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FOREWORD

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Richard J. Lee
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INTRODUCTION:

Among women living in western societies, breast cancer is the most common malignancy with one of the highest mortality rates. Retinoids currently offer a promising alternative modality to antiestrogens. The field of retinoids, analogues of Vitamin A, has been expanding, and these compounds contribute to the growth and differentiation of many normal tissues. Retinoids and antiestrogens are small hydrophobic ligands, and interact with members of the steroid/thyroid receptor superfamily. These nuclear receptors (e.g., estrogen: Estrogen Receptor [ER]; retinoids: Retinoic Acid Receptor [RAR] & Retinoic X Receptor [RXR]) are transcription factors that are activated allosterically by ligand binding turning on their respective transcriptional function. The ligand-bound receptor then forms a hetero- or homo- dimer complex to initiate the transcription of genes that may, in part, mediate the inhibition of breast cancer proliferation. In any form of chemoprevention, acquisition of resistance to retinoids can be a problem. It is plausible that an alteration in the common pathways shared by both retinoids and antiestrogens may develop into simultaneous resistance to both drugs. Coregulators are proteins that are involved in many of the different nuclear receptor pathways. They facilitate the promotion or repression of the transcription of hormone driven genes. Their regulation of transcription may originate from the bridge formation between the nuclear receptors attached to the hormone response elements and the transcriptional initiation complex located on the promoter, yet the exact mechanism remains unclear. We propose to identify these coregulators and retinoid related genes that could contribute to the acquisition of retinoid resistance in breast cancer. We will use the yeast two-hybrid system to find novel and known coregulators and the cDNA microarrays to identify pathways and biomarkers of acquired retinoid resistance and cross-resistance in breast cancer

PURPOSE:

We hypothesize that the loss/gain of coregulator(s) that interact with RAR α is responsible for the acquisition of resistance to RAR/RXR selective retinoids in breast cancer.

TECHNICAL OBJECTIVES:

Specific Aim 1. We will select estrogen independent MCF-7/LCC1 against retinoids, 4HPR [unclear specificity], 9-cis-RA [pan RAR and RXR agonist], and TTNPB [RAR selective agonist]. We will evaluate the resistance and sensitivity of the newly derived and established cell lines to RAR/RXR selective retinoids and antiestrogens, TAM [ER partial agonist] and ICI 182,780 [ER antagonist], and will measure retinoid and estrogen receptor levels. We will use the cell lines to further investigate the pattern of expression of the genes identified in Aim 1.

Specific Aim 2. We will identify novel coregulator(s) that interact(s) with the RAR α (Retinoic Acid Receptor) using the yeast two-hybrid system in the MCF-7 cDNA Library. We will examine their patterns of expression in retinoid sensitive cell lines MCF-7 and MDA435/LCC6 and in the established retinoid resistant cell lines, MDA-MB-231, BT-20, and MCF-7/RR [generated by selection against tamoxifen], and in the newly generated retinoid resistant ER+ cell lines from Aim 1.

SUMMARY

In the past year, Technical Objective 1 has taken the main focus of the project since retinoid generated resistant models are necessary to study acquired resistance pathways. Stock cultures of mammary epithelial cancer cells, MCF-7, were maintained in BioFluids IMEM with phenol red and with 5% fetal bovine serum in a humidified atmosphere containing 5% CO₂ and 95% air 37 °C. RR, MIII, LCC1, LCC20^{HPR}, and LCC21^{9CIS} cells were fed in BioFluids IMEM without phenol red and with 5% Charcoal Stripped Calf Serum Improved Minimal Essential Media (CCS IMEM). All cells were maintained in Falcon T-75 cm² or T-175 cm² flasks until they reached 80%-90% confluence.

