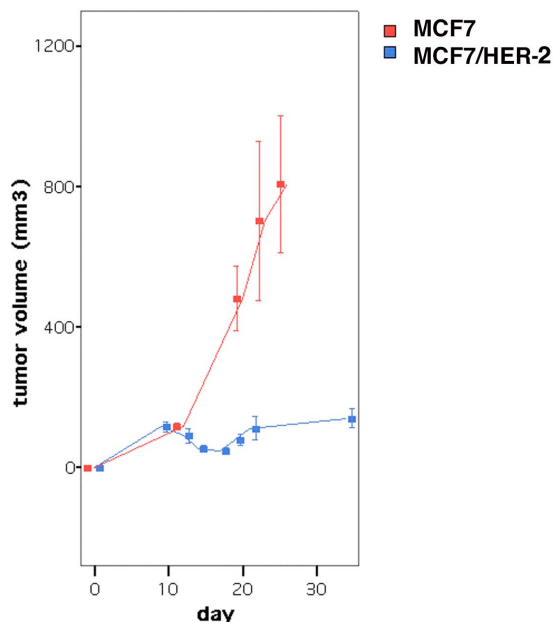
The biological significance of Herstatin-mediated loss of HER-3 expression was shown by loss of Heregulin mediated signaling in the Herstatin expressing cells. MCF7 and the two Hst(1) and Hst(2) transfected cells were treated with 5nM Heregulin and the cells were analyzed for activation of the Akt pathway by blotting with antibodies for the activated form of Akt, P-Akt. In both clonal cell lines, Hst expression blocked HRG activation of the Akt pathway (Figure 2 top panel [WB anti-P-Akt]). Herstatin expression similarly blocked the other major pathway regulated by HRG, the MAP-kinase pathway, demonstrated by loss of HRG stimulated phosphorylated MAPK (Figure 2, lower panel [WB anti P-MAPK]).

Figure 2



The next step in this project was to inject nude mice with the different cell lines to establish xenografts and then to determine whether MCF7/HER-2 cells exhibit tumorigenic growth in the absence of exogenous estrogen. Once tumors formed, the next step planned was to examine growth of xenografts with and without tamoxifen.

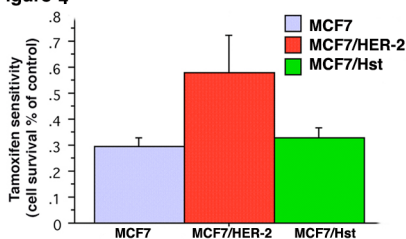
Figure 3



Surprisingly, the MCF-7/HER-2 cells were not

tumorigenic when implanted into nude mice compared to the MCF7 parental cell lines, even in the presence of estrogen. While the MCF7 parental tumors grew as previously reported in numerous studies, the MCF7/HER-2 cells failed to grow for up to 45 days following introduction of tumor cells (Figure 3). Moreover, a second clonal MCF7/HER-2 cell line generated in our laboratory also failed to proliferate as a xenograft. Our findings indicate that while HER-3 is upregulated (Figure 1), and cells become tamoxifen resistant in the HER-2 overexpressing cells (Figure 4), that low levels of expression of HER-2 actually inhibit tumorigenic growth. This result was unexpected. Unpublished information from other laboratories that have

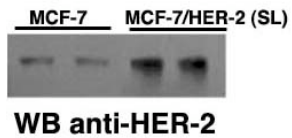
Figure 4



investigated MCF7/HER-2 cells lines suggest that HER-2 overexpression at very high levels is required to generate a tumorigenic model. Next we obtained a MCF7/HER-2(SL) cell line from Dr. Shih Wen Luoh, at VA Medical Center in Portland, Oregon. We characterized these cells for HER-2

receptor levels and found about fivefold higher amounts than in parental cells. Figure five illustrates the HER-2 levels in replicate cell extracts

