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TITLE: Defining the Molecular Actions of Dietary Fatty Acids in Breast Cancer: Selective Modulation of Peroxisome Proliferator-Activated Receptor Gamma

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**14. ABSTRACT**
Stark differences in the actions of linoleic acid (LAA), an omega-6 fatty acid, and eicosapentaenoic acid (EPA), an omega-3 fatty acid, on breast cancer tumors have been described. We propose that transactivation of peroxisome proliferators-activated receptor gamma (PPARγ) mediates the physiological effects of different dietary fatty acids on breast cancer. PPARγ plays a role in the development and progression of breast cancer tumors. We have shown that individual ligands of PPARγ can selectively activate PPARγ in three different ways. Selective activation of PPARγ by a single ligand occurs between tissue types and between individual breast cancer cell lines. Also, unique ligands selectively activate PPARγ within a single cell type. We propose that fatty acids will elicit their effects on breast cancer cells in a similar manner. Using both pharmaceutical and molecular approaches we have demonstrated that PPARγ serves as a molecular target for both LAA and EPA. Our data shows that fatty acids utilize PPARγ to activate a PPAR response element reporter system and that the receptor is both sufficient and necessary to observe this response. Also, EPA treatment increases the ability of PPARγ to bind to DNA. Furthermore, through multiple approaches we have determined that fatty acids do not need to be converted to prostaglandins but themselves can function as PPARγ ligands. To date, we have determined that both LAA and EPA act as PPARγ agonists. The objective of future studies will be to demonstrate that LAA and EPA act as selective PPARγ modulators (SPARMs) in breast cancer cells. We hypothesize that, though both LAA and EPA are PPARγ agonists, they function as SPARMs by causing unique gene expression and that this is in part the mechanism responsible for the different physiological actions of these fatty acids. Most recently, we have demonstrated that rosiglitizone, a PPARγ ligand, also serves as an agonist of the estrogen receptor in breast cancer cells.

**15. SUBJECT TERMS**
Breast Cancer, Dietary Fatty Acids, PPAR, Nuclear Receptor, Molecular

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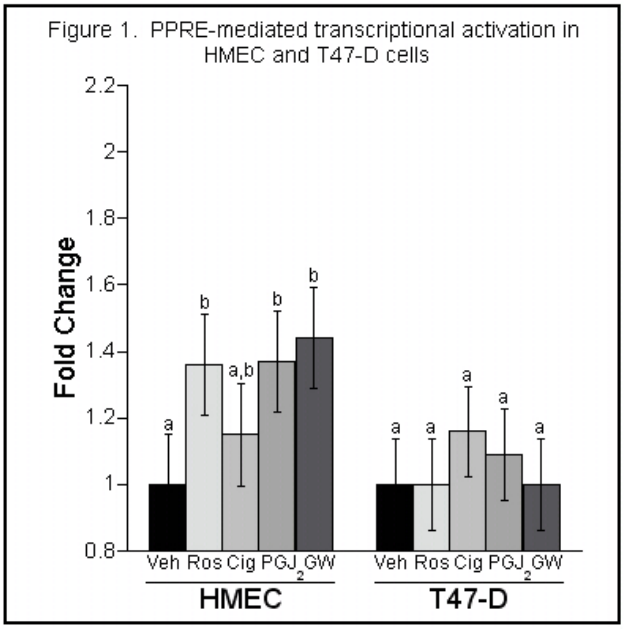
# Introduction

Breast cancer remains one of the leading forms of cancer in American women. One in every eight women in the United States will be diagnosed with breast cancer, a 5-fold higher rate than that observed in women living in Japan and China. Studies examining women that emigrate from Asian countries to the United States have found similar breast cancer risk as American women within 40 years suggesting that genetics alone can not account for differences in breast cancer incidence between these populations (*1*). A number of environmental aspects are being explored to elucidate factors that might influence breast cancer risk. Though controversial, it has been estimated that diet influences the development of up to 50% of all breast cancer cases in American women (*2*). A growing body of literature indicates that the type of dietary fat consumed (diets high in omega-3 fatty acids versus diets high in omega-6 fatty acids) influences breast cancer (*3, 4*) suggesting that consumption of specific fatty acids may impact breast cancer differently. Animal studies, have also provided convincing evidence of a correlation between types of fats ingested and mammary tumor development and growth (*5-8*). The focus of this proposal is to define the molecular link between specific fatty acids and the progression of breast cancer. We are exploring the possibility that fatty acids may elicit their effects in breast cancer cells by acting as ligands of the peroxisome proliferator-activated receptor gamma (PPARγ). Our laboratory has previously demonstrated that individual fatty acids can activate a PPAR-response element (PPRE), but whether this effect was the direct result of PPARγ activation has been left unexplored (*9*). Evidence suggests PPARγ is involved in the initiation (*10, 11*) and progression (*12-14*) stages of breast cancer. The objective of this proposal is to determine the mechanism of action that individual fatty acids use to either positively (increase cellular differentiation and/or decrease cellular proliferation) or negatively (increase cellular proliferation and/or tumor metastases) impact breast cancer cells. We propose that PPARγ is the molecular target responsible for the physiological effects of different dietary fatty acids on breast cancer.

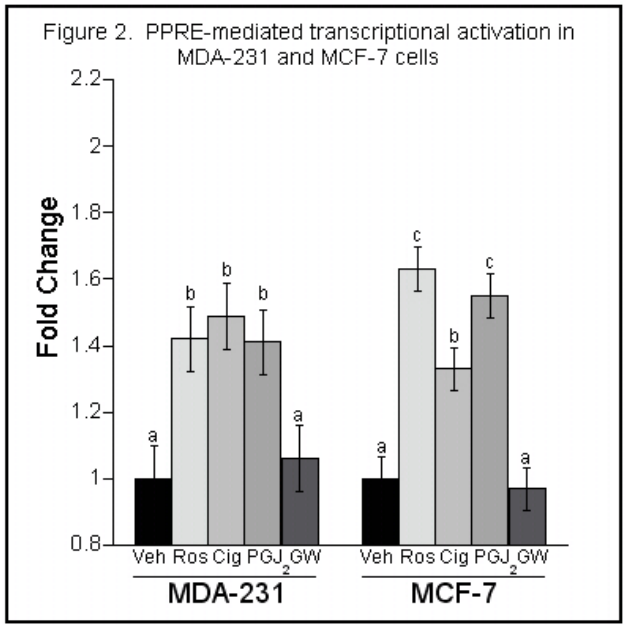
# Body

The orphan nuclear receptor, PPARγ is one of three in a family of receptors (PPAR α, β, and γ) (*15-17*). It is expressed in numerous cell types including adipocytes, epithelial cells of the breast, colon, and lung, and macrophages among others (*18-22*). Several ligands of PPARγ have been identified including 15-deoxy-Δ12,14-prostaglandin $J_2$, linoleic acid (LAA), lysophosphatidic acid, and the thiazolidinedione class of anti-diabetic drugs such as ciglitazone and rosiglitazone (Ros) (*9, 23-26*). Transactivation of the receptor requires ligand binding, heterodimerization with retinoid X receptor alpha (RXRα), and binding of this complex to PPAR-specific response elements (PPREs) in the promoter regions of target genes (*22, 27*).

To begin to explore the role that individual fatty acids might play in the progression of breast cancer we wanted to first demonstrate that individual ligands of PPARγ could selectively modulate the receptor. The ability of individual ligands to selectively mediate the activity of a nuclear receptor dependent on the tissue type examined has been used to develop compounds that act as selective estrogen-receptor modulators (SERMs). Tamoxifen, which was originally described as an estrogen-receptor antagonist, has been found to act as an agonist in several different tissue types (*28-30*). It has been proposed that individual ligands may be able to act as selective PPARγ modulators (SPARMs) in a manner similar to the way other compounds function as SERMs (*31*).



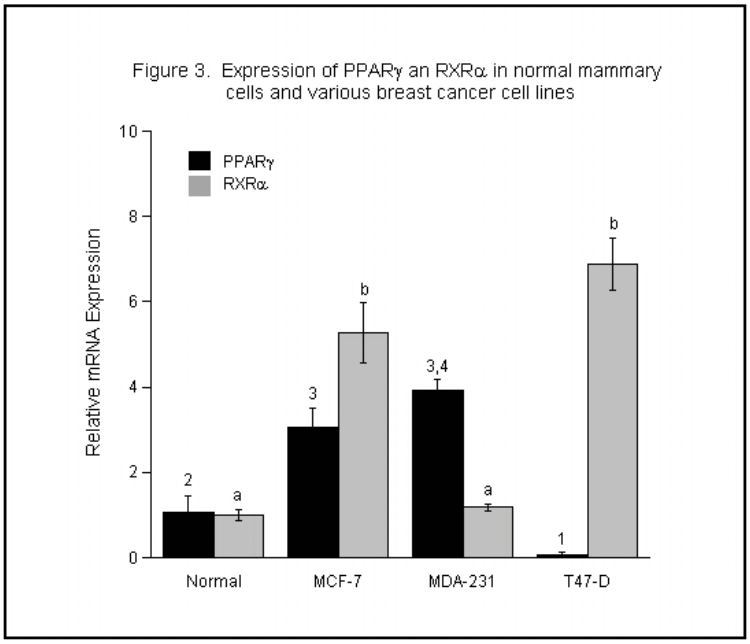

To address these fundamental questions we have conducted a series of experiments that tested the ability of individual PPARγ ligands to selectively activate PPARγ. To this end we have utilized a PPRE-reporter construct transfected into the cells prior to ligand treatment. The data from these studies has been collected, analyzed, and the resulting manuscript was published in *Molecular and Cellular Endocrinology* (see appendix).




