Cancer Preventive Rexinoid Modulates Neutral Lipid Content of Mammary Epithelial Cells through a PPARγ-dependent Mechanism

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Abstract
Synthetic rexinoids effectively suppress both estrogen receptor-positive and –negative mammary tumors in animal models, making them prime candidates for a novel class of cancer preventive agents. When used in combination with chemotherapy in non-small cell lung cancer, the rexinoid bexarotene was most effective in patients who developed hypertriglyceridemia as a side-effect. While serum triglycerides originate from the liver, the effect of bexarotene on lipogenesis in breast epithelial cells is not known. Gene expression studies of normal mammary epithelial cells (HMECs) indicated that rexinoids modulate lipid metabolism in particular, enzymes involved in triglyceride synthesis. High content analysis revealed dose-dependent accumulation of neutral lipids within adipophilin (ADFP)-associated cytoplasmic lipid droplets following long-term bexarotene treatment. Bexarotene also induced the mRNA and protein levels of the peroxisome proliferator-activated receptor gamma (PPARγ), while selective knock-down of PPARγ attenuated the induction of both lipid droplets and ADFP. Pharmacologic activation of PPARγ, but not PPARα or retinoic acid receptors (RARs), effectively induced lipid accumulation. Furthermore, combining the PPARγ agonist rosiglitazone with bexarotene synergistically suppressed the growth of HMECs, and revealed a strong non-linear inverse correlation of cell growth to lipid droplet accumulation in the cell population. These findings indicate that rexinoids activate a lipogenic program in mammary epithelial cells through an RXR/PPARγ–mediated mechanism. Importantly, combining low doses of bexarotene with the PPARγ agonist rosiglitazone provides effective growth suppression of mammary epithelial cells, potentially dissociating systemic adverse effects associated with standard bexarotene treatment from the anti-proliferative effects on the mammary epithelium.
Introduction

The feasibility of the chemoprevention of estrogen receptor (ER)-positive breast cancers has been established by the use of selective estrogen response modifiers (SERMs) (Cuzick et al., 2003) and the demonstration that ligand-dependent transcription factors are ideal targets for cancer preventive agents (Uray and Brown, 2006). However, effective preventive agents for ER-negative breast cancers still need to be developed (Uray and Brown, 2011). Retinoids selectively activating retinoid X receptors (rexinoids) efficiently suppress the development of mammary tumors in animal breast cancer models (Gottardis et al., 1996), both alone or in combination with agents of different mechanism of action. Unlike anti-estrogenic compounds, rexinoids prevent the development of both ER-positive and ER-negative breast tumors (Bischoff et al., 1999; Wu et al., 2002). Bexarotene is a synthetic rexinoid which has been approved for the treatment of refractory cutaneous T-cell lymphomas and tested against other cancer types in combination with various chemotherapeutic protocols, with moderate success. While the cancer preventive potential of bexarotene exceeds its effectiveness in the treatment of existing cancers, its clinical use is affected by dose-limiting side-effects, primarily hypertriglyceridemia arising from elevated hepatic very low density lipoprotein (VLDL) production (de Vries-van der Weij et al., 2009). Interestingly, a Phase III clinical trial comparing the effects of chemotherapy with chemotherapy plus bexarotene in advanced non-small-cell lung cancer patients found that the occurrence of high-grade hypertriglyceridemia correlated with increased survival in bexarotene-treated patients (Blumenschein et al., 2008), suggesting a connection between lipid metabolism and cell growth control. Conversely, while inducing tumor regression in several rodent mammary carcinoma models, its antitumor effects correlated with the induction of adipocyte-specific gene expression (Agarwal et al., 2000). In contrast to the causes for elevated systemic triglycerides, the consequences of rexinoid treatment on the lipid metabolism of the epithelial cells, the actual targets of cancer prevention, are not well characterized. Our previous studies have indicated that bexarotene regulates the expression of genes involved in lipid metabolism (Abba et al., 2008; Kim et al., 2006). Differentiation and lactation in the mammary gland are also associated with lipid accumulation and expression of perilipins, highly phosphorylated adipocyte proteins localized at the
surface of lipid droplets, in secretory cells as a result of a concerted, developmentally regulated program to increase the availability of fatty acids necessary for lipid synthesis (Russell et al., 2007). Thus, we adopted a high throughput image-based assay (e.g., high content analysis, HCA) to quantitatively evaluate the effect of rexinoids on lipid metabolism, proliferation and nuclear receptor levels in mammary epithelial cells. A further goal of this study was to elucidate whether the systemic side effects of bexarotene could be dissociated from its growth suppressive effect on the mammary epithelium.

The cancer preventive effects of rexinoids are largely attributed to their ability to elicit cell cycle arrest and inhibit mammary epithelial cell growth both in vitro and in vivo (Li et al., 2007; Wu et al., 2006). Therefore, proliferation markers currently serve as surrogate biomarkers of a cancer preventive effect in the breast. Bexarotene-induced hypertriglyceridemia is controlled by dose adjustment of the drug or the addition of lipid-lowering therapy (Assaf et al., 2006); however, it remains to be shown whether or not the treatment will retain its chemopreventive effect at reduced dosage.