Estrogen independent MCF-7/LCC1 was selected stepwise against 4HPR [unknown receptor specificity] and 9-cis-RA [K_d (nM) RARα=11, β=7, γ=22; RXRα=9, β=11, γ=16]. 4HPR is presently used in phase II clinical trials because of its low toxicity and effectiveness. Veronesi et al. from Milan, Italy (AACR Proceedings: Volume 40, March 1999, p. 304, Abstract # 2016) reported that 4-HPR is effective in reducing local recurrence and contralateral breast cancer in premenopausal women with early breast cancer. 9-cis-RA is a potent pan agonist for the RAR and RXR. These two drugs, 4-HPR and 9-cis-RA, were ideal choices to create cell line models of acquired retinoid resistance for human breast cancer due to their potential use in the clinics. The two generated cell lines were renamed as LCC20^{4HPR} and LCC21^{9-cis}. LCC20^{4HPR} was selected stepwise against 4HPR up to a concentration of 15 μM, and then the drug was removed from the cells. LCC21^{9-cis} was selected stepwise against 9-cis-RA up to a concentration of 10 μM and 9-cis-RA was removed from the cells. After removal of the drug from the two different variants, the cells were allowed to grow without the presence of the drug for 30-60 passages. A proliferation assay was necessary to verify the stable resistance of the newly

