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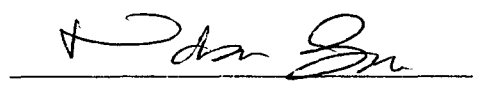

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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this research was to investigate the specific mechanisms by which transcriptional pathways may become more responsive to retinoids in cells that express the estrogen receptor (ER). To determine which region of ER is required for retinoid sensitivity, several deletion mutants of ER were subcloned into a retroviral vector and stable ER-deletion mutant human breast cancer cell lines were derived. Studies with these stable cell lines indicate that the N-terminal region of ER is required to potentiate the retinoid response. Like wild-type ER positive cells, C-terminal deletion mutants are growth inhibited by retinoids and give a greater than 100 fold induction on a RARE compared to the retroviral control. These results indicate that the restored response to RA is mediated by the N-terminal of ER. Blocking the AF1 domain with ICI diminishes the effect of ER on transcription from a RARE. Activation of AF1 may play an important role in the cross-talk between ER and RAR pathways. We are currently investigating the phosphorylation state of ER in the N-terminal and using kinase inhibitors in combination with RA to determine if these two agents could synergize to inhibit breast cancer cell growth.

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INTRODUCTION

This project explores the interaction between the expression of estrogen receptor in human breast cancer cells and their response to retinoids. It is well documented that estrogen receptor augments the growth inhibition caused by retinoids. We are studying why estrogen receptor positive cells respond to retinoids and, more generally, how related steroid hormones may act together to promote or inhibit cell growth. The proposed research may illuminate mechanisms by which steroid hormone receptors interact to control transcription, as well as suggest directions for the use of retinoids in the treatment of breast cancer.

BODY

The objective of this award was to study the mechanism by which growth of human breast cancer cells can be inhibited by retinoids. Retinoids are derivatives of Vitamin A that are of increasing interest in the treatment of cancer patients. Retinoic acid (RA) acts by binding to a family of nuclear receptors (RAR/RXR) that in turn bind as heterodimers to response elements (RAREs) in the promoters of various genes to activate transcription. Transcription from retinoid response elements can be modulated by transcriptional intermediates that can directly stimulate or inhibit transcription in a ligand-dependent fashion. For example, in the absence of RA, SMRT and N-CoR proteins interact directly with RAR and RXR in an inhibitory manner to prevent transcription from the RAREs. Conversely, AIB1, SUG1, TIF2 and CBP are stimulatory intermediates that increase transcription in the presence of RA.

In general, ER+ breast cancer (ERα-positive) cells are growth inhibited by retinoids, whereas ER- cells are resistant. Prior studies in this laboratory have indicated that the retinoid response observed in ER+ cells is independent of its ligand, estrogen, thereby suggesting an original molecular mechanism for its interaction with the retinoid response. To understand the role of the estrogen receptor, we first wished to establish comparable cell lines. For this purpose, we compared MDA-231, an ER-negative breast cancer cell line, with S30 in which ER was stably transfected with the estrogen receptor. Any differences in the retinoid response when comparing the ER-positive (S30) and the parental ER-negative (MDA-231) cell lines will therefore be due to estrogen receptor only.

The role of the estrogen receptor in RA response is further complicated by the recent discovery of another estrogen receptor, ERβ. ERβ can heterodimerize with ERα, interact with the same ligands and shares greater than 90% homology with the DNA-binding domain of ERα. The level of ERβ in the ER-negative and ER-positive cells lines was assessed by RNAse protection assay and correlate well with the literature. ERβ levels were found to be slightly higher in the ERα-negative cells MDA-MB-231 and its ERα transfected stable cell lines, while it was barely detectable in the ERα-positive cells MCF-7.