In these studies, we first tested the ability of several different PPARγ ligands to activate the PPRE-reporter in either normal mammary epithelial (HMEC), estrogen-dependent breast cancer cells (T47-D and MCF-7), or estrogen-independent breast cancer cells (MDA-MB-231). Following transfection with a PPRE reporter plasmid, HMEC, T47-D, MDA-MB-231, and MCF-7 cells were treated with either vehicle control or PPARγ ligands for 18 hours. For the four cell lines, differences in ligand activity were observed. In the HMEC, Ros and $PGJ_2$ both significantly increased reporter activity over control (Fig. 1). Interestingly, GW, a known antagonist of PPARγ, also significantly stimulated reporter activity in HMECs. GW treatment did not change reporter activity compared

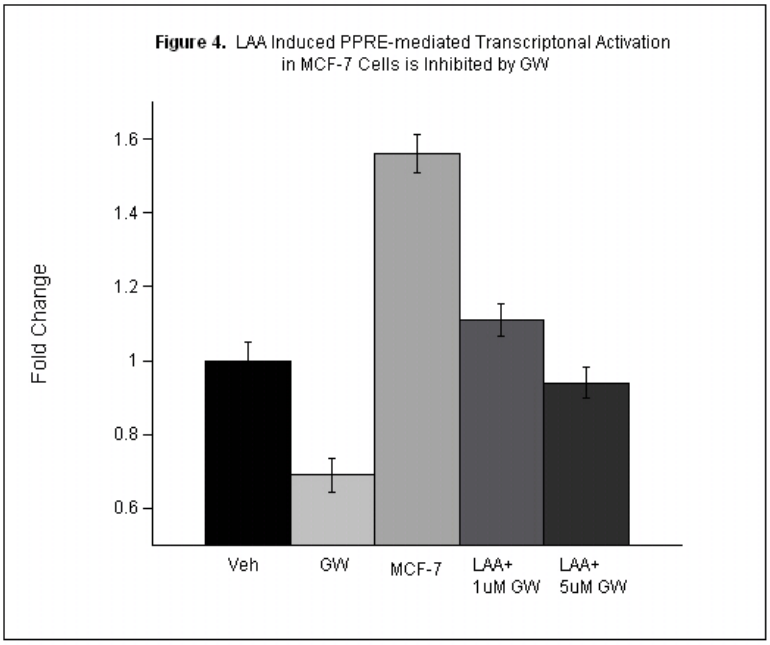
to control in any of the other breast cancer cell lines. No treatments significantly increased reporter activity in the T47-D cells (Fig. 1). In MDA-MB-231 cells Ros, Cig, and $PGJ_2$ all significantly enhanced PPARγ activation over control, while these same three treatments also increased reporter activation in MCF-7 cells when compared to control (Fig. 2). Both Ros and $PGJ_2$ treatments resulted in significantly higher activity than Cig in MCF-7 cells.

Next we wanted to determine if mRNA levels of PPARγ and its heterodimic partner, RXRα, in cells were predictive of how the breast tumor cells would respond to PPARγ ligands. To do this, mRNA concentrations of PPARγ1 and RXRα were measured in all four cells lines (HMEC, T47-D, MDA-MB-231, and MCF-7) used previously in the transfection assays in the absence of ligand treatment. Data is presented as fold change in expression compared to the HMECs. T47-D cells had significantly lower levels of PPARγ than all of the cell lines (Fig. 3). However, these cells had the highest expression of RXRα among all cell lines. MCF-7 cells express significantly higher levels of PPARγ expression than the HMECs or T47-Ds and higher RXRα than all of the cells tested except T47-Ds. MDA-MB-231 cells had PPARγ mRNA levels similar to MCF-7 cells, but had lower RXRα expression.



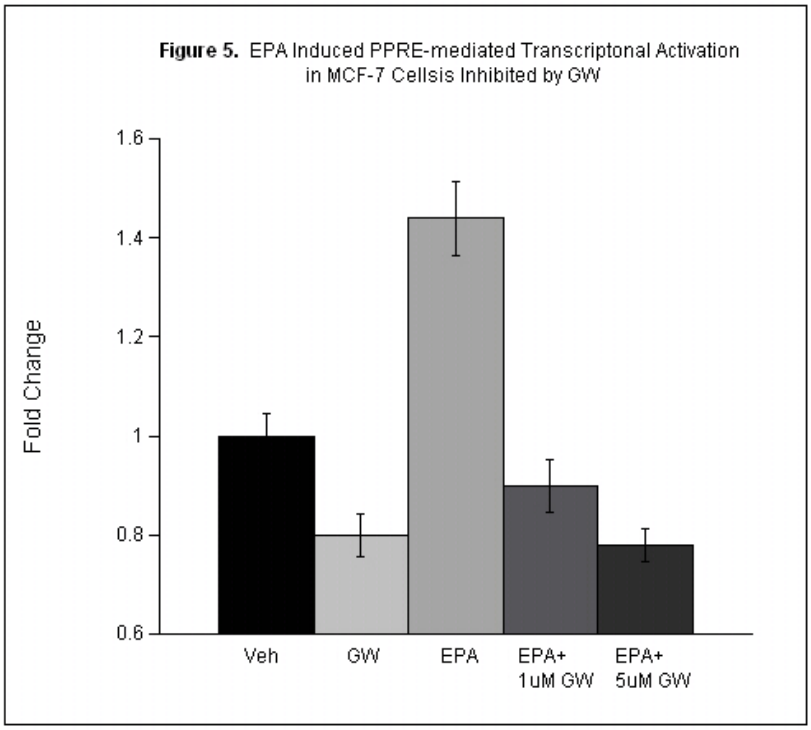

Data from these experiments demonstrated that selective activation of PPARγ occurs in multiple ways. Distinct ligands selectively activate PPARγ dependent on the tissue type from which the cell line was derived (data not shown, see MCE manuscript for full description). SPARM activity was also observed between different cell lines of the same tissue origin. Specifically, normal mammry cells and breast cancer cells responded differently to individual ligands and differences were observed between unique breast cancer cell lines. Also, individual ligands selectively activated the PPRE reporter within single breast cancer cell lines.

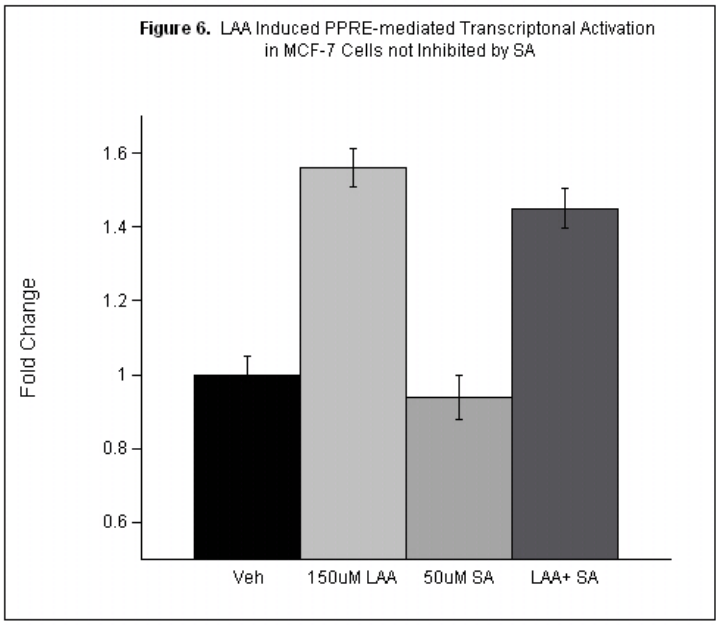
We have now begun to determine if individual fatty acids utilize PPARγ as a molecular target in breast cancer cells and if like other ligands they to can function as SPARMs resulting in diverse physiological effects. Although fatty acids have been shown to activate PPRE-reporter assays in a number of different cell types (*9, 17, 32*), left unexplored was whether PPARγ serves as a mediator of these responses. Defining this mechanism is critical to the greater understanding of how and if fatty acids function directly through PPARγ in breast cancer cells. To study this, we have utilized both pharmaceutical and molecular approaches. In the first approach, we used GW, the PPARγ specific antagonist, to block the ability of individual fatty acids to increase PPRE-reporter activity. We have previously established the antagonistic actions of GW in this system by inhibiting the PPRE reporter activation induced by Ros, a selective PPARγ agonist (data not shown). We then wanted to determine if the PPRE activation which results from treatment of either LAA, an *n*-6 fatty acid, or eicosapentaenoic acid (EPA), an *n*-3 fatty acid, could be inhibited by GW. For these experiments, MCF-7 cells were transiently transfected with a PPRE reporter construct and subsequently treated with either LAA or EPA in combination with GW for 18 hrs. Treatment with 150μM LAA significantly




increased PPRE reporter activity and this effect was inhibited in a dose response pattern when the cells were co-treated with GW (Fig. 4). Addition of 100μM EPA also significantly enhanced reporter activity in the MCF-7 cells compared to vehicle control (Fig. 5). Co-treatment of these cells with EPA plus GW significantly decreased reporter activity compared to EPA treatment alone to levels not significantly different from control.