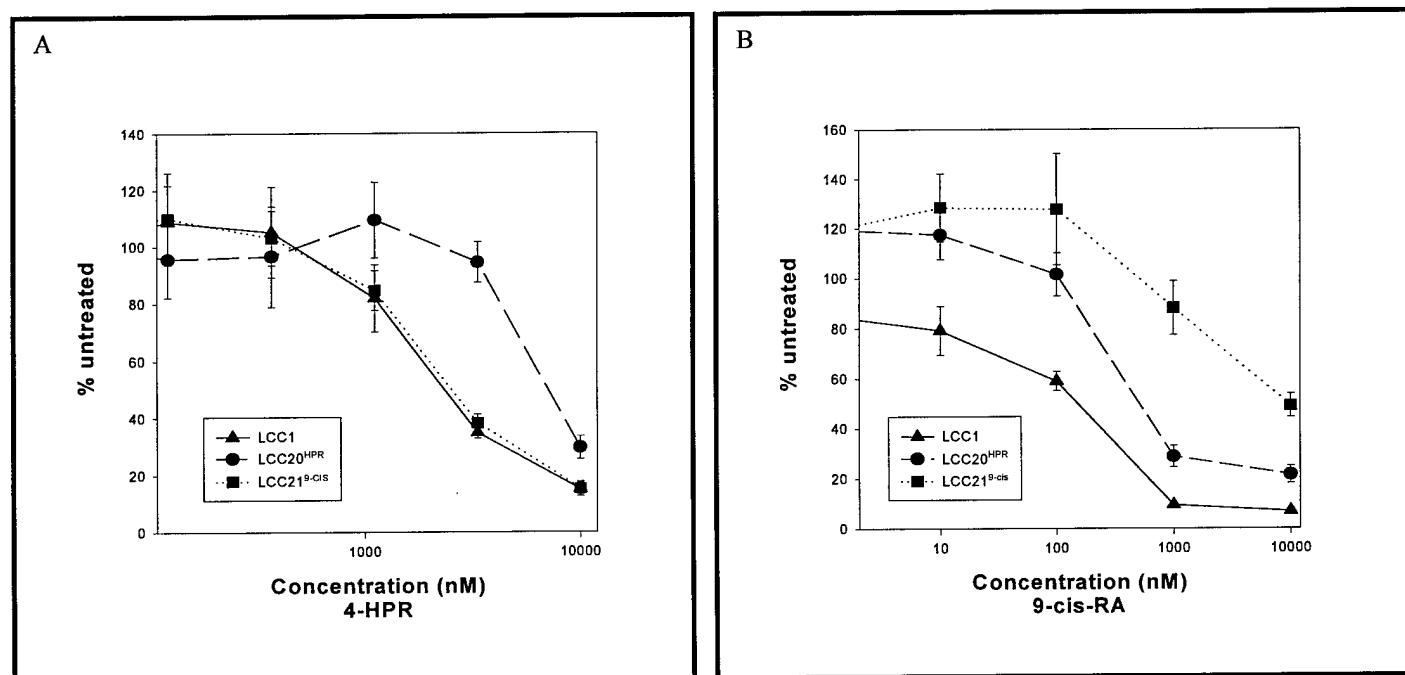


Figure 1: Dose response effects of A) 4-HPR and B) 9-cis-RA on the anchorage-dependent growth of LCC1, LCC20^{4HPR}, and LCC21^{9-cis} after 6 days of treatment. The data are the means $\pm$ SD of 6 replicate wells from a representative experiment.

generated MCF-7 derivatives. Their acquired retinoid resistance patterns were verified by crystal violet assay that measures the endpoint of growth proliferation. Cells were seeded in Falcon- 96 well plates at an optimum density found from the 8-day growth study. There were 10 treatments (6 replicates per treatment) of logarithmic increasing concentrations for each retinoid or antiestrogen. For retinoids (9-cis-RA and 4HPR),

controls contained the vehicle (0.1% ethanol). Cells were fed and treated every three days. On the seventh day, plates were fixed and stained with a crystal violet solution, dried for at least 24 h, and the crystal violet resuspended in 0.1 M Sodium Citrate in 50% ethanol. Absorbance for each plate was measured with DYNATECH's MR 700 Microplate Reader at 570 nm.

LCC21^{9-cis} showed, not surprisingly, 10 to 100-fold resistance to 9-cis-RA, but showed no sign of cross-resistance to 4-HPR with respect to its parental cell line MCF-7/LCC1 [Figure 1A,B]. However, preliminary data suggests that LCC20^{4HPR} is 3 to 5-fold (a half log) resistant to 4-HPR, and interestingly had a half log shift cross resistance to 9-cis-RA with respect to its parental cell line LCC1 [Figure 1A,B]. The cross-resistance data suggests that the pathway in acquiring resistance to 4HPR may overlap the pathway in acquiring resistance to 9-cis-RA. Currently, the LCC1 cell line is being selected against other clinically useful retinoid drugs TTNPB [K_d (nM) RAR α =20, β =39, γ =51; RXR α =8113, β =4093, γ =2566] and LG1069 [K_d (nM) RAR α >5000, β >5000, γ >5000; RXR α =27, β =44, γ =44]. Under treatment of TTNPB, cells' morphology is altered. They appear enlarged and flattened as the cells adapt to its treated environment.

Measurements of retinoid receptor expression profiles are currently in progress on LCC20^{4HPR} and LCC21^{9-cis}. Receptor profiles are measured with RNase Protection Assays using RNA radiolabeled probes. Presently, RNase Protection Assays of the retinoid resistant cell lines with RAR α and RXR α riboprobes have thus been completed. At least three independent experiments were performed. For the RPA, all cells were fed and stripped with 5% CCS IMEM. Stripping MCF-7 was done as follows; for three days, MCF-7 was washed with 5% CCS IMEM three times on the first day of stripping and washed stepwise down for the following two days. On the fourth day, RNA was extracted from the cells. RNA was obtained using Trizol Reagent (Life Technologies, Inc., Grand Island, NY) according to manufacturer's instructions. The RAR α and RXR α probes were kindly provided by Dr. Marco Gottardis and Dr. William Lamph from Ligand Pharmaceuticals. The RAR α and RXR α probe were made from 125 bp of the RAR α cDNA and 95 bp of the RXR α cDNA, respectively, and the 36B4 loading control was obtained similarly from 220 bp of the 36B4 cDNA. Riboprobes were labeled with [α -³²P]UTP. Briefly, RNA (30 μ g), 36B4 probe, and retinoid probes were hybridized overnight at 50 °C followed by digestion with RNase A. Protected fragments were run on a 6% acrylamide Tris-Borate-EDTA/UREA gel (NOVEX, San Diego, CA). The gel was then vacuum dried at 80 °C for 1 hr. Protected and labeled bands were quantified by phosphoimaging screens and visualized with autoradiography