Task #1 addressed the RA-induced transcriptional activation in ER+ and ER- cell lines. RA binds RAR and forms a heterodimer with RXR on the promoter of certain genes containing retinoic acid response elements (RARE). One such element, βRARE, contains a DR5 (sequence AGTTCAn_5GTTCA) located in the promoter of the RARβ gene. We have tested this reporter, as

well as various other reporter plasmids driven by direct repeats (DR) and inverted repeats (palindromes). While the presence of the estrogen receptor does not appear to affect transcription from promoters containing inverted repeats, the activity from promoters containing direct repeats are markedly affected. Cells that express ER exhibit an increase in fold induction from the RARE (DR5) as compared to ER-negative cells. This transcriptional effect is observed for a minimal promoter containing the βRARE linked to TK-CAT, and also for the 5kb natural promoter. We observed that the fold induction from this promoter was greater than 100 times higher for ER-positive cell lines as compared to the ER-negative lines (FIG 1). However, the increase in fold activation was not caused by an increase in activity in the presence of RA, but rather by a significant decrease in baseline activity from the promoter in the absence of any ligand. This indicates that ER modifies the transcriptional response from RAREs in absence of RA and may be a mechanism by which ER renders the breast cancer cell more sensitive to the growth inhibitory effects of RA.

To confirm that ER alters the retinoid response and is not an artifact of other regulatory sites contained in the TK part of the reporter construct, transfections were also performed using a TK-CAT plasmid. The presence of ER, and addition of RA do not alter the transcription activation from the TK-CAT construct, thereby confirming that the decrease in baseline activation seen on the βRARE is specific for this promoter. To evaluate the possible effect of ER on other promoters containing direct repeats, we used both VDR and TR response elements. ER does not alter the transcriptional response from these promoters containing DR3 and DR4 respectively. The effect of ER therefore appears to be specific for DR5s, or RARE-containing promoters.

To confirm an in-vivo role for ER on RARE containing promoters, we evaluated the expression of RARβ by RNAse protection assay. As stated above, the RARβ gene contains a RARE in its promoter and is activated by retinoids. In ER-positive cells, the baseline expression of this gene (in absence of RA) was more than 10 fold decreased when compared to the expression in ER-negative cells (FIG 2). This data confirms that observed in the transcriptional assays. Therefore, the effect of ER is not to alter the transcription in the presence of RA, but rather to decrease the baseline expression in absence of ligand.

ER typically recognizes inverted repeats with a sequence containing AGGTCA. This sequence is similar to that of the RAREs that have a half site sequence of AGTTCA. We hypothesized that ER may bind to a part of the DR5 promoter, thereby blocking the RAR/RXR proteins until a ligand is bound to RAR. To test this hypothesis, we performed DNA binding experiments using the DR5 as bait. We found, using various binding conditions, that ER does not alter the binding of RAR or RXR to the response element and that ER does not bind directly to the DR5. The role of ER therefore does not appear to be on the binding of the nuclear receptors to their response elements.

Once bound to their response elements, nuclear receptors recruit coactivators or corepressors. The effect of ER on these transcriptional intermediates was explored in Aim #2. Northern or Western blots were performed to determine the level of expression of the inhibitory and stimulatory transcriptional intermediates. Northern blots or Western blots of the coactivators AIB1, TIF2, SRC1 and CBP, as well as the corepressor SMRT did not indicate any significant difference in expression for ER-positive versus ER-negative cells.

To address Task 2a, or the hypothesis that ER may compete with cofactors required by RAR/RXR, we transiently transfected increasing amounts of cofactor and observed the effects on transcription from a βRARE in absence of RA. We noticed that although coactivators increased transcription and corepressors repressed baseline activity, the effect was similar for ER-positive versus ER-negative cell lines. There is a possibility that ER binds novel cofactors and to address this possibility, we have generated GST-RAR to identify protein bands that are different in ER-positive and ER-negative cells. This research is currently ongoing.

In Aim #3, we wished to contrast the expression levels of the orphan receptors Nur77 and Coup-TF that can bind to RAREs and alter baseline transcription. While these 2 proteins were found to alter the effect of RA in human lung cancer cells, we did not notice any difference in expression levels of these proteins in our ER-positive and ER-negative breast cancer cell lines.