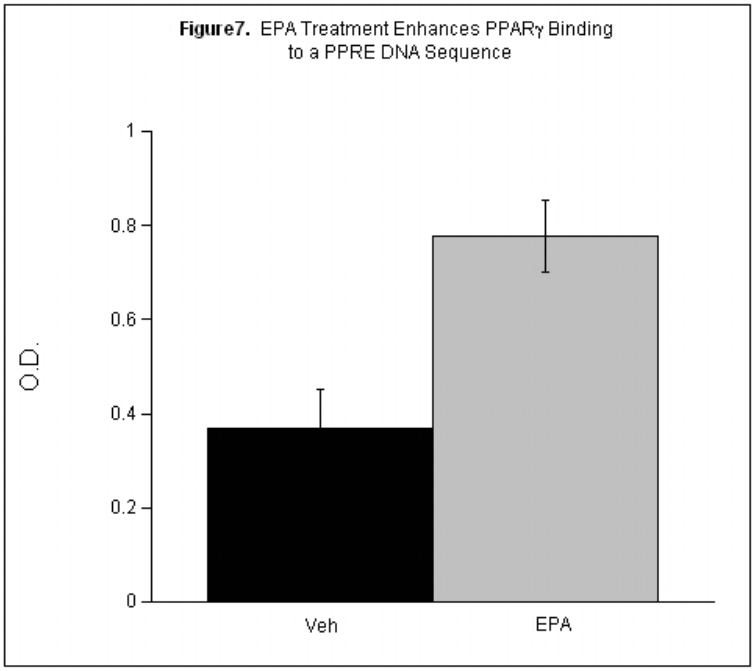
In addition to examining the molecular pathways of LAA and EPA in MCF-7 cells, we wanted to determine if these fatty acids were themselves the functional ligand of PPARγ. Fatty acids are metabolic precursors of prostaglandins that are formed at the cellular level (*33*) and individual prostaglandins have been shown to be ligands of PPARγ (*23, 34*). Therefore, we sought to determine whether LAA itself or a prostaglandin formed from LAA was responsible for the increased activity in the PPRE-reporter assay in MCF-7 cells. Cyclooxygenase (COX) enzymes utilize LAA and other fatty acids as substrates in the formation of prostaglandins (*35, 36*). In order to determine whether LAA is itself a ligand of PPARγ rather than an upstream metabolic precursor of the ligand, we co-treated MCF-7 cells with LAA and salicylic acid (SA). Salicylic acid has been shown to effectively inhibit COX activity (*37, 38*). In these studies, cells treated with SA alone, at an optimal dose for inhibiting COX activity, had no increased PPRE-reporter activation (Fig. 6). MCF-7 cells co-treated with LAA and SA had increased reporter activity similar to that of cells treated with LAA alone. We have confirmed this data in other systems in which LAA and EPA enhance PPRE reporter activity (data not shown). For the EPA studies, asprin (acetyl salicylic acid) which has also been shown to inhibit COX activity was used to inhibit the enzyme. Collectively these studies indicate that the conversion of fatty acids to prostaglandins is not required for PPRE activation.






To further evaluate the ability of LAA and EPA to influence PPARγ's activation of a PPRE, we then employed a PPARγ negative cell line (22Rv1). In these studies, we co-transfected 22Rv1 cells with both the PPRE-reporter construct and a PPARγ1 expression plasmid and then treated the cells with either LAA or EPA for 18 hrs. In the absence of PPARγ expression, neither LAA nor EPA influenced the PPRE-reporter, but activity was enhanced in cells co-transfected with PPARγ (data not shown). This activation was inhibited by the addition of GW. Taken together, these data demonstrate the molecular consequence of either LAA or EPA exposure in these cells results in the direct transactivation of PPARγ and the upregulation of the PPRE-reporter. However, this data falls short of identifying PPARγ as the only molecular target of these ligands. Individual fatty acids have been shown to bind the other isoforms of PPAR (α and δ) (*32*). So, while we have not ruled out the possibility that LAA and/or EPA may also elicit responses through PPAR α or δ, we have, through both pharmacological and molecular approaches, clearly identified PPARγ as a molecular target of these fatty acids in MCF-7 cells.

In addition to PPRE activation, we have shown that EPA increases binding of PPARγ to a DNA. For these studies, recombinant PPARγ and RXRα proteins were incubated with either vehicle or EPA. An ELISA-based methodology was used to quantify PPARγ binding to a PPRE following EPA treatment. Either vehicle or 100μM EPA was incubated with recombinant PPARγ and RXRα proteins *in vitro*. These reaction mixtures were then added to 96-well plates seeded with an oligonucleotide containing a PPRE. PPARγ specificity and DNA binding was quantified by incubating with a primary antibody for PPARγ and colorimetric analysis, respectively. EPA significantly increased the ability of PPARγ to bind the PPRE when compared to vehicle control (Fig. 7). This data is critical in demonstrating that EPA can influence the actions of un-ligand bound PPARγ. To our knowledge, this is the first data to show that addition of a ligand enhances the ability of PPARγ to bind DNA. Furthermore, since this is a cell-free

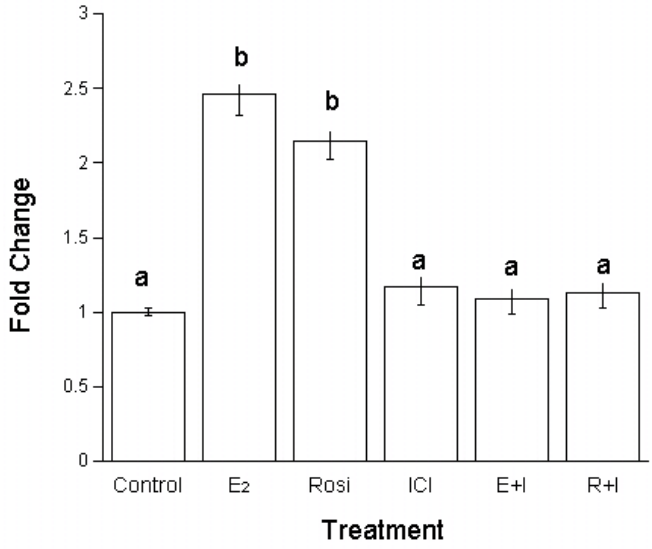


system, these data further support the notion that fatty acids themselves are functional ligands of PPARγ.

To date, we have demonstrated that PPARγ can serve as a molecular target of both *n*-3 and *n*-6 polyunsaturated fatty acids. In fact, both LAA and EPA enhance PPRE-reporter activity. However, epidemiology, animal, and cell line data all suggests that these two fatty acids result in opposing effects in breast cancer cells. Therefore, we hypothesize that while both compounds activate PPARγ that the molecular and cellular changes that occur in response to PPARγ activation is quite different for each compound. To begin to explore this, we have collected RNA from MCF-7 cells treated with vehicle, 150μM LAA, or 100μM EPA and then analyzed changes in gene expression utilizing microarray technology. We have begun to analyze this vast collection of data. Preliminary information suggests that the two treatments result in over 5,000 genes that are significantly changed dependent on treatment. The next step in this process will be to identify PPARγ response genes. Once this analysis is complete, genes will be verified using rt-PCR. We anticipate that the data will enable us to determine how two ligands that activate the same receptor result in drastically different physiological responses. We hypothesize that, though both LAA and EPA are PPARγ agonists, they function as SPARMs by causing unique gene expression and that this is in part the mechanism responsible for the different physiological actions of these fatty acids.

In recent studies, we have begun to explore the possibility that some compounds known to bind PPARγ may also act as ligands of estrogen-receptor alpha (ERα). Approximately 65% of all breast cancers are ER positive and are diagnosed as estrogen-dependent cancers. As such, it is critical to identify compounds that stimulate ER activity. Ros is a thiazolidinedione drug that we have studied in the past with regards to its ability to function as a SPARM. However, recently we have explored the ability of Ros to activate an ER response element (ERE) in MCF-7 cells. To do this, MCF-7 cells were transiently transfected with a luciferase reporter under the control of an ERE. Cells were then treated with estradiol ($E_2$) or Ros for 18h (Fig. 8). $E_2$ treatment significantly increased ERE reporter activity. Interestingly, Ros treatment also resulted in a significant increase in ERE reporter activity. ICI 182,780 (ICI), a pure ERα antagonist, was used to determine whether Ros activation of an ERE reporter is dependent on the ER signal transduction pathway. ICI treatment alone did not alter ERE reporter activity but ICI completely blocked ERE reporter activation by both $E_2$ and Ros. On going experiments are exploring the ability of Ros to influence cellular proliferation of MCF-7 cells and the molecular pathways responsible for these actions. The ability of Ros to activate both PPARγ and ERα could have important implications for the use of these drugs in the treatment of diabetes in patients at risk of developing breast cancer.

**Figure 8. ERE-Mediated Reporter Activity in MCF-7 Cells**



## Methods:

### Cells and cell culture

HMEC, MDA-MB-231, T-47D and MCF-7 were obtained from the ATCC (Rockville, MD) and maintained as described in the attached manuscript. Two cell lines were used in experiments investigating fatty acids. Breast epithelial adenocarcinoma cells (MCF-7) were used in most experiments. Human prostate carcinoma cells (22Rv1) were used as PPARγ negative cells. Both cell lines were cultured in medium that provided optimal conditions for their growth. Cells were maintained in medium containing 10% fetal bovine serum (FBS0 and were grown in medium lacking phenol red at 37ºC in a 5% $CO_2$ atmosphere. Cells were grown in T-75 flasks before being transferred to 24-well plates in preparation for transfection.

### Fatty acid preparations

LAA and EPA were purchased in pure fatty acid form and then dissolved in hexane to create a fatty acid stock solution. The stock solutions were maintained under nitrogen gas at all times and fresh fatty acid preparations were made before every experiment. For transfection assays, appropriate volumes of the stock solutions were then combined with calculated volumes of 6N NaOH to form fatty acid salt complexes. The preparations were then dried under nitrogen gas until no fluid remained. The fatty acid salt was then dissolved in cell culture media containing 10% FBS. It has been reported that the availability of free fatty acids in the body is dependent on the presence of albumin and therefore depends on albumin concentration (*39*). As a result, the chosen FBS conditions were necessary to form fatty acid/ albumin complexes. Once the fatty acid was completely dissolved in the media, hydrochloric acid was used to balance the pH and the media was filter sterilized through a 0.2μm syringe filter. For the DNA binding studies, EPA was purchased and used in sodium salt form.