Our data show that the RXR-selective retinoid bexarotene induces the accumulation of neutral lipid-containing cytoplasmic droplets by activating an RXR/PPARγ-dependent lipogenic program in mammary epithelial cells. This increase in neutral lipid content is concomitant with the up-regulation of PPARγ levels as well as the enzymes required for triglyceride synthesis. The data also demonstrates that combination of low-dose bexarotene with the PPARγ agonist rosiglitazone acts synergistically to suppress the growth of mammary epithelial cells. As marked lipid accumulation occurs at higher bexarotene doses, potentially adverse responses may be dissociated from the anti-proliferative effects of bexarotene when combined with rosiglitazone.
MATERIALS AND METHODS

Cell culture

Normal human mammary epithelial cells (HMECs) were derived from healthy women who had undergone reduction mammoplasties and were purchased from Clonetics Corporation (San Diego, CA). Cells from at least five different isolates were used in the experiments, with their passage numbers kept below 12. HMECs were maintained in Mammary Epithelial Basal Medium (MEBM) supplemented with 50 µg/mL bovine pituitary extract, 5 µg/mL insulin, 10 ng/mL human recombinant epidermal growth factor, 0.5 µg/mL hydrocortisone, 30 µg/mL gentamicin, and 15 ng/mL amphotericin-B (Clonetics Corporation). Cells were cultured in a humidified environment at 37°C with 5% CO₂ in the air.

Ligands

The synthetic RXR-ligands bexarotene (Targretin, LGD1069) and LGD100268 were a kind gift from Dr. Reid Bissonnette at Ligand Pharmaceuticals. Rexinoids were dissolved and kept in 50/50% dimethyl sulfoxide (DMSO)/ethanol solvent and used at 1 μM final concentration, unless otherwise indicated. siRNA duplexes were designed and synthesized by Sigma-Proligo (Sigma-Aldrich, St. Louis, MO) and Dharmafect 1 siRNA Transfection Reagent was purchased from Thermo-Fisher Scientific Inc. (Pittsburgh, PA).

Fluorescence microscopy

For general fluorescent imaging purposes HMECs were seeded on 0.17 mm glass coverslips 24 hours before treatment. For high throughput, high content applications HMECs were plated on Greiner 96-well or Aurora 384-well optical plastic bottom test plates at densities of 2,000 and 500 cells per well, respectively. Cells were then exposed to bexarotene or solvent, or other nuclear receptor ligands for 24 hours. Compound dilutions and addition to multi-well plates were performed using a Beckman NX liquid handling system. On completion of the treatment plates were washed with cold PBS and fixed for 20 minutes at room temperature using 4% formaldehyde. After fixation, cells were briefly permeabilized (2 min) with 0.1% Triton-X100 and prepared for imaging by washing in PBS and adding a 1 μg/ml DAPI
solution. For the specific detection of triglycerides (TG) as dominant neutral lipids within rexinoid-treated cells we used the green fluorescent dye LipidTox (Invitrogen, Carlsbad, CA) at 1:500 neutral lipid stain. For the immunofluorescent detection of proteins cells were incubated for 30 minutes in 5% non-fat milk in Tris-buffered saline, followed by incubation with antibodies against PPARγ (1:200), RXRα (1:500) or ADFP (1:200) overnight at 4°C. Fluorescent images were captured using an IC 100 automated cytometer (Beckman Coulter, San Diego, CA) running CytoShop 2.0 or using an ImageXpress Micro (Molecular Devices Inc., Sunnyvale, CA) high throughput imager. Three 8-bit images per field were acquired with a Nikon S Fluor 20X/0.75NA or S Fluor 40X/0.90NA objective and 2x2 binning (DAPI, GFP/Alexa488, Alexa594) and resulted in 0.344x0.344 μm²/pixel grayscale bitmapped images. The used fluorophores: DAPI, LipidTox and Alexa594-labeled secondary antibody were excited at 358 nm, 488 nm and 594 nm, respectively, using appropriate band-pass filters. In general, 12 to 64 fields, with a minimum of 200 cells imaged per well for analysis. Apoptotic and fragmented cells, as well as non-segmentable structures were excluded from the analysis. High-resolution, 3D images were acquired and deconvolved with a DeltaVision Core Fluorescent Image Restoration System using 40X/0.95 NA “dry” objective or a 60X/1.3NA oil objective (Applied Precision Instruments, Issaquah, WA).

**Image analysis and quantitation**

Commercially available image analysis algorithms specifically developed to accurately identify and quantitate subcellular structures were used to create overlay masks, thereby allowing the measurement of lipid droplets and nuclear structures as well as the colocalization of lipid-associated proteins. Analysis of microscopy data was carried out by the Lipid Droplet and the Colocalization algorithms of the CyteSeer image analysis software (Vala Sciences Inc, San Diego, CA) as described before in detail (McDonough et al., 2009). In brief, after segmenting cell nuclei using the DAPI channel, pixels identified as above threshold in the lipid or the antibody image by each algorithm were defined as the “lipid mask” or the “protein mask”, respectively. Lipid content of a cell was measured by the total integrated intensity of the lipid signal (in the FITC channel) under the lipid mask (e.g., segmented lipid droplets; see Figure 2). Lipid droplet-derived and all other (>60) measurements from the areas of each mask were reported on a
per cell basis. The resulting numerical data included both single cell and well-by-well readouts, representing data averaged across all cells imaged within each well. Cells whose nuclei crossed the edge of an image were excluded from measurements. Further, as a measure of health/toxicity, cells with nuclear DNA content <50% of the average value corresponding to G0/G1 were excluded from further analysis. To quantitatively assess the suitability of the assay for subsequent use in high content screening experiments, we determined Z-factor (Z′) values for the various measurements available through the image analysis algorithms. The Z′ statistic is commonly used to quantify the robustness of assays (Zhang et al., 1998). 