My research has focused mainly on identifying the functional domain of ER required to confer retinoid sensitivity (Aim #4). For this purpose, ER- cell lines (MDA-MB-231) were stably transfected with estrogen receptor or deletion mutants. The ER wild-type and mutants were subcloned into a retroviral vector containing a packaging site, multiple cloning site, internal ribosomal entry site (IRES) and enhanced green fluorescent protein (GFP) located between two long terminal repeats (LTS). Following the subcloning step, viral producer cells were transfected with the ER-containing vector. Positively transfected cells (green fluorescent cells) were cloned and expanded to maximize the percentage of viral producers. When induced to produce virus, the stably transfected cells shed virus containing ER-GFP RNA, reverse transcriptase, integrase and protease. Media containing the virus was collected, concentrated and used to infect MDA-231 target cells. The RNA, converted to DNA by reverse transcriptase, gets integrated at random sites within the genome and a mixed population of ER (or deletion mutant)-positive green fluorescing cells is generated. The GFP does not have any deleterious effect on cellular function and provides and easy mechanism for detection of infected cells.

My first goal was to establish an ERα (wild-type)-positive stable cell line. The presence of ER was confirmed by Northern blot but ER was undetectable by Western analysis using a C-terminal antibody. However, ER was detected by immunohistochemistry using a N-terminal antibody. Upon sequencing of the ER, this stable cell lines was found to contain a base pair deletion, leading to a frameshift and a STOP codon at amino acid 346. The first stable cell line to be derived was thus labelled MDA-231/ER_{345}. The ER_{345} stably transfected cells survive both in stripped and full calf serum. This is in contrast to many reports in the literature which state that ER(wild-type)-reconstituted cells are growth inhibited by full calf serum and must be grown in stripped serum only. The ability of the 231/ER_{345} cell line to grow in serum may be a result of its inability to bind ligand.

The growth rates of ER_{345}-transduced cells and the control empty-vector transduced ER-negative cells were studied to determine if the reconstitution of the ER deletion mutant restored RA sensitivity. Results from a growth curve comparing the two cell lines indicate that the ER_{345}-transduced cells were growth-inhibited by retinoids, whereas the parental cells were not. Since the only variation between these cell lines is the presence of ER deletion mutant, this clearly indicates a role for the estrogen receptor in RA-mediated growth inhibition and retinoid pathways.

Using the retroviral technology described above, I successfully generated breast cancer cell lines stably containing various ER-deletions (FIG 3). However, the difficulties of generating a full length ER stable cell line has been well documented in the literature (1-3) and indeed, I was unable to generate such a cell line. As such, I used the S30 cell line (an ER-positive clone derived by C. Jordan from the ER-negative cell line MDA-MB-231) as a positive control for full length ER.

When the transcriptional response to RA was observed using a βRARE reporter construct, significant differences were observed for the various ER mutant cell lines (FIG 4). We noted that the ER-ligand binding domain was not necessary for the transcriptional effect. This is consistent with our findings that ER ligands do not modify the RA response. Furthermore, MDA-MB-231 cells stably transfected with N-terminal deletion mutants exhibited the same transactivation profile as the mock-transfected cells. However, the fold induction on the βRARE reporter was greatly increased (>100 fold) when full length or C-terminal deletion mutants were transfected, thereby indicating that cross-talk between ER and RARs occur via the N-terminal domain. These results suggest a novel interaction between the N-terminal domain of steroid hormone receptors and nuclear receptors that may prove important when targeting specific nuclear receptor pathways. The generation of these cells lines therefore provides a valuable tool for understanding the role of ER in the RA-mediated response.

The ER C-deletion mutant stable cells lacking domain E (ER345 and HE15) respond to retinoids as well as the ER-positive cells. Studies with the ER C-terminal deletion mutant cell line imply that the C-terminal is not required for potentiating the RA response and suggest that the effect of ER on growth inhibition and transcription is in part due to the N-terminal. The N-terminal of ER contains the activation function 1 (AF1) domain that can interact with co-regulatory molecules. We postulated that this domain may be involved in potentiating the retinoid response in ER-positive breast cancer cells by sequestering co-regulatory molecules required for basal activation of a RARE. AF1 is an important region of the N-terminal of the ER whose activity is constitutive, ligand-independent, and mediates the activity of growth factors and MAP-kinase pathways (FIG 5). Unlike tamoxifen, which blocks only the activation function 2 (AF2) of the estrogen receptor, the anti-estrogen ICI blocks both the AF1 located in the A/B domain, and AF2, located in the E domain.

