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13. ABSTRACT *(Maximum 200 words)*

We have created a series of breast cancer cell lines, transfected with green fluorescent protein (GFP) that can be use to examine the effects of anti-estrogens on the invasive potential of breast cancer cells that survive anti-estrogen therapy. We have tested 20 of these cell lines to establish that they have retained their sensitivity to tumor necrosis factor-α (TNFα) and to several anti-estrogens (tamoxifen, 4-hydroxytamoxifen and ZM 182,780). Most, but not all of the cell lines induce apoptosis after treatment with anti-estrogens. After 72 h of treatment 60-70% of the transfected cells die by apoptosis, while 40% of the cells survive. During the apoptotic process the cells induce the transcription of several extracellular matrix proteases (particularly cathepsin B and MMP-9) and down regulate the expression of at least one matrix metalloprotease inhibitor (TIMP-1). These data provide support for our hypothesis that while anti-estrogen therapy induces cell death in the majoroty of hormone responsive cells, a same population of cells may survive the treatment but may induce the enzymes required for invasion. If further experimentation demonstrates directly that there is an increase in the invasive potential of the surviving cells it will suggest anti-estrogen therapy for early stage disease or as a chemo-preventive strategy is contra-indicated.

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Mark Ironswood 10/20/98

PI - Signature Date

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Annual Report for Grant Number DAMD 17-97-7298

Title: Apoptosis and Tumor Invasion in Breast Cancer

The research outlined in this application is designed to test the hypothesis that the acquisition of the invasive phenotype may result if apoptosis is initiated but endonuclease activation is abrogated.

In the original application there were two specific aims:

Specific Aim 1. *To characterize the ability of anti-estrogens to induce apoptosis and an invasive phenotype in a sub-population of cells in vitro.*

Specific Aim 2. *To compare thesensitivity of MCF-7$^{ae}_{inv}$ sublines to other agents that induce apoptosis and determine if multiple agents can act synergistically to induce apoptosis.*

Prior to the initiation of the research we were asked to describe how we would pursue this work in vivo as part of a follow up study. At that time we added specific aim 3 to the research program, with the understanding that this specific aim was outside the scope of the two funding period.

Specific Aim 3: *To determine whether treatment with anti-estrogens or other apoptosis inducing reagents (such as vitamin D analogs) alters the rate and/or sites of metastasis and whether invasive cells generated by treatment with one reagent are sensitive to treatment with other reagents that induce cell death in vivo.*

While specific aim 3 was clearly not part of the original application, our proposal to use MCF-7 cells transfected with GFP (green fluorescent protein) to monitor invasive cells both *in vitro* and *in vivo* suggested to us that we would be well advised to create these MCF-7^{GFP} sublines before proceeding to examine the effects of anti-estrogens on these breast cancer cell lines to ensure that the transfected cells retained their normal estrogen dependence and their responsiveness to anti-estrogens.

We have therefore modified our original experimental timetable to include an additional preliminary task: the development of estrogen sensitive clonal cell lines expressing enhanced Green Fluorescence Protein (GFP). These cell lines have been developed by transfecting estrogen dependent MCF-7 cell with pEGFP-c1, a plasmid containing the enhanced green fluorescent protein under the control of the constitutive CMV promoter, and an ampicillin selectable marker. These cells were cultured for 7 days, and then sorted on a Becton-Dickinson fluorescence activated cell sorter into 96-well culture plates using the autosort facility of the machine. The cells in individual wells were clonally were expanded to produce a series of high, medium and low GFP-expressing

[This page contains unpublished data]

sublines by growth in G418. A total of 20 sublines have been stabilized, characterized and frozen in liquid nitrogen for further use. Phase contract and fluorescence photomicrographs of one of these sublines is shown in Figure 1, demonstrating that virtually all of the cells within the selected subline express GFP at approximately the same level. [A color version of Fig 1 is provided in appendix 1.]

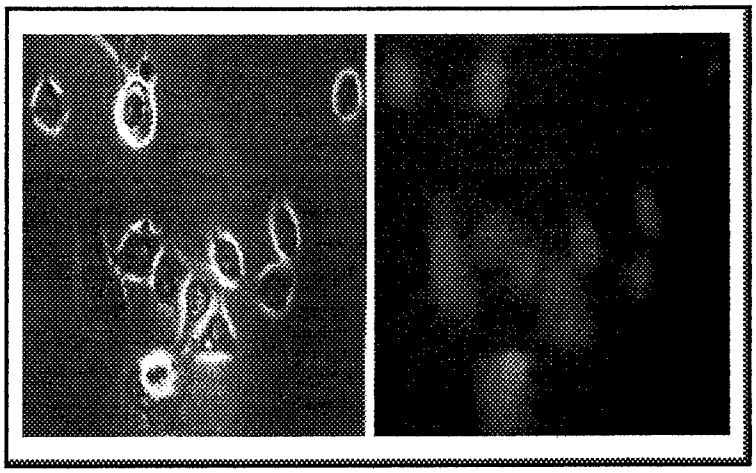


Figure 1: Phase contrast (left panel) and fluorescence microscopy (right panel) of MCF-7 cells transfected with a plasmid expressing green fluorescent protein (GFP). MCF-7^{GFP}cells were grown for 72 h in serum free defined medium prior to photography.