\[ Z' = 1 - 3 \times (SD_{max} + SD_{min})/(X_{max} - X_{min}) \]

where SD = standard deviation, and X = mean. For Z′ to be positive (e.g., Z′ > 0), the sum of the SD’s from the maximum and minimum measurements must be less than 1/3th the range of the assay (range = Xmax – Xmin). For an ideal assay in which there is no variation in the determinations (i.e., SDs = 0), \( Z' = 1 \). Z′ values above 0.2 are considered “screenable”, a Z′ > 0.5 is considered excellent. The Z′ score for the measurements of both cell counts and total cellular lipid signal intensity under the lipid mask exceeded Z′ = 0.55, indicating a very low chance of false positive hits arising from random variation in a high throughput screening application.

**RNA extraction and measurement of transcript levels**

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Reverse transcription (RT) was performed in triplicate, with a “non-RT control” lacking reverse transcriptase in parallel, to control amplifications due to genomic DNA contamination. Gene specific primers and dual-labeled TaqMan probes in qRT-PCR assays to measure mRNA levels of lipogenic genes are listed in Table 1. Transcript quantitation based on real-time monitoring of amplification was carried out using an ABI PRISM 7900 HT Sequence Detection System performing 40 cycles of 95°C for 12 seconds and 60°C for 30 seconds. Values of transcripts in unknown samples were obtained by interpolating their Ct (PCR cycles to threshold) values on a standard curve derived from known amounts of cognate, amplicon-specific synthetic oligonucleotides. Transcript levels were normalized to the level of cyclophilin mRNA.

**Western-blot analysis**
Proteins extracts were solubilized and fractionated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% bovine serum albumin, the membrane was probed with antibodies against stearoyl-CoA desaturase 1 (SCD1) (1:200; sc14715, Santa Cruz Biotechnology Inc., Santa Cruz, CA), ACSL1 (1:1000; 4047, Cell Signaling Technology, Inc., Danvers, MA) or DGAT1 (1:200; SAB2500307, Sigma-Aldrich Co., St. Louis, MO) and subsequently incubated with peroxidase-conjugated secondary antibody (1:5000; GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Signals were visualized by ECL detection (Thermo Fisher Scientific, Rockford, IL). After stripping (30 min at 50°C in 2% SDS and 100mM β-mercapto-ethanol) the same membrane was re-probed with anti-β-actin antibody (1:5000; Sigma-Aldrich Co., St. Louis, MO). Densitomeric quantitation was performed using Image J, a public domain, Java-based image processing program developed at the National Institutes of Health. The protein levels of lipogenic enzymes were normalized to β-actin.

Selective gene knock-down experiments

To assess the effect of gene silencing on target gene expression and lipid accumulation, 10⁴ cells were seeded on 24-well tissue culture plates 24 hours prior to transfection. Transfection conditions were optimized for highest level of knock-down at the lowest toxicity using pools of three gene-specific siRNAs at 10 nM concentration and Dharmafect 1 transfection reagent (Dharmacon, Inc., Lafayette, CO). Suppression of transcript levels exceeding 70%, as measured by real-time quantitative RT-PCR, was considered acceptable.

Statistical analysis

Statistical significance was determined using the student’s t-test or two-tailed analysis of variance (ANOVA). A p value of <0.05 was considered statistically significant. Values are presented as means +/- SD. Synergy between two agents was quantitatively assessed by constructing isobolograms with the CalcuSyn software (Biosoft, Cambridge, UK) which performs drug dose-effect calculations using the median effect method described by Chou and Talalay (Chou and Talalay, 1984). Distinction from additivity and statistical significance of the synergistic effect was established by direct comparison of the
combination with the relative effects of both individual drugs at their respective effective dose (the dosage that produces a desired effect in half the test population (ED50)) as described by Laska et al. (Laska et al., 1994). Comparisons between groups were carried out by two-way Student’s t-tests.

RESULTS

It is now well established that rexinoids induce changes in the cell cycle and suppress the growth of non-transformed mammary epithelial cells (HMECs) through the modulation of growth regulatory transcription factors and signaling molecules (Uray et al., 2009; Wu, DuPre et al., 2006). However, the growth suppressive effects of these agents do not strictly correlate with their chemopreventive potential, suggesting that other factors also contribute to the cancer preventive activity of rexinoids. In addition to regulating cell growth, our previous studies have also indicated that the synthetic rexinoid bexarotene (Bex, LGD1069, Targretin) preferentially regulates the expression of genes involved in lipid metabolism (Abba, Hu et al., 2008; Kim, Kong et al., 2006). Quantitative RT-PCR analysis of mRNA levels showed that the expression of key enzymes involved in TG synthesis, such as the long chain acyl-CoA synthase 1 (ACSL1), stearoyl-CoA dehydrogenase 1 (SCD1) and diacylglycerol acyl transferase (DGAT1), were significantly up-regulated after 24 hours bexarotene treatment (Figure 1A). To detect changes in the protein levels of these lipogenic enzymes we performed Western-blot analyses using total cell extracts from HMECs treated with solvent or 1 μM bexarotene for 48 hours (Figure 1B). Densitometric analysis revealed that all three of these enzymes ACSL1, SCD1 and DGAT1 were, in fact, induced by bexarotene 5-fold (p<0.005), 2.3-fold (p<0.05) and 1.75-fold (p<0.005), respectively (Figure 1C). Furthermore, when we examined the lipid content of HMECs on long-term treatment with 1 μM bexarotene using the general lipid dye Oil Red O, qualitative analysis by brightfield imaging indicated an overall increase in cytoplasmic lipid content after a period of no less than four days bexarotene treatment (data not shown).