Using these ER ligands to monitor βRARE transcriptional activity, we determined that the AF1 domain is indeed important in the cross-talk between ER and RAR pathways (FIG 6). Both ICI and Tamoxifen bind to the C terminal of ER and thus, as expected, had no effect on the HE15 and ER345 mutants. However, in the ER-positive MCF7 cells, blocking the AF1 domain with ICI inhibited ER-mediated transcriptional inhibition and restored transcriptional activity to the same level as that observed in the ER-negative cell lines. It is not surprising that this effect was not observed for ERβ stable cell line since the AF1 activity of ERβ has been reported to be weaker than that of ERα. Furthermore, tamoxifen has been shown to block both the AF1 and AF2 domain of ERβ (4)

There are many examples in the literature where growth factor phrophorylation cascades lead to potentiation of cancer cells. For example, excessive activity of EGFR, a membrane tyrosine kinase receptor, leads to increased phosphorylation and enhanced proliferation (5). Activation of these pathways occurs via a signaling cascade which are dependent on the Shc/Grb2/Ras pathway, as

well as PI3K and ERKs. These mitogenic pathways can lead to increased phosphorylation of the estrogen receptor, particularly in the N-terminal region (6). To determine if the AF1 domain of ER must be phosphorylated in order to activate the cross-talk between ER and RAR, we are currently generating more targeted point mutations in the N-terminal of ER. Furthermore, we are focusing on inhibiting the above mitogenic pathways via specific kinase inhibitors such as PD98059 (MAPK inhibitor) and SB203580 (p38 Kinase inhibitor). Our aim is to observe the proliferation and transcriptional activity of ER-positive and ER-negative cells in the presence of RA and the kinase inhibitors to determine if they would synergize with RA in inhibiting breast cancer cell proliferation.

Given that RA can inhibit the growth of ER-positive breast cancer cells, we wished to determine the genes induced by RA treatment. Activating these genes in breast cancer cells defective in RA-regulated pathways could then prove to be an important means of growth suppression. Although not mentioned as a specific aim, finding downstream target of RA was an integral part of this research project. To identify the genes involved in the growth suppression effects of RA, we used the Clonetech Cancer Array. We probed the array using the ER-positive stable cell line S30. (FIG 7) We discovered a series of genes that are activated by RA and are currently testing the activation of these genes in ER-negative cell lines. This may give us an insight into which genes are required for RA-induced growth inhibition and these genes could be activated by other means in order to regulate cell proliferation.

KEY RESEARCH ACCOMPLISHMENTS

- Generated stably-transduced cell lines from the ER-negative parental line MDA-231 by infection with virus containing ER-wild-type, ER-deletion mutants and empty retroviral vector.

- Determined the ERβ expression by RNAse protection assay in ER-negative and ER-positive cell lines.

- Established that the expression of transcriptional intermediates is similar in ER-positive and ER-negative cell lines and that ER does not appear to sequester any of the known cofactors common to both the RAR and ER pathways.

1. Determined that the N-terminal region of ER, and not the C-terminal (or ligand-binding domain) alters the transcriptional activation of a βRARE. Other promoters that utilize RXR as a partner (ex. VDR and TR) are unaffected by the presence of ER.

2. Discovered 3 genes important for RA-induced growth inhibition.

REPORTABLE OUTCOMES

- Cell lines

  Stably-transduced cells derived from the ER-negative parental line MDA-231 were generated by infection with virus containing ER-wild-type, ER-deletion mutants and empty retroviral vector.

1. Presentations/Abstracts

Mini-Symposium	**The American Association for Cancer Research 92nd Annual Meeting**, March 24-28, 2001, New Orleans, Abstract #1449 "The N-terminal Region of the Estrogen Receptor Alpha (ER) Mediates Enhancement of the Transcriptional Response to Retinoic Acid in Human Breast Cancer Cells"
Poster Presentations	**The 3rd Annual Medical Science Conference between McGill University and University of Toronto**, Ste-Lucie-des-Laurentides, PQ November 5-7, 1999 "Novel Interactions Between the Estrogen and Retinoic Acid Receptor in Human Breast Cancer Cells."
	DOD Era of Hope, Atlanta, Georgia

June 8-11, 2000
"Estrogen Receptor Alters the Sensitivity to Retinoic Acid in Human Breast Cancer Cells."