In our initial experiments we have compared the sensitivity of these sublines to TNF-α with that of the parental MCF-7 wild type cells (MCF-7^{WT}). Without exception, the MCF-7^{GFP}, retain their sensitivity to TNF-α and demonstrate a very similar dose response to the drug as the parental wild type MCF-7 cells, suggesting that the expression of GFP in these cells does not influence the ability of the transfected cells to undergo apoptosis after the appropriate stimulation (Fig. 2).

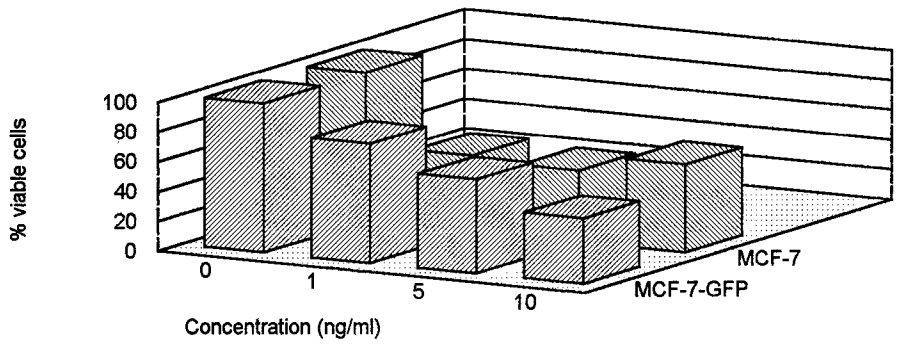


Figure 2: Comparison of sensitivity of MCF-7^{GFP} and parental MCF-7^{WT} to increasing doses of TNF- α. The two cell lines were grown for 4 days and then treated with increasing doses of TNF-α. After 72 h the number of surviving cells was estimated using crystal violet, and compared to untreated controls. Results are the mean of three independent experiments.

[This page contains unpublished data]

Most, but not all of the transfected sublines have also retained their sensitivity to anti-estrogens and induce cell death (as monitored by DNA fragmentation (TUNEL positivity) and cell viability as monitored by crystal violet or flow cytometry. The relative sensitivity of the subline shown in Fig 1 and 2, to ZM182,780, tamoxifen and 4-hydroxytamoxifen is shown in Fig 3. The results demonstrate that this particular subline retains its sensitivity to all three anti-estrogens, although unlike the parental MCF-7 cell line appears to be sensitive to the pure anti-estrogen ZM 182,780 only at higher concentrations (> 1 μM), even though the overall rate of cell death appears to be of the same order of magnitude (approximately 40% of the cells appear to survive anti-estrogen therapy for 72h). Most of the sublines display similar sensitivities to the individual anti-estrogens although we have not yet determined whether the (minor) variations we see are reproducible or biologically significant.

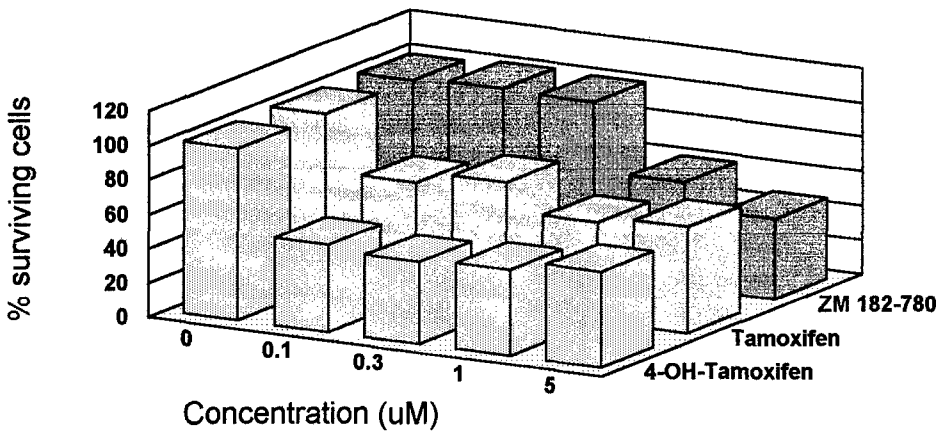


Figure 3: Dose response of MCF-7 ^{GFP} cells to ZM182,780, tamoxifen or 4-hydroxytamoxifen. MCF-7^{GFP} cells were incubated with each of the indicated anti-estrogens for 72h. The cells were harvested and the cell viability was assessed using crystal violet and compared to untreated cells incubated for the same length of time. Results are average of two independent experiments.