To better discriminate and quantify cellular phenotypic changes in response to rexinoids on a cell-by-cell basis, we developed a high-content image-based assay that simultaneously measures cytoplasmic
lipid features (e.g., lipid droplet number and size), nuclear DNA content, expression of lipid associated proteins, and transcription factors that regulate lipid metabolism. High resolution deconvolution microscopy and 3D viewing confirmed the presence of elevated levels of neutral lipids and indicated the formation of small adipocyte-like lipid droplets in the cytoplasm of HMECs treated with bexarotene (Figure 2A, B). Quantitative assessment of the rexinoid response based on changes of the cellular phenotype was achieved by specific segmentation of lipid droplets, cell nuclei, cell boundaries and quantitation of RXRα immunolabeling (Figure 2C). Cell-by-cell analysis following automated high throughput microscopy (HTM) revealed that, in over 90% of the cells, neutral lipid content exceeded the average of the untreated cells after bexarotene treatment and was overall 7-fold higher (Figure 2D). These data indicated a robust lipid content increase, albeit heterogeneous among individual cells. Importantly, we observed similar levels of neutral lipid accumulation in five different batches of primary mammary epithelial cells, derived from different individuals.

The image data further demonstrated that the increase of intracellular lipid droplet counts (Figure 2E) was also associated with small but statistically significant increases in both droplet diameter (Figure 2F) and surface area (data not shown). The product of the changes in these parameters and the increased lipid droplet counts amounted to a marked increase in total cellular neutral lipid content (Figure 2G) upon bexarotene treatment. Lipid droplet counts were highly correlated with the total intensity of the lipid signal (r²=0.78, p<0.0001, figure not shown). Furthermore, using deconvolution-based 3-D imaging, we verified that quantitation of both integrated pixel intensities and lipid droplet counts provide a good estimate of the accumulated lipid mass.

Using a three compartment masking approach (e.g., nuclei-DAPI, lipid droplet-LipidTox, and cell boundary-CellMask, a general protein dye), fluorescently-labeled cytoplasmic lipid droplets were shown to predominantly colocalize with immunolabeled adipocyte differentiation-related protein (ADFP, ADRP, adipophilin), a protein known to play a role in adipogenic differentiation (Russell, Palmer et al., 2007). Further, total and lipid droplet-associated ADFP levels were also markedly increased in bexarotene-treated cells (Figure 2H-J). Bexarotene also exponentially induced ADFP mRNA levels 48 hours
following drug treatment (Figure 2K). In summary, elevated triglyceride production appears to primarily result in an increase in the number of lipid droplets, rather than a marked increase in their size.

To assess the sensitivity of HMECs to bexarotene, cells were treated with escalating doses of the drug over a period of four days (Figure 3A). Lipid accumulation occurred in a dose-dependent manner and a significant increase could be defined with bexarotene levels under 0.1 μM, a concentration lower than needed to significantly suppress growth in these cells. Immunofluorescent labeling of RXRα indicated its ubiquitous expression and RXRα levels in the nuclei at the time of imaging were unaffected by bexarotene doses up to 1 μM (data not shown). The ratio between nuclear and cytoplasmic fractions of RXRα was determined from the integrated pixel intensities under the nuclear versus the cytoplasmic masks. As expected, the majority of RXRα was always in the nuclear fraction, although some cytoplasmic labeling was also consistently detected. However, neither the number of lipid droplets nor the amount of cytoplasmic lipids showed any correlation with RXR expression.

Rexinoids can activate a number of RXR-containing permissive heterodimers to regulate the expression of target genes of their partners. Thus, we compared the effects of agonists of PPARs and retinoid receptors on lipid accumulation in HMECs. Rosiglitazone is an insulin sensitizer thiazolidinedione clinically used for the treatment of type II diabetes that selectively activates the PPARγ receptor (Kahn and McGraw, 2010). We found that both RXR agonists bexarotene (Bex) and LGD100268 (LG268), as well as the PPARγ agonist rosiglitazone (Rosi), increased the number of lipid droplets in primary HMECs, whereas a PPARα agonist, WY14643, (WY) and a pan-RAR agonist (TTNPB) were ineffective (Figure 3B). Further, immunofluorescent quantitation of PPARγ expression following bexarotene treatment showed that both the nuclear PPARγ protein and its mRNA levels increased (Figure 3C, 3D left panel). Next, siRNA transfections were performed to verify that PPARγ was required for both lipid accumulation and droplet formation. Knock-down of PPARγ in HMECs markedly reduced the mRNA levels of PPARγ, versus that observed with the control (siNT) (Figure 3D, right panel). Transfections of siRNAs against PPARα, PPARδ or RXRα resulted in over 80% reduction
in their respective target mRNA levels, but had no impact on PPARγ expression, as measured by qRT-PCR (data not shown). Figure 3E shows the comparison of the neutral lipid contents of HMECs treated with vehicle or bexarotene after siRNA mediated knock-down of RXRα, PPARα, PPARδ and PPARγ. Bexarotene induced a significant induction of lipid content in all knock-down groups, however the extent of the changes were markedly different, depending on the selective suppression of the receptors. Statistical tests (ANOVA and Dunnett’s multiple comparison post-hoc test) comparing the changes in lipid content reveal that the bexarotene-induced change in lipid content in the control knock-down groups (siNT) were significantly (p<0.05) different from all other groups transfected with siRNAs against RXRα, PPARδ and PPARγ, but not PPARα (Figure 3E), indicating that suppression of RXRα and PPARγ diminished the ability of bexarotene to induce lipid accumulation. In addition, silencing of PPARγ also abrogated the induction of ADFP mRNA (Figure 3F).