2. Awards

2001-2002 - Fonds FCAR – Fonds pour la formation de chercheurs et l'aide a la recherche. Studentship from the Government of Quebec. $20 000/yr

2001 – McGill Alma Mater Student Travel Grant - $250 for travel to AACR conference in New Orleans.

2001 - AACR-Aventis Scholar in Training Travel Award to present my research at AACR.

TRAINING EXPERIENCE

The following courses were completed as required by the Department of Medicine at McGill University.

516-604D – Recent Advances in Cellular and Molecular Biology
516-511B – Joint Venturing with Industry
516-608A – Molecular and Cellular Biology Topics
516-616A – Molecular Embryology
516-602B – Advanced Techniques in Molecular Genetics
516-701D – Comprehensive Examination

During the course of this grant, my work was reviewed yearly by my Thesis Committee (4 faculty members of the Department of Medicine and 1 external member) who helped to suggest ideas and approved the scientific development of my thesis project. In May 1999, I passed my Comprehensive Examination and in May 2000, completed the required oral presentation to the Department of Medicine. At my last committee meeting, in May 2001, it was suggested that I organize my experiments such that I can start writing my thesis in the summer of 2002.

During the 3 years that this grant was awarded, in addition to presenting my research to the post-docs, technicians, and other graduate students in my lab, I presented at various department meetings and yearly student seminars in the Department of Medicine at McGill University, in the Molecular Oncology group at the Lady Davis Institute, and at joint lab meetings with Dr. Sylvie Mader at the Universite de Montreal. This has developed my ability to effectively present my results to diverse audience.

CONCLUSION

Cross-talk between nuclear receptors is a topic of increasing interest in the field of nuclear receptors. Normally, this cross-talk occurs via the C-terminal of the receptor and involves

recruitment of cofactors (7-11). The fact that we found this cross-talk to occur in the N-terminal is novel and potentially very interesting. The N-terminal region of receptors requires no ligand and been reported to be modulated by phosphorylation alone (6,12). Given that phosphorylation is important to cellular proliferation and that many cancer cells have kinase activity, then one can postulate that phosphorylation in the N-terminal region of receptors may be important in the cross-talk between the ER and RAR receptors.

The mechanism of action of retinoids and traditional ER inhibitors such as tamoxifen are already known to be different and thus potentially additive or synergistic. Clinical trials are currently underway to evaluate the effectiveness of retinoids and combination retinoid/tamoxifen in vivo. (13-15). Our current results and the future work leading from this proposal will aid in understanding the signaling pathways between ER and retinoids and may help in providing a strategic approach to the development of therapies for the treatment or prevention of breast cancer.