Figure 5



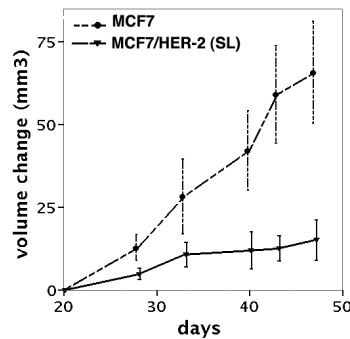
(Figure 5). We also found in the last funding period that this cell line, with elevated HER-2, did not exhibit the upregulation of HER-3 that we found in the HER-2 transfected cell lines we isolated (Figure 6). Moreover, *in vivo* growth of the MCF7/HER-2(SL) in xenograft models was significantly reduced relative to

Figure 6



the parental MCF7 cells (Figure 7). Repeated measures ANOVA of volume change indicated a significantly different growth rate ($P=0.021$). This is interesting in the context that there are few tumorigenic breast cancer cell lines that overexpress HER-2. For example the BT474 and SKBR3 breast cancer cells form tumors very inefficiently in nude mice (Clinton, unpublished observations). Moreover, this study illustrates the variability in HER receptor content found in HER-2 transfected cell lines, and the differential effect of HER-2 overexpression on HER-3 levels within the context of MCF7 cells.

Figure 7



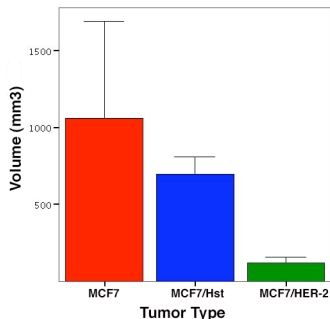
Task. Examine the tumorigenic growth of MCF7 and MCF7/Hst with and without tamoxifen.

The purpose of this task was to determine whether Herstatin confers tamoxifen sensitivity to breast cancer cells that have low levels of HER-2. We have previously shown that Herstatin blocks HRG signaling (Figure 2) and HRG-mediated growth in MCF7 cells. Our more recent studies also demonstrate that Herstatin expression blocks EGF signaling in these cells.

Therefore, it was possible that Herstatin may enhance sensitivity of ER positive breast cancers regardless of overexpression of HER-2. We examined this possibility *in vitro* in cultured cells. While HER-2 overexpression conferred tamoxifen sensitivity, there was no significant effect of Herstatin expression on tamoxifen sensitivity in MCF7 cells (Figure 4). Therefore, in the context of breast cancer cells that do not overexpress HER-2, Herstatin did not further enhance tamoxifen sensitivity even though it blocked signaling of multiple members of the EGFR family.

Figure 8

Error bars show mean and S.D.



We next examined the tumorigenic growth of MCF7/Hst versus MCF7 cells. The MCF7 and MCF7/Hst tumors were not significantly different in size ($P=0.45$), whereas both MCF7 and MCF7/Hst were significantly larger than the MCF7/HER-2 ($P=0.02$ and $P<0.001$ respectively) (Figure 8). These findings show that Herstatin expression did not significantly inhibit the tumorigenic growth of the MCF7 cells. Although we found that Herstatin inhibited