### Plasmids

The PPRE-reporter construct, 3XPPRE-TK-pGL3, contains three copies of a PPRE sequence (AGGACAAAGGTCA ) upstream of the mTK promoter between the Xho I and Hind III restriction enzyme sites of the pGL3 basic vector. CMV promoter controlled β-Galactosidase (β-GAL) expression vector was a kind gift from Dr. Melinda Wilson. pBluescript cloning vector plasmid was purchased from Stratagene.

### Transfection assays

In most cases, cells were transiently transfected with 3μg of PPRE-reporter and 1μg of β-GAL plasmid per 24 well plate. For 22Rv1 experiments, cells were transfected with 3μg PPRE-reporter, 1μg β-GAL, and either 1μg bluescript or 1μg PPARγ1 per 24 well plate. For studies evaluating the ability of Ros to act as a ligand of ER we utilized and ERE-reporter instead of the PPRE-reorter. Plasmids were transfected into cells using ESCORT transfection reagent over a four hour period. Cells were subsequently treated with 150μM LAA, 100μM EPA and/or other compounds (GW9662, Ros, ICI, etc…) for 18 hrs. Following treatment, cells were lysed in 50μl passive lysis buffer. The quantification of induced Firefly (*Phontius pyralis*) luciferase protein was performed using the reagent found in the Luciferase Assay System Kit according to the manufacturer's instructions. Luminometry was performed on a Berthold Lumat 9507. β-GAL activity was utilized as a constitutively active reporter. β-GAL activity was measured using a β-GAL Enzyme Assay System according to manufactures instructions. Mean fold induction was obtained by dividing the RLU/β-GAL ratio data from each treatment well by the mean values of the vehicle control appropriate for each treatment. Each set of treatments were performed in replicates of 6 in 3 separate experiments.

**RT-PCR Analysis**

Real-time PCR was performed on total RNA using the TaqMan One-Step RT-PCR Master Mix Kit purchased from Applied Biosystems and used according to manufacturers instructions. Commercial FAM labeled probe/primer pairs constructed by Applied Biosystems using the Celera genomic database were used to asses PPARγ and RXRα mRNA levels. Quantitation of mRNA was performed using an ABI Prism 7700 Sequence Detection System and the TaqMan methodology, which uses the 5' nuclease activity of the Taq DNA polymerase to generate a real-time quantitative DNA assay. Data were analyzed using a $C_t$ cycle method. At the completion of the amplification (40 cycles), the amount of target message in each reaction was recorded as a threshold cycle number ($C_t$), which is inversely correlated to the abundance of the initial message level. $C_t$ measures the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The amount of target was normalized to the endogenous reference target, human GAPDH, again using a FAM labeled Taqman probe/primer solution available from Applied Biosystems. This normalized target $C_t$ value was then set relative to a normalized calibrator sample (i.e. untreated normal cell type) as given by the equation $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct$ represents $\Delta Ct$, target sample minus $\Delta Ct$, calibrator. Finally, this value was then used to produce a relative quantity by comparison to an appropriate control sample.

**Quantification of PPARγ binding to DNA**

To determine if EPA influenced PPARγ's ability to bind to a PPRE an enzyme-linked immunosorbent assay (ELISA)-based protocol was used. Prior to using this TransAM PPARγ kit an *in vitro* reaction was performed. For this reaction, either vehicle (methanol) or 100μM EPA sodium salt (final concentration) was incubated with 100ng each of PPARγ and RXRα recombinant proteins at room temperature for 20 min. The TransAM PPARγ kit was then performed according to kit instructions. Briefly, 3μl aliquots of each reaction were added to the 96-well ELISA plate in triplicate. The wells of the ELISA plates were coated with an immobilized oligonucleotide that contains a PPRE (5'-AACTAGGTCAAAGGTCA-3'). After incubation with the ligand/protein reaction mixture, the wells were washed and a primary antibody recognizing an accessible epitope on PPARγ protein upon DNA binding was added. Incubation with an appropriate secondary antibody conjugated to horseradish peroxidase was then performed following another wash. This step was followed by a colorimetric reaction which was quantified using spectrophotometry.

# Key Research Accomplishments

- We have demonstrated that individual PPARγ ligands can selectively activate the receptor in cancer cell lines derived from different tissues.
- We have shown that selective modulation of PPARγ occurs between normal mammary epithelial cells as well as different breast cancer cell lines when the cells are treated with PPARγ ligands. These data suggest that breast tumors in individual patients may respond to PPARγ differently.
- We have shown that individual PPARγ ligands can selectively modulate the receptor within a single cell line.
- We have demonstrated that distinct expression patterns of RXRα and PPARγ mRNA in tumor cells may be predictive of how they will respond to PPARγ ligand treatment, but further investigation is necessary to better define this approach.
- We have demonstrated that both LAA and EPA can induce PPRE-reporter activity in breast cancer (MCF-7) cells and that this effect is inhibited when cells are co-treated with a PPARγ specific antagonist.
- We have shown that inhibiting prostaglandin formation in MCF-7 cells does not significantly change the ability of fatty acids (LAA) to induce a PPRE-reporter. This data suggests that the fatty acids themselves are ligands of PPARγ and need not be metabolized to elicit their response on the receptor.
- Using a PPARγ negative cell line (22Rv1), we have confirmed that expression of PPARγ is required for either LAA or EPA to induce PPRE-reporter activity clearly defining that PPARγ can serve as a molecular target of fatty acids.
- We have demonstrated that EPA treatment increases the ability of PPARγ to bind to DNA. This is the first time a ligand has been shown to enhance binding of PPARγ to its response element. Furthermore, because this is a cell-free system, these data support the conclusion that metabolism of fatty acids to other compounds is not required for them to interact with PPARγ.
- We have completed microarray studies in which MCF-7 cells were treated with either LAA or EPA. Ongoing analysis of these data will identify genes that are selectively modulated by PPARγ following treatment with the different fatty acids.
- We have determined that Ros, like $E_2$, can activate an ERE-reporter. Our data indicates that this activation is the result of Ros serving as a ligand of ERα. Ongoing experiments will determine if Ros induces MCF-7 cell proliferation in a manner similar to $E_2$ and its molecular actions.

# Reportable Outcomes

## Training and Employment

- In addition to completing studies outlined in this grant, supporting funds have enabled the PI to participate in scientific endeavors that are beyond the scope the original grant. The PI has participated in collaborative projects related to breast cancer research which have resulted in presentations and eventual manuscripts (see details below). Additional manuscripts are in early stages of preparation and as such are not listed below, but will be credited to this grant.
- In January 2006, Dr. Allred was invited to interview for a tenure-track faculty position at Texas A&M University. Since that time, Dr. Allred has accepted that faculty position in the Department of Nutrition and Food Science and has relocated to College Station, Texas. In this position he will develop an independent research program.

## Manuscripts

- **Allred, C.D.** and Kilgore, M.W. Selective Activation of PPARγ in Breast, Colon, and Lung Cancer Cell Lines. *Molecular and Cellular Endocrinology*. 235: 21-29. 2005.
- Wang, X., **Allred, C.D.**, Southard, R.C., Wilson, M.E., and Kilgore, M.W. Myc-Associated Zinc Finger Protein Mediates the Overexpression of PPAR gamma in Human Breast Cancer Cells by Driving Promoter Switching. Submitted for publication in *Cancer Research*.
- **Allred, C.D.,** Talbert, D.R., and Kilgore, M.W. Modulation of Peroxisome Proliferator-Activated Receptor Gamma by Linoleic Acid in MCF-7 Cells. Manuscript in Preparation.

## Abstracts presented

- **Allred, C.D.** and Kilgore, M.W. Selective Activation of PPARγ Demonstrates Tissue Specificity Between Tumor Cell Lines. Presented at the Keystone Research Conference: Orphan Nuclear Receptors in 2004 in Keystone Colorado.
- **Allred, C.D.**, Talbert, D.R, and Kilgore, M.W. Selective activation of PPARγ in Cancers of the Breast, Colon, and Lung. Presented at the Twenty Fourth Annual University of Kentucky Symposium in Reproductive Sciences in 2005 in Lexington, Ky.
- **Allred, C.D.**, Talbert, D.R., and Kilgore, M.W. Defining the Molecular Actions of Linoleic Acid in Colon Cancer: Modulation of Peroxisome Proliferator-Activated Receptor Gamma. Presented at the Endocrine Society's Eighty Seventh Annual Meeting in 2005 in San Diego, CA.
- **Allred, C.D.**, Talbert, D.R., and Kilgore, M.W. Defining the Molecular Actions of Linoleic Acid in Breast Cancer: Modulation of Peroxisome Proliferator-Activated Receptor Gamma. Presented at the Era of Hope Meeting in 2005 in Philadelphia, PA.
- Talbert, D.R., **Allred, C.D.**, and Kilgore, M.W. The Antidiabetic Thiazolidinedione Rosiglitazone Transactivates ER and Induces Proliferation in Breast Cancer Cells. Presented at the American Association for Cancer Research annual meeting in 2006 in Washington, D.C.
- **Allred, C.D.**, Talbert, D.R., and Kilgore, M.W. Peroxisome proliferator-activated receptor gamma as a molecular target of polyunsaturated fatty acids in breast cancer cells. Presented at the Experimental Biology Meetings in 2006 in San Francisco, CA.
- Talbert, D.R., **Allred, C.D.**, and Kilgore, M.W. Rosiglitazone Activation of ERα induces proliferation in MCF-7 breast cancer cells and is dependent on the ERK-MEK pathway. Presented at the Endocrine Society's Eighty Eigth Annual Meeting in 2006 in Boston, MA.