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APPENDIX

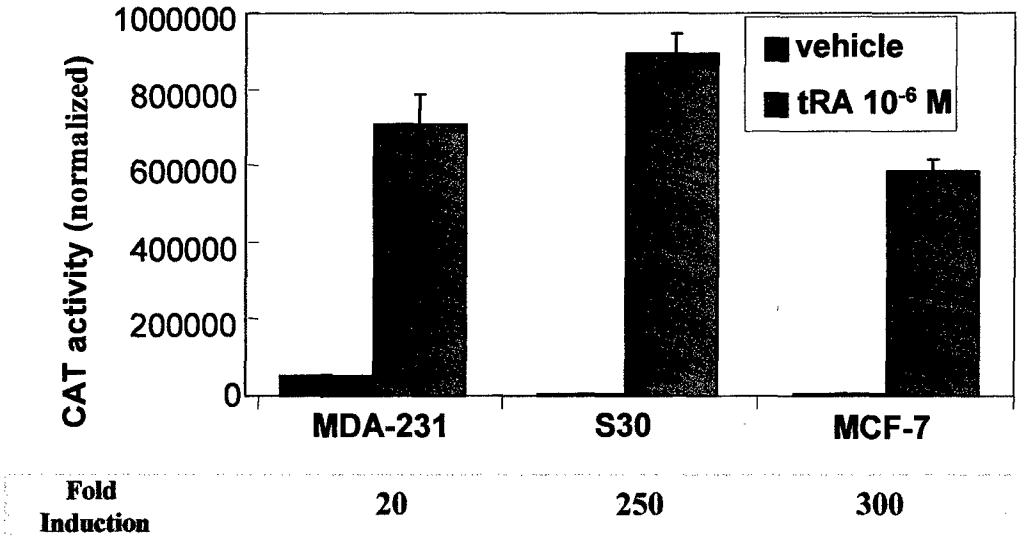


FIG1: Transcriptional activity on the βRE-TK-CAT reporter contruct in the ER-negative (MDA-231) and ER-positive (S30 and MCF7) cells.