Individual ligand experiments were then followed by combined treatments of rosiglitazone and bexarotene. As shown in Figure 4A, bexarotene (1 μM) and rosiglitazone (2 μM) in combination were more than twice as effective as either drug alone. The statistical analysis indicated a significant difference between cellular lipid contents of control and bexarotene-treated cells, and also control versus bexarotene plus rosiglitazone-treated cells. In comparison, the extent of change upon bexarotene alone was significantly different from the change induced by the combination of bexarotene plus rosiglitazone. In contrast, the amount of cellular neutral lipid content was inversely proportional to cell counts at the end of the six day treatment (Figure 4B). While rosiglitazone was only moderately growth suppressive by itself, it enhanced the growth suppression elicited by bexarotene. To determine whether the effects of the two ligands were additive or synergistic, we performed combined dose response experiments in a matrix of varying dose combinations, including combinations at nanomolar concentrations. Cell proliferation was determined by image-based cell counting (triplicate wells with 45 imaged microscopic fields per well) after four days of treatment (Figure 5A). The cell proliferation dose-response curve of HMECs to bexarotene alone indicated an EC50 of 0.1 μM and rosiglitazone alone showed an EC50 of 3.1 μM. The isobologram resulting from the combinations of incremental doses of these two agents indicated that one
or both constituents contributed to the growth suppressive effect to a greater degree than its own potency, resulting in superadditive, or synergistic effects (Figure 5B). The synergistic combinations of rosiglitazone and bexarotene identified by the Calcusyn algorithm include 0.2, 0.66 and 2 micromolar rosiglitazone and 0.01-0.1 micromolar bexarotene, respectively (table in Figure 5C). To validate these findings, the effects of combinations were compared to the ED50 effects of bexarotene and rosiglitazone applied individually. The combinations significantly different from the ED50s of both individual compounds are shown in Figure 5D. Notably, the various combinations of bexarotene and rosiglitazone were not synergistic across the entire dose range, but primarily with low doses (0.01-0.1 μM) of bexarotene and 0.66-2 μM doses of rosiglitazone.

The combination of bexarotene and rosiglitazone also resulted in enhanced lipid accumulation, although the interaction between the two agents did not appear synergistic with respect to lipid content. Overall, there was a marked non-linear inverse correlation between cell counts and the mean cellular lipid content after four days of treatment when comparing various bexarotene and rosiglitazone combinations (p<0.001, Figure 5E). Markedly, the increase in bexarotene concentration from 0 to 10 nM causes a downshift of the rosiglitazone dose-response curve toward lower cell counts (less proliferation, y axis) and a slight shift to the right (x axis), indicating a minimal increase in lipid content (arrow “a”). In contrast, the incremental rosiglitazone doses combined with 100 nM bexarotene resulted in a minor decrease in cell counts, but a greater shift to the right (arrow “b”), indicative of higher lipid content. Subsequent higher bexarotene doses (0.33, 1 μM, etc.) cause further shifts to the right, indicating an even stronger lipid-inducing effect, and less growth suppression. Taken together, the dose pairs of low dose bexarotene and rosiglitazone with synergistic efficacy for growth suppression did not result in additive lipid accumulation, indicating that effective growth suppression of mammary epithelial cells can be achieved using significantly reduced doses of bexarotene combined with rosiglitazone, without a proportionate elevation in lipid levels.
DISCUSSION

RXR-selective retinoids such as bexarotene (Bex, LGD1069, Targretin) have proven very effective agents in the prevention of both ER-positive and ER-negative breast cancers in various animal models, and are currently being tested in clinical trials. However, the effective dose of bexarotene is associated with side-effects that limit its use as a chemopreventive drug, therefore the development of dose-reduction strategies has become particularly important. While the causes for elevated serum triglyceride levels are known, the consequences of rexinoid treatment on lipid metabolism within epithelial cells, the actual target cells of cancer prevention, have not been elucidated. In the present study we applied a high content analysis approach to define rexinoid-induced changes in lipid metabolism and cell growth of breast epithelial cells on a cell-by-cell basis, rather than the usual approach of qualitative assessment of lipid content in bulk populations. We show that effective growth suppression of mammary epithelial cells may be achieved using low doses of bexarotene when combined with the PPARγ agonist rosiglitazone, thereby dissociating the adverse systemic effects of bexarotene from the therapeutic anti-proliferative effects on the mammary epithelium.