**Invited Lectures**

- Invited oral presentation at Twenty Fourth Annual University of Kentucky Symposium in Reproductive Sciences May 2005
- Invited oral presentation at Texas A&M University January 2006

## Conclusions

PPARγ is highly expressed in breast cancer tumors and treatment of these cells with known PPAR agonists *in vitro* have been shown to suppress tumor cell growth. This has led to the possibility that PPARγ may be utilized as a therapeutic target in the treatment and prevention of breast cancer. Through a combination of pharmacological and molecular approaches we have now demonstrated that PPARγ serves as a molecular target of both *n*-6 (LAA) and *n*-3 (EPA) fatty acids. Future studies, beginning with microarray analysis, will test the hypothesis that these compounds act as SPARMs. We propose that both LAA and EPA stimulate PPARγ mediated gene transcription, but that each compound induces unique gene expression patterns that result in very diverse physiological responses in breast cancer cells. This mechanism in part would explain how consumption of diets high in either *n*-6 or *n*-3 fatty acids have opposing effects on breast cancer development and progression. The end result of these studies will be a stepping stone toward developing dietary recommendations for fatty acid consumption for patients with breast cancer as well as those at high risk of developing the disease.

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# Abbreviations

- PPARγ- peroxisome proliferator-activated receptor gamma
- ERα- estrogen receptor alpha
- PPRE- PPARγ response element
- ERE- ER response element
- RXRα- retinoid X receptor alpha
- TZD- thiazolidinedione
- Ros- rosiglitazone
- Cig- ciglitazone
- $PGJ_2$- 15-deoxy-delta 12,14-$PGJ_2$
- GW- GW9662
- LAA- linoleic acid
- EPA- eicosapentaenoic acid
- $E_2$- estradiol
- RLU- renilla units
- rt-PCR- real-time polymerase chain reaction
- $C_t$- cycle number

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# Selective activation of PPARγ in breast, colon, and lung cancer cell lines

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**Abstract**

Peroxisome proliferator-activated receptor gamma (PPARγ) plays a critical albeit poorly defined role in the development and progression of several cancer types including those of the breast, colon, and lung. A PPAR response element (PPRE) reporter assay was utilized to evaluate the selective transactivation of PPARγ in 10 different cell lines including normal mammary epithelial, breast, lung, and colon cancer cells. Cells were treated with one of four compounds including rosglitizone (Ros), ciglitizone (Cig), 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ ($PGJ_2$), or GW 9662 (GW). We observed differences in transactivation between cell lines from different tissue origin, across cell lines from a single tissue type, and selective modulation of PPARγ within a single cell line by different ligands. Interestingly, GW, a PPARγ antagonist in adipocytes, enhanced PPRE reporter activation in normal mammary epithelial cells while it had virtually no effect in any of the cancer cell lines tested. Within each cancer type, individual cell lines were found to respond differently to distinct PPARγ ligands. For instance, Ros, Cig, and $PGJ_2$ were all potent agonist of PPARγ transactivation in lung adenocarcinoma cell lines while these same ligands had no effect in squamous cell or large cell carcinomas of the lung.

Message levels of PPARγ and retinoid X receptor alpha (RXRα) in the individual cell lines were quantitated by real time-polymerase chain reaction (RT-PCR). The ratio of PPARγ to RXRα was predictive of how cells responded to co-treatment of Ros and 9-*cis*-retinoic acid, an RXRα agonist, in two out of three cell lines tested. These data indicate that PPARγ can be selectively modulated and suggests that it may be used as a therapeutic target for individual tumors.


*Keywords:* PPAR; Thiazoladinediones; Breast cancer; Colon cancer; Lung cancer

## 1. Introduction

The American Cancer Society estimated that collectively cancers of the breast, colon and lung accounted for 42% of all cancer deaths in men and 50% of all cancer deaths in women in 2004. In fact, breast, lung, and colon cancer rank as the top three types of malignancies identified in women today and one out of every eight women will develop breast cancer. In men, lung cancer is the most prevalent cause of cancer related death with malignancies of the prostate and colon following as next most common. A wide variety of chemotherapeutic options are being explored to treat these diseases. Novel therapeutic targets are being developed in an effort to identify endogenous, hormonal targets to either suppress cancer cell growth or induce apoptosis. One of the emerging targets for such treatments is peroxisome proliferator-activated receptor gamma (PPARγ).

The orphan nuclear receptor, PPARγ, is one of three of a family of receptors (PPARα, β, and γ) (Dreyer et al., 1992; Issemann and Green, 1990; Kliewer et al., 1994). It is expressed in numerous cell types including adipocytes, epithelial cells of the breast, colon, and lung, and macrophages among others (Braissant et al., 1996; Kilgore et al., 1997; Lemberger et al., 1996; Nagy et al., 1998; Tontonoz et al., 1994). Several ligands of PPARγ have been identified including 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ ($PGJ_2$), linoleic acid, lysophosphatidic acid, and the thiazolidinedione class of anti-diabetic drugs such as ciglitazone (Cig) and rosiglitazone (Ros) (Forman et al., 1995; Kliewer et al., 1997; Larsen

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et al., 2003; McIntyre et al., 2003; Thoennes et al., 2000). Transactivation of the receptor requires ligand binding, heterodimerization with retinoid X receptor alpha (RXRα), and binding of this complex to PPAR-specific response elements (PPREs) in the promoter regions of target genes (Kliewer et al., 1992; Tontonoz et al., 1994).

Recent evidence demonstrates that PPARγ is overexpressed in many different tumor types (DuBois et al., 1998; Tontonoz et al., 1997).In the breast, adenocarcinoma cells from patients expressed higher levels of PPARγ than normal epithelial cells from the surrounding mammary gland (Elstner et al., 1998). Similarly, in the colon, expression of PPARγ protein is significantly higher in human colon cancer sections when compared with non-tumor tissue (Chen et al., 2002). PPARγ has also been identified in both adenocaricoma and squamous cell carcinomas of the lung (Theocharis et al., 2002). Exposing cancer cells to PPARγ ligands produces physiological effects that may be exploited for treatment purposes. In culture, synthetic PPARγ ligands have been shown to inhibit growth of several tumor cell lines (Brockman et al., 1998; Elstner et al., 1998; Mueller et al., 1998). A number of studies have determined that PPARγ ligands induce cellular differentiation and/or apoptosis in breast, colon, and lung cancer cells (Chang and Szabo, 2002; Elstner et al., 1998; Mueller et al., 1998; Sarraf et al., 1998). The combination of receptor overexpression in tumors and known physiological effects of its ligands on cancer cells makes PPARγ a viable target of future chemotherapeutic agents.

The ability of individual ligands to selectively mediate the activity of a nuclear receptor dependent on the tissue type examined has been used to develop compounds that act as selective estrogen-receptor modulators (SERMs). Tamoxifen, which was originally described as an estrogen-receptor antagonist, has been found to act as an agonist in several different tissue types (Fisher et al., 1998; Jordan and Morrow, 1999; Levenson and Jordan, 1999). It has been proposed that individual ligands may be able to act as selective PPARγ modulators (SPARMs) in a manner similar to the way other compounds function as SERMs (Sporn et al., 2001). We previously demonstrated that individual fatty acids can selectively activate a PPRE-reporter assay in estrogen-dependent breast cancer (MCF-7) cells (Thoennes et al., 2000). Specifically, omega-3 fatty acids inhibited transactivation of PPARγ to levels below control while omega-6, monounsaturated and saturated fatty acids stimulated the activity of the PPRE reporter. These data demonstrated that individual compounds can selectively activate PPARγ within the context of a single breast cancer cell line. However, compounds have yet to be identified that act as PPARγ agonists in one tissue while functioning as antagonists of the receptor in other tissues.

In the studies presented here, we sought to determine if distinct ligands could selectively activate PPARγ across different cell lines of mammary, colon, and lung origin. To this end we have utilized a PPRE-reporter construct transfected into the cells prior to ligand treatment. Data from these experiments demonstrated that selective activation of PPARγ occurs in multiple ways. Distinct ligands selectively activate PPARγ dependent on the tissue type from which the cell line was derived. SPARM activity was also observed between different cell lines of the same tissue origin and individual ligands selectively activated the PPRE reporter within single cell lines. These data indicate that it may be possible to design PPARγ ligands that can be used to selectively mediate receptor activity and thus customize treatment regiments against specific cancers.

## 2. Materials and methods

### 2.1. Reagents

All PPARγ ligands were purchased from Cayman Chemical Company (Ann Arbor, MI). Ciglitazone (Cig) and GW9662 (GW) were solubilized in ethanol purchased from Aaper Alcohol and Chemical Company (Shelbyville, KY). Rosiglitazone (Ros) was dissolved in dimethyl sulfoxide (DMSO) and $PGJ_2$ was solubilized in methyl acetate purchased from Sigma (St. Louis, MO).

### 2.2. Cells and cell culture

Ten individual cell lines were used in these experiments. Four mammary cell lines including normal mammary epithelial (HMEC) and three breast cancer cell lines (MCF-7, T47-D, and MDA-MB-231). Two colon cancer cell lines (Caco-2 and HT-29) and four lung cancer (A549, H358, H520, and H1299) were utilized. HMEC were purchased from Cambrex (Rockville, MD) while the MCF-7, T47-D, MDA-MB-231, H358, H520, and H1299 cells were all purchased from American Type Culture Collection (Bethesda, MD). The HT-29 and A549 cells were generously provided by Dr. David Kaetzel (University of Kentucky, College of Medicine) and the Caco-2 cells were a gift from Dr. Charlotte Kaetzel (University of Kentucky, College of Medicine). All cells were cultured in medium previously described to provide optimal conditions for their growth. When possible multiple cell lines were maintained in the same medium to reduce error when comparing across cell types. Cells were maintained in medium containing 10% FBS. All cell types were grown in medium lacking phenol red at 37 °C in a 5% $CO_2$ atmosphere. Cells were grown in T-75 flasks before being transferred to 12-well plates in preparation for transfection.