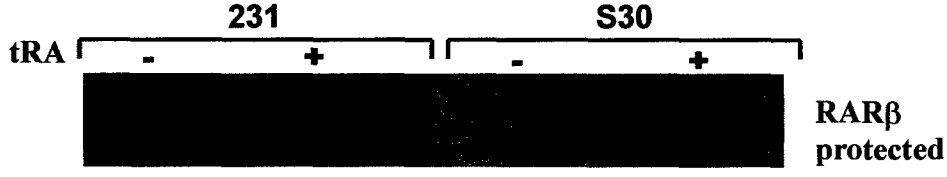


FIG 2: RARβ gene expression as shown by RNAse Protection Assay

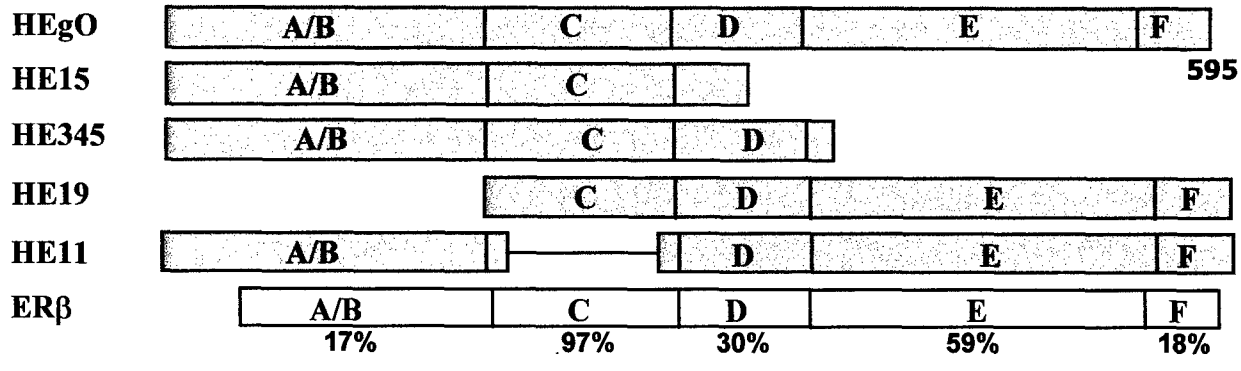


FIG 3: ER-deletion mutants used to generate stable transfectants

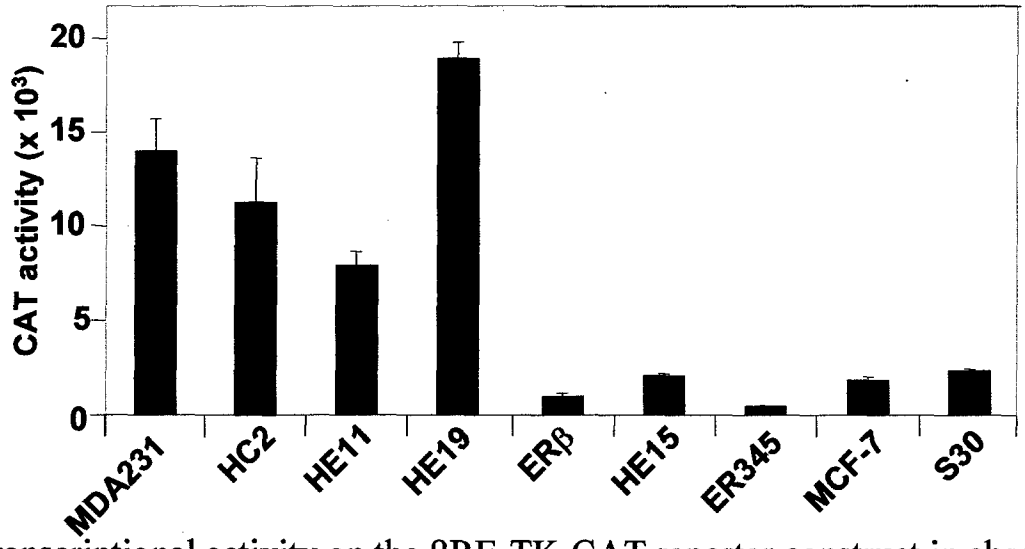


FIG 4: Transcriptional activity on the βRE-TK-CAT reporter construct in absence of RA.

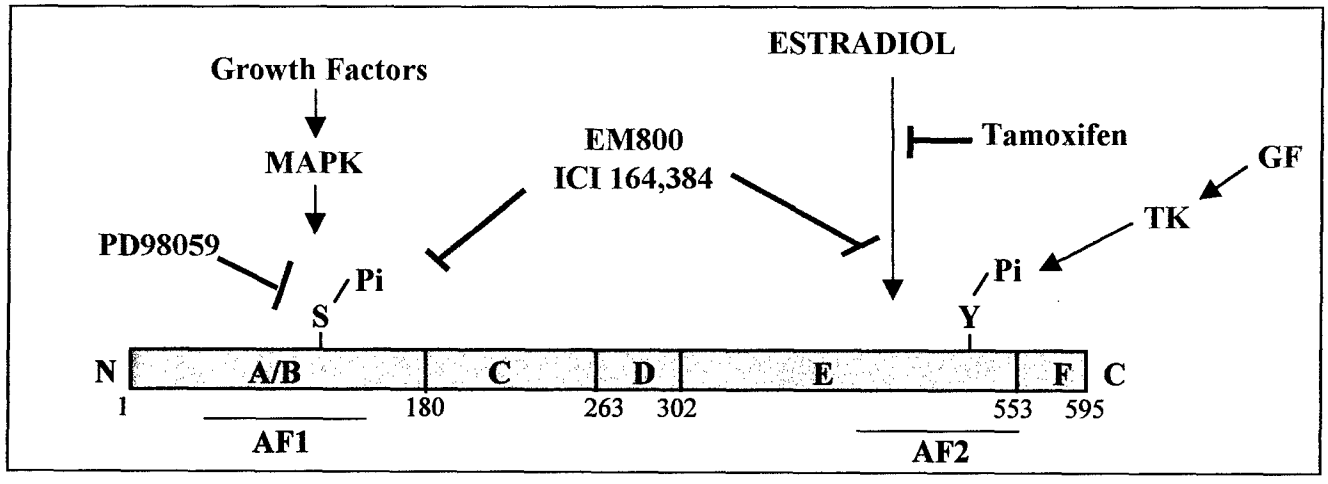


FIG 5: The activity of the estrogen receptor can be modulated in the AF1 and AF2 domain.