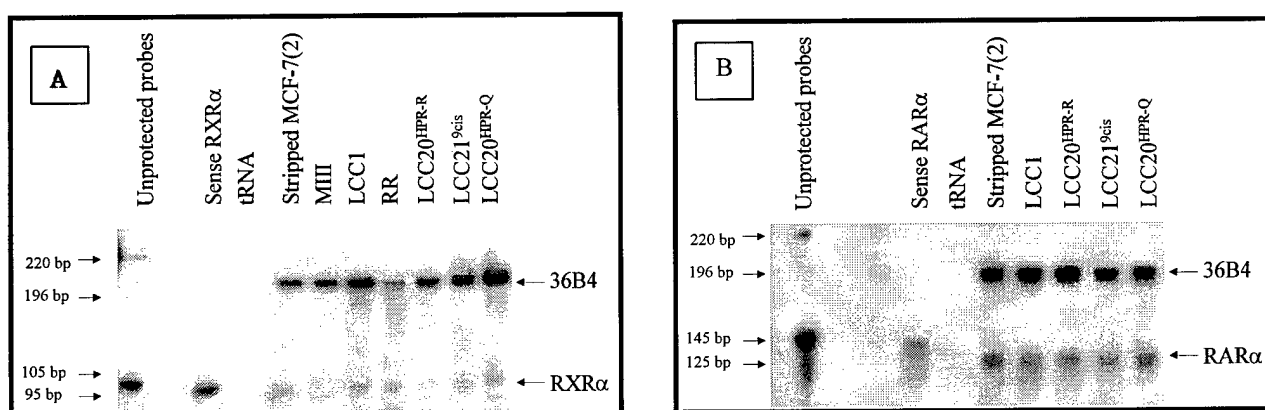


Figure 2: RNase Protection Assays of MCF-7 and its retinoid resistant variants probing with the A) RXR α and B) RAR α riboprobes.

performed at -70 °C between two Chromex Quanta III intensifying screens.

The RAR α and RXR α levels appear unaltered in the retinoid resistant cell lines, LCC20^{4HPR}, and LCC21^{9-cis} from the parental cell lines LCC1, after at least three repetitions from different pools of RNA. Densitometric analyses confirm that there are no changes in RAR α and RXR α expression among the parental and resistant cell

lines. There may be, however, a 1.6-fold induction of RXR α in the RNA from LCC1 to stripped clone 2 MCF-7; we believe that this induction may be due to a variability of a specific MCF-7 clone, and thus may not be biologically relevant. The loss of retinoid receptors RAR α and RXR α expression is thus not responsible for the acquired resistance to retinoids. Further work on the other retinoid receptors is currently underway in the description of the retinoid resistant cell lines.