### 2.3. PPRE reporter plasmid

The reporter construct, 3XPPRE-TK-pGL3, contains three copies of a PPRE sequence (AGGACAAAGGTCA) upstream of the mTK promoter between the *Xho*I and *Hin*dIII restriction enzyme sites of the pGL3 basic vector (Promega, Madison, WI). *Bam*HI and *Bgl*II were then used to release the 2.2 kb fragment containing the 3XPPRE-mTK-Luciferase. This fragment was ligated into the BamH I receptor site

of pRL-TK plasmid (Promega) completing the new reporter which contains both Luciferase and Renilla in a single expression plasmid. Renilla expression was used as a transfection efficiency control.

### 2.4. Transfection assays

Cells were transiently transfected with 5 μg of PPRE reporter plasmid per 12-well plate. Cells were transfected with ESCORT transfection reagent for 4 h. Cells were subsequently treated with either 10 μM Ros, 10 μM Cig, 1 μM $PGJ_2$, or 1 μM GW for 18 h. In all cases, PPARγ ligand concentrations for each compound used were those shown to be maximally effective following dose reponse studies (data not shown). Proper vehicle controls including ethanol, DMSO, and methyl acetate were run for each treatment group. Following treatment, cells were lysed in 50 μl passive lysis buffer and treated according to manufacturer's instructions (Promega dual luciferase assay kit). Luminometry was performed on a Berthold Lumat 9507 and data calculated as raw Luciferase Units (RLUs) divided by raw Renilla units. Mean fold induction was obtained by dividing the RLU data from each treatment well by the mean values of the vehicle control appropriate for each treatment. Each set of treatments were performed in replicates of six in three separate experiments.

### 2.5. RNA preparation

Total RNA was isolated from cultured cells utilizing an RNeasy® Mini Kit (Qiagen, Valencia, CA) according to manufactures instructions. Untreated cells from each cell line were used for RNA isolation. All cell lines were maintained in optimal growth conditions prior to RNA collection. RNA was stored at −80 °C and concentration was measured at by spectrophotometry.

### 2.6. Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time PCR was performed on total RNA using the TaqMan One-Step RT-PCR Master Mix Kit purchased from Applied Biosystems (Foster City, CA) and used according to manufacturers instructions. Commercial FAM labeled probe/primer pairs constructed by Applied Biosystems using the Celera genomic database were used to asses PPARγ (cat#: Hs00234592_m1) and RXRα (cat#: Hs00172565_m1) mRNA levels. Quantitation of mRNA was performed using an ABI Prism 7700 Sequence Detection System and the TaqMan methodology, which uses the 5′-nuclease activity of the Taq DNA polymerase to generate a real-time quantitative DNA assay. Data were analyzed using a $C_t$ cycle method.

At the completion of the amplification (40 cycles), the amount of target message in each reaction was recorded as a threshold cycle number ($C_t$), which is inversely correlated to the abundance of the initial message level. $C_t$ measures the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The amount of target was normalized to the endogenous reference target, human GAPDH (cat#: Hs99999905_m1), again using a FAM labeled Taqman probe/primer solution available from Applied Biosystems. This normalized target $C_t$ value was then set relative to a normalized calibrator sample (i.e. untreated normal cell type) as given by the equation $2\text{-}^{\Delta\Delta}C_t$, where $\Delta\Delta C_t$ represents $\Delta C_t$, target sample minus $\Delta C_t$, calibrator. Finally, this value was then used to produce a relative quantity by comparison to an appropriate control sample.

### 2.7. Statistical analysis

As previously described (Thoennes et al., 2000), fold changes in luciferase to renilla ratios were subject to a two-factor analysis of variance (ANOVA) hypothesis testing ($\alpha = 0.05$) based on the two nominal variables of treatment and experimental date using a custom designed program running on the StatServer 6.1 (Insightful, Seattle, WA) server housed in the University of Kentucky's Department of Statistics. In every case, the post-hoc test, Tukey's pair-wise comparison, was performed to identify significant differences between the various treatments within a cell line. Briefly, the Tukey methodology simultaneously determined the presence of significant differences between individual treatment mean estimations across the entire balanced set of pairwise comparisons using the studentized range distribution, $q$. Mean fold changes in luciferase/renilla ratios of treatments compared to vehicle controls were displayed by column graph with one-half of the critical value for comparison from the Tukey's comparison as an estimation of error. Significant differences within those comparisons for a single cell line are designated by an alpha-numeric system.

For RT-PCR analysis, cycle threshold measurements, $C_t$, for the mRNA targets of both PPARγ and RXRα were repeated in triplicate within each cell line. The average $C_t$ value for both PPARγ and RXRα in all 10 cell lines were subjected to analysis of variance hypothesis testing (ANOVA) using Microsoft Excel v10.0 at $\alpha = 0.05$ significance threshold. Following ANOVA, Fisher's least significant difference, LSD, pair-wise comparison was implemented post-hoc. Briefly, the LSD test determines a single critical value based on the mean squared error within groups and a critical value ($\alpha = 0.05$) found in the $t$ distribution. If the average absolute difference between any two groups was greater than the LSD critical value, then the pair-wise comparison for those two groups were found to be significantly different at ($p < 0.05$).

## 3. Results

### 3.1. Effect of PPARγ ligands on reporter activation in breast cancer cells

Following transfection with a PPRE reporter plasmid, HMEC, T47-D, MDA-MB-231, and MCF-7 cells were

treated with either vehicle control or PPARγ ligands for 18 h. For the four cell lines, differences in ligand activity were observed. In the HMEC, Ros and $PGJ_2$ both significantly increased reporter activity over control (Fig. 1A). Interestingly, GW, a known antagonist of PPARγ, also significantly stimulated reporter activity. GW treatment did not change reporter activity compared to control in any of the other breast cancer cell lines. No treatments significantly increased reporter activity in the T47-D cells (Fig. 1A). In MDA-MB-231 cells Ros, Cig, and $PGJ_2$ all significantly enhanced PPARγ activation over control, while these same three treatments also increased reporter activation in MCF-7 cells when compared to control (Fig. 1B). Both Ros and $PGJ_2$ treatments resulted in significantly higher activity than Cig in MCF-7 cells.

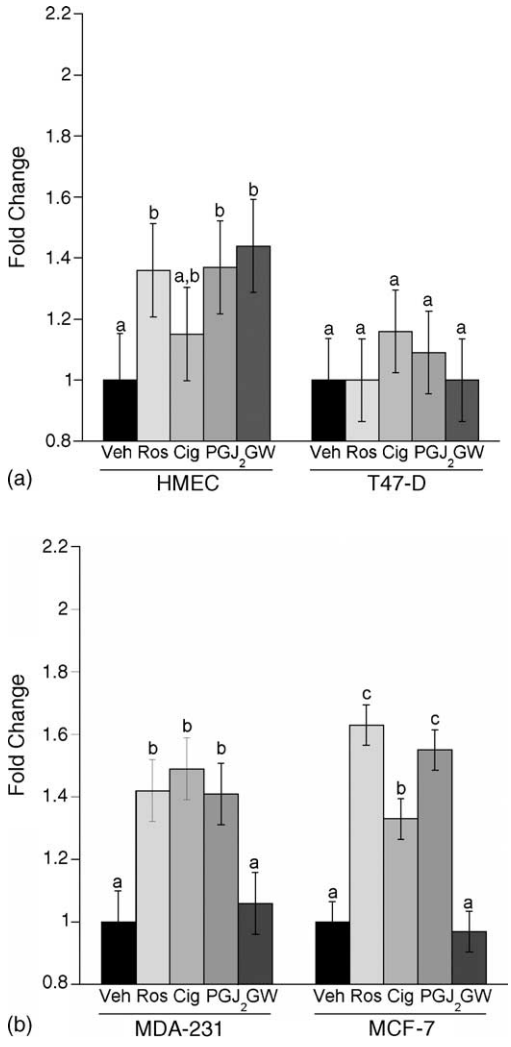


Fig. 1. (A and B) Effect of PPARγ ligands on reporter activation in breast cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPARγ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

### 3.2. Effect of PPARγ ligands on reporter activation in colon cancer cells

Two colon cancer cell lines (HT-29 and Caco-2) were also tested in the same manner described for the mammary cells (Fig. 2). In general, HT-29 cells were more responsive to the PPARγ agonist than the Caco-2 cells. In the Caco-2 cells, only the Ros treatment caused significant increases in PPRE reporter activity when compared to control. Alternatively, in HT-29 cells Ros, Cig, and $PGJ_2$ treatments all resulted in significantly higher reporter activation when compared to vehicle control whereas, GW treatment was not significantly different from control in either colon cell line.