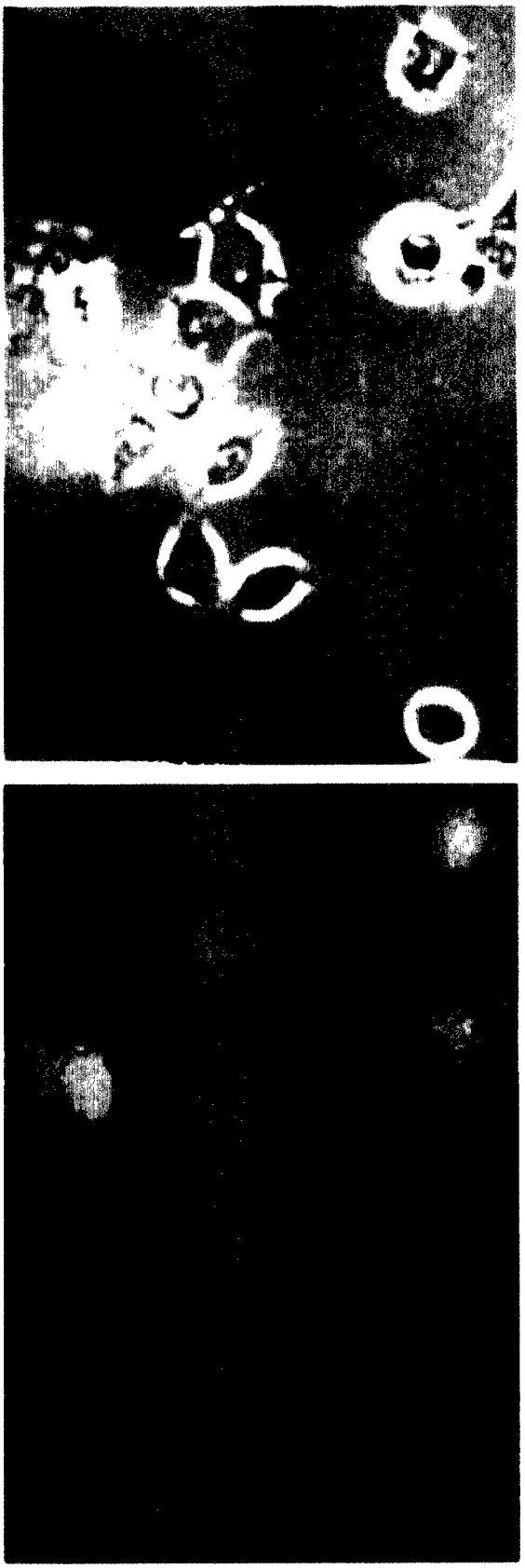
Our preliminary data, based on RT-PCR, also demonstrate that anti-estrogen (ZM 182,780) treatment of these sublines induces the synthesis of several extracellular proteases (notably cathepsin B) while down-regulating the synthesis of at least one inhibitor of the matrix metalloproteases (TIMP-1). While these data clearly offer support to our hypothesis, they need to be repeated under rigorous experimental conditions to confirm this observation and to determine which other extracellular proteases (and acid hydrolases) are induced after treatment with anti-estrogens and which inhibitors of matrix metalloproteases and cathepsins are simultaneously down regulated.

The transfer of our laboratory from the Adirondack Biomedical Research Institute (formerly the W. Alton Jones Cell Science Center) in Lake Placid to the Department of

Biological Sciences at the University of Notre Dame has resulted in a short hiatus . However the new laboratory is now operational, and the MCF-7^{GFP} cells have been resurrected and are now are being grown up to ensure the rapid continuation of the research. We do not anticipate any major problems, and we expect to complete the first two specific aims on schedule.

Our next goal is to confirm that a small population of cells that survive anti-estrogen therapy induce the proteases and acid hydrolases required for the acquisition of the invasive phenotype. We will measure the ability of the MCF-7^{GFP} cells to invade through Matrigel in a modified Boyden chamber assay, by monitoring their intrinsic fluorescence as they pass through the Matrigel and membrane of the chamber before and after treatment with anti-estrogens. We will then establish whether or not other modalities that induce cell death (TNFα and vitamin D analogs) also induce the same phenotypic and epi-genetic changes as seen with the anti-estrogens.

We are also laying the ground work for the third specific aim, since we have initiated an *in vivo* protocol designed to demonstrate that MCF-7^{GFP} cells, when grown as xenografts in the mammary fat pads of nude mice, grow at the same rate as wild type cells.



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