Apart from refractory cutaneous T-cell lymphomas, bexarotene monotherapy failed to demonstrate significant anti-tumor efficacy or improve overall survival in most cancers, however, combinatorial chemotherapeutic applications of bexarotene appear more promising in several tissues (Cesario et al., 2006; Dragnev et al., 2005; Khuri et al., 2001; Yen et al., 2004). Interestingly, rexinoids proved highly efficacious chemopreventive agents in a number of preclinical rodent models of breast cancer (Gottardis, Bischoff et al., 1996; Li, Zhang et al., 2007; Wu, Kim et al., 2002). It was noted early on that the anti-tumor effects correlated with the induction of adipocyte-specific gene expression (Agarwal, Bischoff et al., 2000). However, the effects of rexinoid treatment on lipid metabolism in breast epithelial cells have not been characterized.

The ability to perform quantitative high content analysis in multiwell format facilitated acquisition of both singular and combinatorial data using bexarotene and the synthetic PPARγ agonist rosiglitazone. Our data shows that bexarotene induces the accumulation of cytoplasmic lipid droplets and this increase
in neutral lipid content is associated with an up-regulation of the adipocyte differentiation-related protein (ADFP, ADRP, adipophilin). In fat and liver cells, the expression of ADFP has been shown to directly correlate with fat storage (Brasaemle et al., 1997; Imamura et al., 2002). ADFP is localized at the surface of lipid droplets in adipocytes and a variety of other cells, including fibroblasts, macrophages, hepatocytes and mammary epithelial cells (Brasaemle, Barber et al., 1997) and stimulates lipid accumulation when overexpressed (Imamura, Inoguchi et al., 2002). It has been recently reported that the human ADFP gene is a direct LXR target; since an RXR/LXR heterodimer may be activated through the RXR partner, ADFP could potentially be up-regulated by rexinoids. Interestingly, knock-down of either LXRα or LXRβ was unable to block lipid droplet formation in HMECs (data not shown), suggesting that the induction of ADFP is not the driving force behind the accumulation of lipid droplets in HMECs. Rather, given the 24 hour time-frame needed for its induction, the up-regulation of ADFP and bexarotene may not have a direct transcriptional effect on the ADFP gene itself. Nevertheless, the association of the lipid droplets with ADFP raises the possibility that the metabolic changes observed in response to bexarotene are associated with the activation of differentiation processes.

Although the suppression of RXRα by RNAi indicated that RXRα was required for the activation of the lipogenic pathway, no correlation was observed between RXR expression levels and lipid accumulation when examined on a cell-by-cell basis at the time the biochemical changes were observed. However, it should be noted, that the functional impact of receptor levels and localization upon accumulation of lipid droplets cannot be readily studied in the same experiment, due to the differential time frames of the two processes. Lipid droplet formation as a biochemical consequence follows receptor activation by several days. On the other hand, given the abundant levels of RXRα in mammary epithelial cells, the data also suggests that it may not be a limiting factor in the signaling pathway, and/or falls under little or no regulation during lipid accumulation. Peroxisome proliferator-activated receptors (PPARs) are intricately involved in the regulation of lipid metabolism and preadipocyte differentiation (Hartig et al., 2011). A recent report on a genome-wide search identifying direct targets during adipogenesis for RXR/PPARγ binding sites showed that these sites are predominantly present in the class of genes
involved in lipid and steroid metabolism (Hamza et al., 2009). While PPARγ activity enables ErbB2-positive breast cancer cells, which produce high levels of fat, to convert fatty acids to triglycerides and thereby evade lipid-induced apoptosis (Kourtidis et al., 2009), other studies suggest that PPARγ is involved in regulating the growth and differentiation of a number of different cancer cells (Michalik et al., 2004; Rosen and Spiegelman, 2001). The ability of rosiglitazone to prevent hyperplasia indicated its potential use as a chemopreventive agent (Sporn et al., 2001; Wu et al., 2008). Our data shows that in non-malignant breast epithelial cells bexarotene, while suppressing cell growth, also activates a PPARγ-mediated pathway responsible for the conversion of fatty acids to triglycerides. This feed-forward mechanism resulting in the induction of PPARγ may partially explain the synergistic relationship of bexarotene and rosiglitazone. On the other hand, the elevated cellular lipid content produced in response to various concentrations of bexarotene show a strict inverse correlation to the proliferative ability of primary mammary cells. Thus, neutral lipid content should be considered and studied as a novel diagnostic or potentially predictive marker of chemopreventive activity. Similar to chemotherapy, the idea of combination treatment to achieve cancer prevention has been proposed, with promising preclinical studies demonstrating the potential therapeutic benefits associated with rexinoids administered in combination with drugs acting through alternative mechanisms (Brown et al., 2008; Liby et al., 2006; Sporn, 1980). Some early studies have indicated the potential chemopreventive effects that combined retinoid and troglitazone could have on chemically-induced preneoplastic lesion formation (Mehta et al., 2000). However, it is the concept of using a markedly reduced dose of the highly effective chemopreventive agent bexarotene in combination with a synergistic drug that will warrant successful long-term treatment of patients for cancer preventive purposes. An in vivo study will need to follow to validate the feasibility of this concept.