### 3.3. Effect of PPARγ ligands on reporter activation in lung cancer cells

To examine PPARγ activation in lung cancer cells, four cell lines were chosen. A549 and H358 cells were derived from adenocarcinoma lung tumors while H520 and H1299 cell lines are non-adenocarcinoma derived cell lines. H520 cells are lung squamous carcinoma cells and H1299 cells

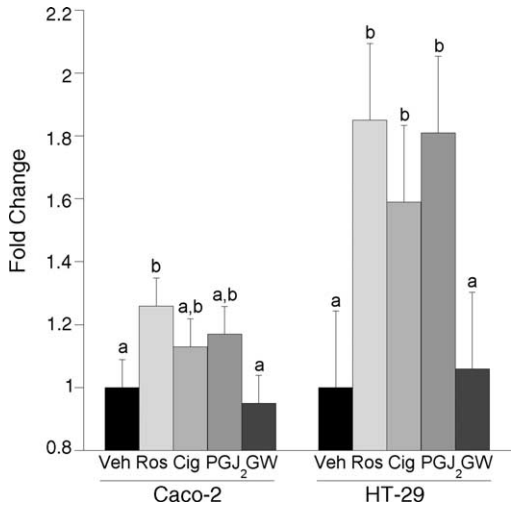


Fig. 2. Effect of PPARγ ligands on reporter activation in colon cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPARγ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

were derived from a metastatic site of a patient with large cell carcinoma of the lung. Cells were transfected and treated in the same manner as the other cancer cell lines. Similar to the breast and colon differences in reporter activity were observed for individual cell lines. In general, non-adenocarcinoma (H520 and H1299) cells did not respond to PPARγ ligands as well as the adenocarcinoma (H358 and A549) cell lines (Fig. 3A and B). Also, as observed in various other cancer cell lines, significant differences between the relative activation of the reporter were seen with individual ligands within single cell lines. In H1299 cells, treatment with Cig, $PGJ_2$, or GW resulted in significantly higher activation of the PPRE reporter when compared to vehicle control while Ros and Cig treatment caused significant increases in activity in H520 cells (Fig. 3A). In H358 cells, Ros, Cig, and $PGJ_2$ all resulted in increased activation when compared to control (Fig. 3B). However, Ros treatment resulted in significantly greater reporter activity when compared to both Cig and $PGJ_2$ as well. Exposure of A549 cells to Ros, Cig, or $PGJ_2$ also caused a significant increase in activation of the PPRE reporter when compared to control (Fig. 3B). However, in these cells reporter activation was greatest in Cig treated cells and the fold change for this treatment was significantly greater than that in $PGJ_2$ treated cells. GW9662 treatment was not significantly different from control in H520, H358, or A549 cells.

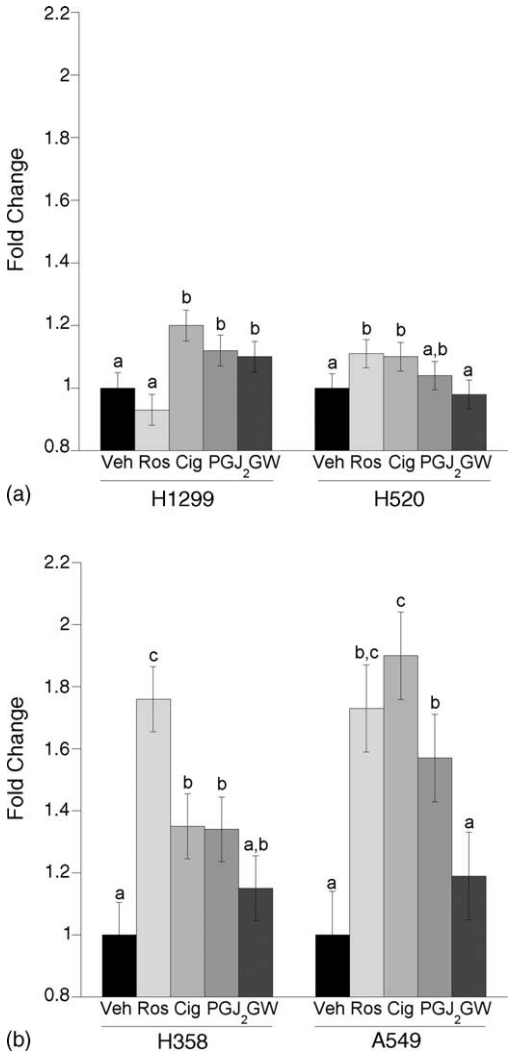


Fig. 3. (A and B) Effect of PPARγ ligands on reporter activation in lung cancer cells. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with one of four PPARγ ligands for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

### 3.4. Expression of PPARγ and RXRα mRNA

mRNA levels of PPARγ and RXRα were measured in all cell lines (Fig. 4). Total RNA was isolated from untreated cells. H1299 had the lowest expression of both PPARγ and RXRα when compared to all other cells. H520 cells had the second lowest levels of PPARγ and RXRα, while HMEC and A549 cells were next highest. H358 cells had similar expression of PPARγ as HMEC and A549 cells, but had significantly more RXRα expression when compared to the same cell lines. T47-D cells had significantly lower levels of PPARγ than all of the cell lines except the H520 and H1299 cells. However, these cells had the highest expression of RXRα among all cell lines. Caco-2 cells expressed the second largest amount of PPARγ mRNA and had high RXRα levels with only MCF-7 and T47-D cells expressing more. HT-29 cells had higher mRNA levels of PPARγ when compared to all other cell lines and RXRα expression similar to Caco-2 and H358 cells. MCF-7 cells express significantly higher levels of PPARγ expression than all but four cell lines and higher RXRα than all of the cells tested except T47-Ds. MDA-MB-231 cells had PPARγ mRNA levels similar to MCF-7 cells, but had lower RXRα expression with only two cell lines having significantly lower levels.

### 3.5. Effect of Ros and 9-cis-retinoic acid co-treatment on reporter activation in selected cell lines

Three cells lines were selected to determine if the relative expression of PPARγ and RXRα are predictive of the effect that co-treatment with a PPARγ agonist (Ros) and RXRα (9-*cis*-retinoic acid) agonist have on the PPRE reporter assay. HT-29, MCF-7, and MDA-MB-231 cells were selected to test this principal because Ros was shown to activate the reporter and the cell lines expressed varying levels of PPARγ to RXRα. MCF-7 cells were found to express more RXRα than PPARγ. Conversely, MDA-MB-231 and HT-29 cells expressed more PPARγ than RXRα. These three cell lines were transfected with the PPRE reporter construct and treated with

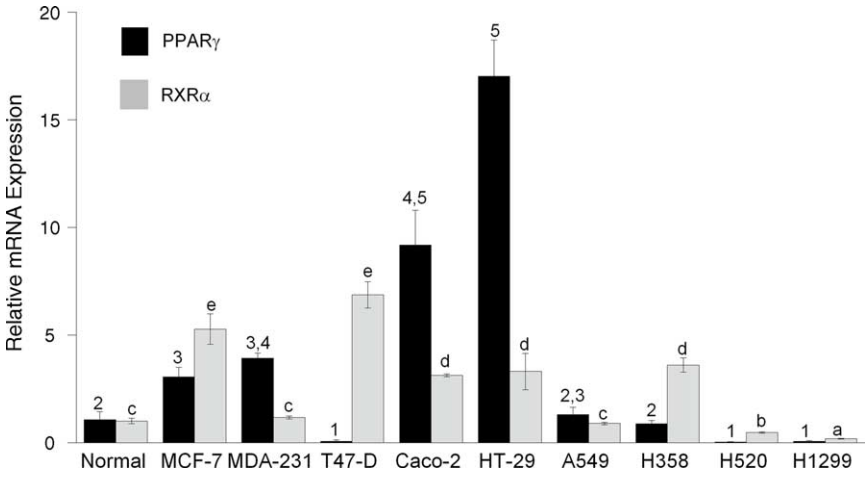


Fig. 4. Relative expression of PPARγ and RXRα cancer cell lines of the breast, colon, and lung. PPARγ and RXRα were detected by real-time PCR. Total mRNA was collected from untreated cells for each of the 10 cell lines. The relative expression levels of PPARγ and RXRα as compared to the endogenous control, human GAPDH were normalized to the expression of the targets in the normal mammary epithelia. Error bars represent the standard error of the mean.

either vehicle, Ros alone, or co-treated with Ros and 9-*cis*-retinoic acid. Ros alone and the co-treatment resulted in significantly higher activation of the reporter in all three cell lines when compared to vehicle controls (Fig. 5). In HT-29 cells, activation of the reporter was higher in the cells treated with Ros alone compared to those receiving the co-treatment. Conversely, co-treatment resulted in significantly greater reporter activity in the MCF-7 and MDA-MB-231 cells when compared to Ros treatment alone.

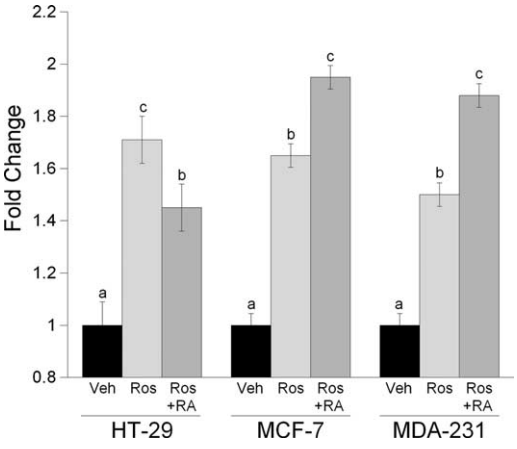


Fig. 5. Effect of cotreatment with Rosiglitazone and 9-*cis*-retinoic acid on reporter activation in various. Cells were transiently transfected with a 3XPPRE-TK-pGL3 reporter vector. The cells were then treated with either rosiglitazone (Ros) or rosiglitazone plus 9-*cis*-retinoic acid (Ros + RA) for 18 h. Luciferase activity was normalized to renilla. Data is expressed as mean fold changes in luciferase to renilla ratios compared to vehicle control for each treatment group. These data are representative of three separate experiments. Error bars represent the critical value for comparison. Statistical comparisons were only made within cell lines and not between. Alphabetical letters are used to signify groups that are statistically different. Error bars that do not share a letter designation were determined to be significantly different. Letter designations between cell lines do not represent statistical differences.