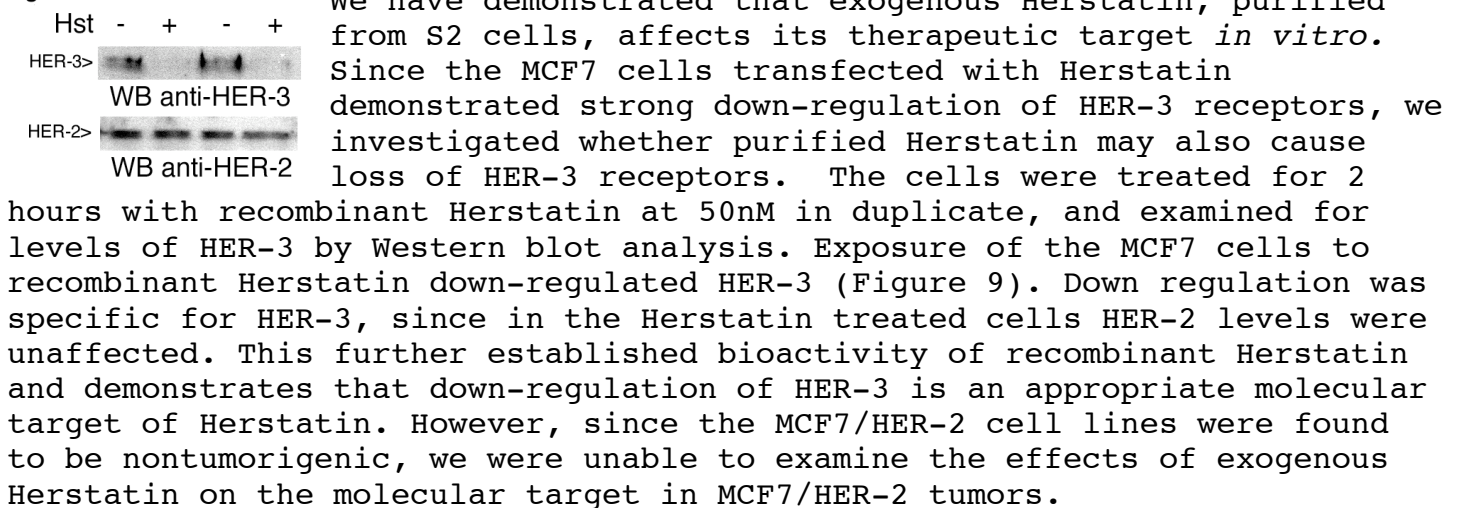
HRG mediated growth of these cells, there is no significant *in vitro* effect of Herstatin on growth of MCF7 cells cultured in low levels of serum (data not shown).

Task. Examine levels of Herstatin secreted from xenografts of MCF7/Hst and effects on therapeutic target in xenografts of MCF7 cells.

We investigated whether Herstatin could be detected in the serum of MCF7/Hst tumor bearing mice. We used a Herstatin ELISA kit available from Upstate Biologicals. This kit detects Herstatin in serum in the range of 4-5 ng/ml. At this level of sensitivity, we were unable to detect Herstatin in the blood of animals bearing tumors of about 700 mm³. This finding suggests that insufficient levels are secreted to examine effects of Herstatin, secreted from the tumor, on a second tumor implanted into the nude mouse.

Task. Examine effects of purified Herstatin on therapeutic target, tyrosine phosphorylated HER-2 and HER-3 in MCF7-HER-2 cells.

Figure 9



KEY RESEARCH ACCOMPLISHMENTS

- Establish and characterize several clonal cell lines of MCF7 cells that overexpress HER-2, Herstatin and HER-2 plus Herstatin.
- Establish that Herstatin inhibition of HRG signaling does not enhance tamoxifen sensitivity unless HER-2 is overexpressed.
- Develop protocols for Herstatin purification and bioactivity measurements.
- Determined that recombinant Herstatin, to be used in tumor models, was effective against HRG stimulated growth of MCF7 cells and that it was effective in down-modulation of the molecular target, HER-3.
- Found that Herstatin conferred tamoxifen sensitivity to HER-2 overexpression, but not to parental MCF-7 cells.

- Determined that Herstatin expression in MCF7 cells does not affect the tumorigenic growth of this cell line.
- Determine that three separate MCF7/HER-2 cell lines ranging from 3-5 fold overexpression of HER-2 are not tumorigenic in estrogen-treated nude mice, even though HRG and EGF-specific signaling are blocked.
- Determine that a HER-2 overexpressing breast cancer cell line, BT474 is sensitive to Herstatin *in vitro*.

CONCLUSIONS

In the final report, we conclude that Herstatin may not be effective as a therapeutic when combined with tamoxifen against ER positive breast cancer cells **that do not overexpress HER-2**. Importantly, Herstatin disrupted key signaling EGF receptor family signaling pathways, inhibited growth, and conferred sensitivity to tamoxifen in HER-2 overexpressing MCF-7 cells. These findings suggest that Herstatin may be effective in enhancing sensitivity of HER-2 overexpressing breast cancer cells to the anti-estrogen, tamoxifen.

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APPENDICES

None

SUPPORTING DATA

None