Rosiglitazone was shown to potentiate the effect of bexarotene in suppressing the proliferation and accumulation of neutral lipids; moreover, combined treatment synergistically inhibited cell proliferation at bexarotene doses markedly lower than its IC50. Clinically, treatment regimens of bexarotene in the range of 100 to 400 mg/m²/day may result in serum drug levels exceeding 1μM (Miller et al., 1997; Rizvi
et al., 1999). This dosage alone may cause mild to severe hyperlipidemia in over 80% of the patients, requiring dose reduction or lipid-lowering treatment (Assaf, Bagot et al., 2006). We show that bexarotene concentrations as low as 10nM effectively inhibit cell proliferation \textit{in vitro} when combined with rosiglitazone. Interestingly, while the addition of rosiglitazone to bexarotene sensitizes breast epithelial cells to the anti-proliferative effect of bexarotene, it appears to blunt these cells' sensitivity to the induction of lipid accumulation. This implies that the genes involved in mediating growth suppression are differentially regulated by the combination of low-dose bexarotene and rosiglitazone from the genes required for triglyceride synthesis. This observation may provide the basis for a combination treatment with low-dose (10-100 nM) bexarotene and other agents similarly synergistic with bexarotene for growth suppression, but either non-additive or antagonistic for lipid accumulation.

In spite of most recent controversies regarding the clinical safety of rosiglitazone, thiazolidinediones remain important means in the treatment of type II diabetes, pending careful assessment of cardiovascular risk (Kahn and McGraw, 2010; Schernthaner and Chilton, 2010). Based on this current report as a proof-of-concept study, other agonists and small molecule compounds activating PPAR\(\gamma\) should be evaluated for their ability to suppress cell growth, inhibit transformation or prevent breast cancer in animal models when used in combination with a rexinoid.
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Authorship Contributions:
- Participated in research design: Uray, Brown and Mancini
- Conducted experiments: Uray and Rodenberg
- Contributed new reagents or analytical tools: Uray, Bissonnette, Brown and Mancini
- Performed data analysis: Uray
- Wrote or contributed to the writing of the manuscript: Uray and Mancini
References


FOOTNOTES:

The authors affirm that this study has been carried out in accordance with the Declaration of Helsinki and followed guidelines promulgated by the U.S. National Institutes of Health.

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The authors further declare no conflict of interest herein.
LEGENDS FOR FIGURES

Figure 1. Quantitation of mRNA and protein levels of enzymes required for triglyceride synthesis in normal mammary epithelial cells. A) HMECs were cultured at low confluence and treated with solvent or 1 μM bexarotene (Bex) for 24 hours. Subsequently, RNA was extracted and quantitative real-time RT-PCR was used to determine the expression levels of stearoyl-CoA desaturase 1 (SCD1), acyl-CoA synthetase 1 (ACSL1) and diacylglycerol acyl-transferase 1 (DGAT1). Molecule numbers were extrapolated from a standard curve and relative mRNA levels were expressed as a percent of the housekeeping gene cyclophilin, ** p<0.005, * p<0.05. B) Western-blot analysis of the protein levels of SCD1, ACSL1 and DGAT1 protein levels in cell extracts from HMECs treated with vehicle or 1 μM bexarotene for 48 hours. C) Densitometric analysis of the Western-blots to quantitate protein expressions of the lipogenic enzymes SCD1, ACSL1 and DGAT1. For all images, ** = p<0.005, * = p<0.05.

Figure 2. Multiparametric assessment of lipid accumulation, the expression and localization of RXRα and ADFP in HMECs in the absence or presence of bexarotene. (A and B) Cells were treated with vehicle or 1μM bexarotene for 96 hours, fixed and stained for RXRα (Alexa Fluor 594, red), neutral lipids (LipidTox, green) and DNA (DAPI, blue). 20 Z-stack images per treatment taken at 60x magnification by a DeltaVision (Applied Precision Instruments Inc., Issaquah, WA), were deconvolved and maximum projected into single plain RGB images for analysis. Scale bars represent 10μm in the sample. C) Analysis of the labeled cellular compartments, by segmentation of the nuclei, lipid droplets and RXRα protein by CyteSeer (McDonough et al., 2009, Vala Sciences, San Diego, CA). D) Cell-by-cell measurement of the neutral lipid content in lipid droplets upon bexarotene treatment. E-G) Lipid droplet metrics in HMECs quantitating: lipid droplet counts formed as a result of the accumulation of neutral lipids (E), average lipid droplet diameter (F) and the average neutral lipid content (G) in HMECs after 4 days of bexarotene. (H-I) Immunofluorescent labeling of ADFP (Alexa594, red channel) localized to the outer rims of lipid droplets (labeled with LipidTox, green channel) in bexarotene treated cells (nuclear DNA stained with DAPI). Scale bars represent 10μm in the sample. J) Quantitation of ADFP...
protein based on cellular integrated signal intensity from deconvolved, projected images at 60x magnification. K) Progression of ADFP mRNA levels in the absence (Vehicle) or presence (Bex) of 1μM bexarotene. For all images, ** = p<0.005, * = p<0.05.

Figure 3. Characterization of the lipid accumulative effect of bexarotene on HMECs. A) Dose-response relationship of total lipid content in HMECs treated with bexarotene for 4 days. Lipid content was measured by HTM (IC-100) based on integrated intensity under the lipid masks. B) Comparison of lipid droplet counts formed in HMECs in response to agonists of the nuclear receptors RXR (bexarotene (Bex) and LGD100268 (LG268)), PPARγ (rosiglitazone (Rosi)), PPARα (WY14643 (WY)) and RAR (TTNPB). C) PPARγ expression levels in cell nuclei upon 4 days of bexarotene treatment. PPARγ protein levels were measured by HTM based on immunofluorescent labeling of the receptor. D) PPARγ mRNA levels after 24 hours of treatment with bexarotene (left side panel) and following knock-down with non-targeting control (siNT) or PPARγ specific siRNAs (siPPARγ). E) Comparison of the relative neutral lipid contents in HMECs treated with solvent (control) or bexarotene for 4 days, following knockdown of the nuclear receptors RXRα (siRXRa), PPARα (siPPARα), PPARδ (siPPARBd) or PPARγ (siPPARγ). F) Comparison of the mRNA levels of ADFP in control or bexarotene-treated cells after knock-down with non-targeting control (siNT) or PPARγ specific (siPPARγ) siRNAs. For all images, ** = p<0.005, * = p<0.05.