## 4. Discussion

Data from the present study demonstrate that individual PPARγ ligands have the ability to selectively activate a PPRE reporter in cancers of the breast, colon, and lung. Differences in PPRE reporter activation were observed between cells derived from different tissue types as well as between cell lines of the same cancer type. Also, within a single cell line, individual ligands selectively induced PPRE reporter activity. Expression of PPARγ and RXRα mRNA were measured in all cell lines in the absence of treatment, but expression was not predictive of how individual cell lines responded to ligand treatment. Finally, differences in how individual cell lines responded to co-treatment with PPARγ and RXRα agonists were observed.

A growing body of evidence indicates PPARγ is involved in both breast cancer development and progression. PPARγ(+/−) mice had almost three-fold increased incidence of mammary adenocarcinomas and decreased survival rate when compared to PPARγ(+/+) litermates (Nicol et al., 2004). Several reports have demonstrated that treating animals with PPARγ ligands prior to chemical induction of mammary tumors is protective against tumor development (Mehta et al., 2000; Suh et al., 1999). It appears that expression and transactivation of PPARγ is protective against breast tumor formation particularly when activated by PPARγ ligands. Our data demonstrate that some ligands are more effective than others in transactivating PPARγ in normal mammary epithelia.

Once a breast tumor has formed, PPARγ appears to have multiple effects. In vitro, treatment of breast cancer cells with troglitazone results in lipid accumulation, changes in gene expression associated with cellular differentiation, reduction in growth rate and clonogenic capacity (Mueller et al., 1998). Others have observed that distinct PPARγ ligands induce apoptosis (Elstner et al., 1998). Conversely, a recent

report by Saez et al. (2004) found that when mice expressing a constitutively active form of PPARγ in the mammary gland were crossed with mice prone to mammary gland cancer, bigenic animals develop tumors that express higher levels of markers of malignancy. The authors conclude that once an initiating event takes place, increased PPARγ signaling serves as a tumor promoter in the mammary gland of these experimental animals. Collectively, these data suggest that the physiological consequence of PPARγ activation is dependent on many factors including the stage of development of the specific breast cancer cell. Our demonstration that individual PPARγ ligands distinctively modulate PPRE reporter activity in breast cancer cell lines differently has implications for breast cancer treatment. Specifically, T47-D cells were fairly unresponsive to any of the three PPARγ agonists tested, whereas, Ros, Cig, and $PGJ_2$ significantly increased reporter activity in MCF-7 and MDA-MB-231 cells. It can be concluded that individual breast cancer cell types are likely to respond to PPARγ ligands in unique physiological ways and our data suggests that, in part, variant cellular responses are the result of selective PPARγ transactivation.

PPARγ also influences colon tumor development and growth. In mice predisposed to the development of intestinal polyps caused by a mutation in the adenomatous polyposis coli (APC) gene, treatment with troglitazone or Ros increases both the number and size of intestinal polyps (Lefebvre et al., 1998; Saez et al., 1998). These data are partially explained by studies demonstrating that PPARγ looses its ability to influence colon tumorgenesis in mice with a mutated APC gene, where as in wild-type APC mice, PPARγ functions as a tumor suppressor (Girnun et al., 2002). Conversely, PPARγ ligands reduce aberrant crypt foci (ACF) formation in mice following tumor induction by azoxymethane (Osawa et al., 2003). Differences in the effects of PPARγ ligands in these two models demonstrates that like mammary cells, colon cancer cells respond to a single PPARγ ligand differently dependent on the cell characteristics. When we examined the ability of PPARγ ligands to activate the PPRE reporter construct in two colon cancer cell lines, differences in cellular responsiveness was revealed. Ros significantly increased reporter activity in both Caco-2 and HT-29 cells though the level of responsiveness was much greater in the HT-29 cells. Furthermore, Cig and $PGJ_2$ did not significantly enhance reporter signal in Caco-2 cells, but were strong agonists in the HT-29 cells. Selective modulation of PPARγ transactivation can explain the variant physiological responses observed in different colon cancer animal models. Differences in ligand activity could have significant impact on colon cancer treatment strategy as a number of studies have shown that PPARγ ligands affect colon tumor cell progression. Treatment of colon cancer cells with PPARγ agonists inhibits their growth in vivo (Brockman et al., 1998; Kitamura et al., 1999; Sarraf et al., 1998; Shimada et al., 2002) and in vitro (Sarraf et al., 1998). Inhibition of growth is often attributed to PPAR induced apoptosis and DNA fragmentation (Chen et al., 2002; Shimada et al., 2002; Yang and Frucht, 2001).

In the lung, epithelial cells possess 15-lipoxygenases which produce a variety of metabolic products including 15(S)-hydroxyeicosatetranoic acid (15(S)-HETE) (Profita et al., 2000). In A549 cells, 15(S)-HETE has been demonstrated to induce apoptosis by binding to PPARγ (Shankaranarayanan and Nigam, 2003). Similarly, treatment of adenocarcinoma (A549) cells with Cig resulted in growth inhibition (Chang and Szabo, 2002); however, this inhibition was not observed in either squamous cell carcinoma (H520) or large cell carcinoma (H1299) cell types (Chang and Szabo, 2002). In the present study, two adenocarcinoma (A549 and H358) and two nonadenocarcinoma (H520 and H1299) cell lines were selected for evaluation. The nonadenocarcinoma cell lines were highly unresponsive to the PPARγ ligands when compared to the adenocarcinoma cells. Ros and Cig significantly increased reporter activity in H1299 and H520 cells, but fold change compared to control was relatively small in these cells. Conversely, A549 and H358 cells were highly responsive to the PPARγ agonists. These data suggest that the varying effects of Cig on adenocarcinoma versus nonadenocarcinoma cells observed in Chang et al., 2002 are likely the down stream result of selective PPRE transactivation.

One focus of these studies was to determine whether individual ligands of PPARγ could act as SPARMs. We present evidence indicating that within each tissue type, individual ligands are capable of selectively activating the PPRE reporter construct dependent on the individual cell line tested. However, individual ligands had unique effects across tissue types as well. For instance, we report that GW, a known PPARγ antagonist in adipocytes (Leesnitzer et al., 2002; Starkey et al., 2003), significantly increased reporter activity in HMECs. This effect was observed in no other cell line except H1299 cells and in those cells the magnitude of change was very small leaving in question its biological significance. These findings are significant because they suggest that an individual compound can function as a PPARγ antagonist in one tissue and as an agonist in other tissues. It is possible that the agonist activity of GW is specific to normal epithelial cells and that changes occur during cancer cell formation that results in the loss of this responsiveness. It is also possible that the actions of GW are mammary specific. Further, investigation is necessary to explore these possibilities.

Another objective of these studies was to determine if PPARγ mRNA expression is predictive of a cell line's responsiveness to PPARγ ligands with regards to PPRE activation. For three of the cell lines (MCF-7, MDA-MB-231, and HT-29) increased relative expression of PPARγ over HMECs correlated with enhanced reporter activity when exposed to the PPARγ agonists. However, A549 and H358 had higher reporter activity in response to the individual PPARγ agonist compared to Caco-2 cells despite the fact that A549 and H358 cells express much lower levels of PPARγ than the Caco-2s. Therefore, PPARγ mRNA levels alone are not predictive of PPARγ mediated PPRE activation. These data led us to ex-

plore the possibility that variances of expression of RXRα, the heterodimic partner of PPARγ, and it's relation to the levels of PPARγ in the different cell lines may play a critical role in PPARγ's ability to activate the PPRE reporter construct. Here we demonstrate that the cell lines differed in their relative expression of PPARγ to RXRα; however, there was no unifying pattern of receptor expression that was predictive of ligand activity in the reporter assay.

Unique expression patterns of PPARγ relative to RXRα in certain cell lines led us to hypothesize that the relative expression of these receptors may be used to identify cell lines in which co-treatment with an RXRα agonist (9-*cis*-retinoic acid) would enhance PPRE reporter activation. Three cell lines were chosen to test this hypothesis. HT-29 and MDA-MB-231 cells express higher levels of PPARγ relative to RXRα and would therefore, not be expected to have increased reporter activity with co-treatment (Ros + 9-*cis*-retinoic acid) compared to Ros alone. Conversely, MCF-7 cells having higher expression of RXRα relative to PPARγ would supposedly demonstrate enhanced reporter activity when treated with both ligands compared to Ros alone. HT-29 cells showed no additional reporter activity in cells co-treated with Ros and 9-*cis*-retinoic acid over those treated with Ros alone. MCF-7 cells had enhanced activation of the PPRE reporter with co-treatment over Ros treatment alone. These two cell lines support the possibility that relative expression levels may be predictive in identifying cells that will more readily respond to co-treatment with both ligands. However, MDA-MB-231 cells did not support this hypothesis. Co-treatment with both ligands had a significantly higher effect on reporter activity when compared to single treatment with Ros even though relative PPARγ expression was higher than RXRα expression in these cells. These data suggest that while this approach may work for certain cancer cell types, receptor expression alone may not be predictive for how cells will respond to co-treatment. A better understanding of how PPARγ and RXRα function in each of the cell lines will be necessary before the predictive value of receptor expression can be realized.

In conclusion, PPARγ ligands, have distinct activities within a cell type, between tumor cells derived from the same tissue, and across distinct tissues. Although we used only one type of PPRE reporter construct in these experiments, the selective modulation of PPARγ within individual cells is likely to be a gene/promoter specific event. Nonetheless, utilizing this reporter of PPAR transactivation, differences were observed in all three cancer types (breast, colon, and lung) where a single ligand enhanced reporter activation in certain cell types, but had minimal to no effect in other cell lines of the same malignancy type. Finally, our data demonstrates that individual PPARγ ligands can selectively activate reporter activity within a single cancer cell type. These data suggest that the effectiveness of PPARγ as a target for chemotherapeutic treatment will greatly depend on the cell that is treated which opens the possibility of utilizing PPARγ for targeted gene therapy.

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