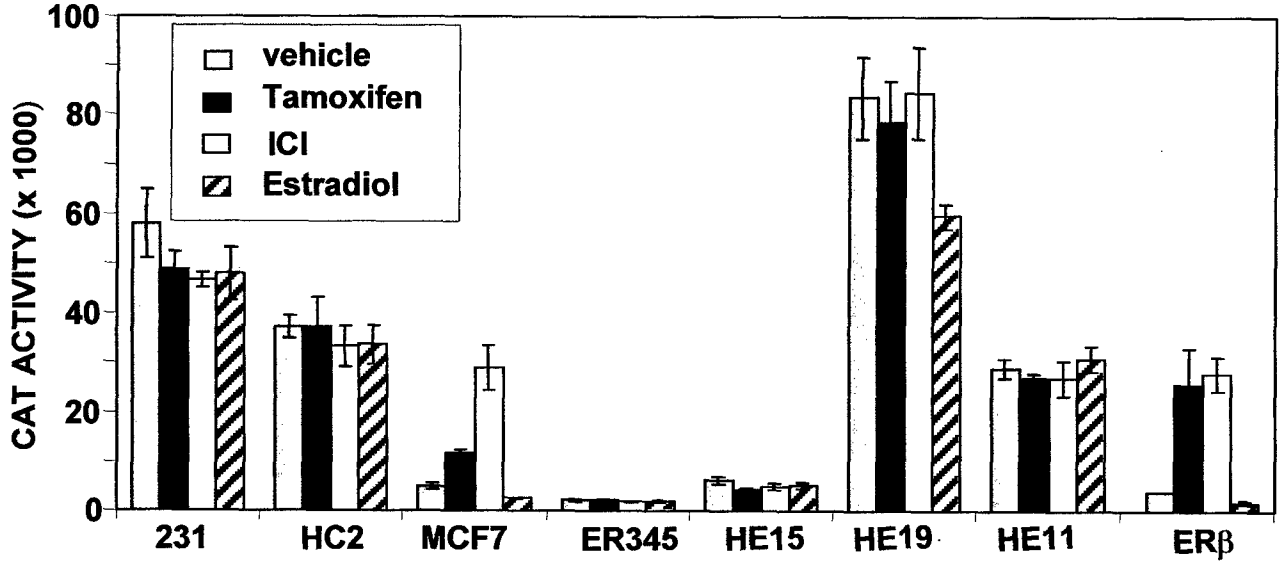
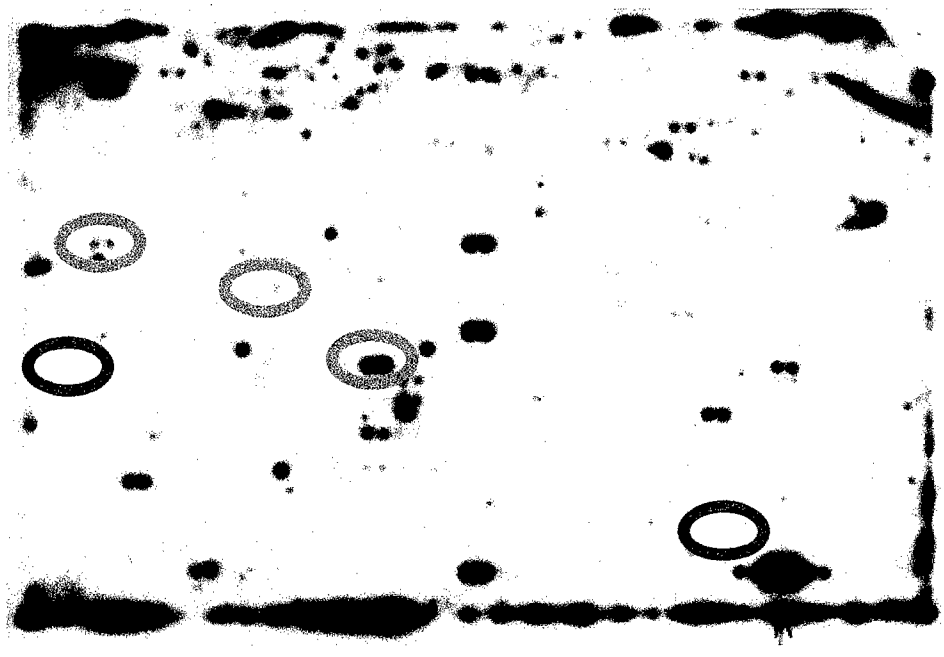


FIG 6: ER ligands alter transcription from the RARE

FIG 7: Clonetech Human Cancer Array comparing the ER-positive S30 cell line in the absence and presence of RA

S30 - RA

S30 + RA
(10^{-5} M 24 hrs)



<u>Genes up-regulated by RA</u>

TIMP-3 (mitogen-inducible gene)
Cyclin D1
Caspase 9 precursor

<u>Genes down-regulated by RA</u>
MIF
CD59

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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