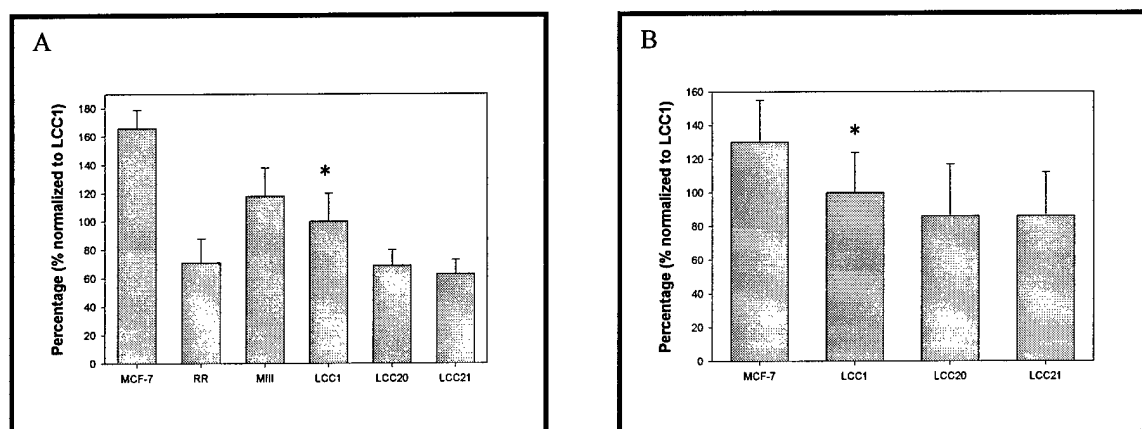


Figure 3: Densitometric analysis of A) RXR α and B) RAR α in the acquired retinoid resistant cell lines and their parental cell lines. The data are the means $\pm$ SD of at least three independent experiments. (*) Parental cell line for LCC20^{4HPR} and LCC21^{9-cis}

To further characterize the cells, we are currently using cDNA microarrays on LCC20^{4HPR} and LCC21^{9-cis}. We are using Clontech's Human Atlas Array that contains human cancer related genes to explore differential expression of genes between the parental cell line LCC1 and its retinoid resistant derivatives. The data from these arrays should help identify genes that contribute to acquired resistance to retinoids in breast cancer cells.

For Technical Objective 2, the yeast two hybrid has not been placed on the priority list due to technical difficulties. There were a considerable high number of false positive clones using the RAR α bait; we have tried to reduce the number of false positives by using a different *Saccharomyces cerevisiae* strain from the Y190. The PJ strain has multiple reporter constructs that may limit the number of false positives; reproducibility was not consistent, so we took the alternative approach by examining the levels of known coregulators. We have taken a fragment from each of the known coregulators (e.g., SRC-1, SMRT, and CBP) through PCR [Polymerase Chain Reaction]. We then inserted the PCR fragments into riboprobe vectors that will be used in RNase protection assays to study known coregulator level changes in the parental and acquired retinoid resistant cells. The assays will be under progress once the retinoid receptor characterization is completed. We have not, however, terminated the yeast two-hybrid project because the technique offers the possibility of discovering novel coregulators.

We will continue to pursue the objectives in Aim 1. In addition, we will use the cDNA microarrays to characterize the newly generated cell lines. Meanwhile, we will examine the role of known and novel coregulators in these acquired retinoid resistant cell lines through RNase Protection Assays and yeast two-hybrid system.

APPENDIX

Bulleted List of Key Accomplishments

- Established Acquired Retinoid Resistant Cell Lines LCC20^{HPR} and LCC21^{9-cis}
- Anchorage Dependent Growth Assays of LCC20^{HPR} and LCC21^{9-cis} with 9-cis-RA and 4HPR
- RNase Protection Assays of retinoid resistant cell lines and parental with RAR α and RXR α
- Constructed SMRT, CBP, and SRC-1 PCR fragments into riboprobe vectors.

List of Reportable Outcomes

Abstracts Presented at

- AACR Proceedings: The Steroid Receptor Superfamily: Celebrating the 10th Anniversary of the AACR Special Conference in Cancer Research: January 8-12, 1999. Renaissance Esmeralda Resort. Indian Wells, CA.
- AACR Proceedings: 90th Annual Meeting: April 10-14, 1999. Volume 40. March 1999. Philadelphia, PA.

Publications

Retinoid cross-resistance to 9-cis-RA and 4HPR is not associated with the loss of RAR α RNA expression. Richard Y. Lee, Todd C. Skaar, Fabio Leonessa, and Robert Clarke. AACR Proceedings: The Steroid Receptor Superfamily: Celebrating the 10th Anniversary of the AACR Special Conference in Cancer Research: January 8-12, 1999. Renaissance Esmeralda Resort. Indian Wells, CA.

The acquisition of retinoid resistance to 4HPR and 9-cis-RA in estrogen independent breast cancer. Lee, R.Y., Skaar, T.C., Leonessa, F., and Clarke, R. AACR Proceedings: 90th Annual Meeting: April 10-14, 1999. Volume 40: Abstract #406, p. 61. March 1999. Philadelphia, PA.

The acquisition of retinoid resistance to 4HPR and 9-cis-RA in estrogen independent breast cancer. Lee, R.Y., Skaar, T.C., Leonessa, F., and Clarke, R. Georgetown University, Department of Physiology and Biophysics, Lombardi Cancer Center, 3970 Reservoir Rd., NW, Washington, DC 20007.

A possible mechanism of tumor progression that allows breast tumors to grow in postmenopausal women, who have low serum levels of estrogens, is through an acquisition of an estrogen independent phenotype. Chemopreventive drugs such as antiestrogens and retinoids are possible modalities to address this particular phenotype. Retinoids, analogs of Vitamin A, inhibit breast cancer cell proliferation and are useful chemopreventive agents for postmenopausal women. These compounds interact with receptors in the super-family of nuclear transcriptional factors. One issue with chemoprevention is the acquisition of resistance, but no established *in vitro* model has addressed the problem in acquiring retinoid resistance.

We have established two stable retinoid resistant cell lines (MCF-7/LCC20^{4HPR} and MCF-7/LCC21^{9cis}) by selecting an estrogen independent MCF-7 variant LCC1 against increasing concentrations of retinoids, N-(4-hydroxyphenyl) retinamide (4-HPR) and 9-cis retinoic acid (9-cis-RA). After growing more than 30 passages without retinoids, MCF-7/LCC20^{4HPR} stably and consistently is 3-5 fold resistant to the drug 4-HPR, but shows a half log shift of cross-resistance to 9-cis-RA. However, MCF-7/LCC21^{9cis} maintains its resistance to 9-cis-RA 100 fold, but exhibits no cross-resistance to 4-HPR. Future studies are presently being directed towards looking at the molecular mechanisms of acquired retinoid resistance.

Retinoid cross-resistance to 9-cis-RA and 4HPR is not associated with the loss of RAR α RNA expression. Richard Y. Lee, Todd C. Skaar, Fabio Leonessa, and Robert Clarke. Georgetown University, Department of Physiology and Biophysics, Lombardi Cancer Center, 3970 Reservoir Rd., NW, Washington, DC 20007.

Retinoids, analogs of Vitamin A, inhibit breast cancer cell proliferation and are useful chemopreventive agents for postmenopausal women. These compounds have varied selectivity for retinoid receptors in the superfamily of nuclear transcriptional factors. 9-cis retinoic acid (9-cis-RA) is a retinoid pan agonist that activates both RAR and RXR isoforms. N-(4-hydroxyphenyl) retinamide (4-HPR) has unclear receptor selectivity, but shows promising clinical activity and is presently used in phase I clinical trials. One issue with retinoid chemoprevention is the acquisition of resistance and possible cross-resistance, but no established *in vitro* model has been developed to study the problem of acquired retinoid resistance for postmenopausal breast cancer patients.

We established an *in vitro* model by generating two stable retinoid resistant cell lines, MCF-7/LCC20^{4HPR} and MCF-7/LCC21^{9cis}. They were generated through selection of an estrogen independent MCF-7 variant (LCC1) against increasing concentrations of 4-HPR and 9-cis-RA. Anchorage-dependent growth assays confirm that MCF-7/LCC20^{4HPR} is stably and consistently 3-5 fold resistant to the drug 4-HPR, but shows a half log shift of cross-resistance to 9-cis-RA after growing more than 30 passages without the drug. However, MCF-7/LCC21^{9cis} maintains its resistance to 9-cis-RA (100-fold), but exhibits no cross-resistance to 4-HPR. To measure the RAR α RNA levels, we used a RNase Protection Assay with a riboprobe that is antisense to the nucleotide region 1798 to 1922 of the RAR α gene. RAR α RNA levels of these retinoid resistant cell lines are unaltered with respect to the parental cells. Future studies are presently being directed towards identifying the molecular mechanisms of acquired retinoid resistance and cross-resistance.