Figure 4. Lipid accumulative and growth suppressive effects of the combination of bexarotene and rosiglitazone in mammary epithelial cells. A) Comparison of total neutral lipid contents in HMECs at the end of a 6-day treatment with bexarotene (1μM), rosiglitazone (2μM), or bexarotene and rosiglitazone in combination. B) Time course growth assay comparing cell counts following treatments with bexarotene (1μM), rosiglitazone (2μM) or the combination of the two drugs on normal HMECs. The level of statistical significance is indicated by ** = p<0.005, * = p<0.05.
Figure 5. Establishing synergistic growth suppressive effects between combined doses of bexarotene and rosiglitazone on HMECs. A) High throughput microscopy was used to compare cell counts following 4 days of treatment with bexarotene, rosiglitazone or the two drugs in combination. Means of average cell counts from triplicate wells with 45 imaged microscopic fields per well are shown for each drug concentration. The data bars corresponding to the six bexarotene-rosiglitazone combinations confirmed in Figure 5D as statistically significantly different from the ED50s of both individual compounds, and thus synergistic, have been circled. B) Isobologram analysis comparing the effects of bexarotene-rosiglitazone dose pairs based on dose-effect calculations obtained through the median effects method (Chou and Talalay, 1984). Dose values of single agents with equal potencies mark the line of additivity, which segregates dose pairs with synergistic and antagonistic interactions between two drugs. C) Table summarizing doses of bexarotene and rosiglitazone and the combination indexes derived from the resulting isobologram (only 0.2, 0.66 and 2 micromolar rosiglitazone containing combinations shown). A combination index value (CI) less than 0.9 indicates synergy, while a CI value greater than 1.3 indicates antagonism. The numeric IDs of combinations found synergistic or antagonistic in the secondary tests (see figure 5D) are highlighted with a light gray background and those antagonistic with dark gray background. D) Statistical evaluation of synergism: significance of the synergistic effect associated with dual bexarotene-rosiglitazone treatment was established by direct comparison of variant dosage combinations by two-tailed Student’s t-tests with the relative effects of both individual drugs at ED50, as previously described by Laska et al. E) Scatter plots of cell growth and lipid accumulation of HMECs treated with 0, 10 or 100 nM bexarotene in conjunction with increasing doses of rosiglitazone (0, 0.2, 0.66, 2 and 6.66 micromolar, represented by data points of proportionally increasing size within each of the three bexarotene treatment groups). The directional shifts of the dose-response curves are indicated by arrows “a” and “b”. Each data point represents the mean of four replicate wells, and is plotted according to average cellular lipid content and cell counts per well.
Table 1. Gene specific primers and dual-labeled TaqMan probes used in qRT-PCR assays.

<table>
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<tr>
<th>Gene Name</th>
<th>Accession #</th>
<th>Forward Primer 5' → 3'</th>
<th>Reverse Primer 5' → 3'</th>
<th>TaqMan Probe 5' → 3'</th>
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<td>SCD1</td>
<td>NM.005063</td>
<td>CACCCCTTCGCTGATGTGCTCT</td>
<td>GTAAGTTTGCAAGCCCTGACC</td>
<td>FAM - ATGACAAAGAACATAGCCCAGGAG - BHQ</td>
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<td>ACSL1</td>
<td>NM.01995</td>
<td>AGATCTCGAGATATTTTTTTTACAA</td>
<td>GGTCGACTATGTTTTGCTGCAGT</td>
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<tr>
<td>DGAT1</td>
<td>NM.01078</td>
<td>TGGAAACCTGCTGGGTGACAG</td>
<td>TGCCCCGTGAAGGAT</td>
<td>FAM - TGGATCTGACAGAATGCTCTAAGGCCC - BHQ</td>
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<tr>
<td>Adip</td>
<td>NM.001222</td>
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<td>Cyclophilin</td>
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<td>ACAGCGGAGGGCTTGG</td>
<td>TTCTGCTGTCTGTGCTTTTCGACCT - BHQ</td>
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</tbody>
</table>

Table 1. Gene specific primers and dual-labeled TaqMan probes used in qRT-PCR assays.

Primers and probes were used in qRT-PCR assays to measure mRNA levels of lipogenic genes. Transcript quantitation based on real-time monitoring of amplification was carried out according to the parameters defined in Materials and Methods.
Figure 1

A. Relative SCD1 mRNA levels (% of cyclophilin) for Control and Bex conditions.

B. Western blot analysis for SCD1, ACSL1, DGAT1, and β-actin proteins.

C. Protein expression (normalized to β-actin) for Control and Bex conditions.
Figure 2
Figure 2

H-I: Immunofluorescence images of cells treated with DAPI, LipidTox, and Adfp-Alexa 594.

J: Bar graph showing total Adfp expression with Vehicle and Bex treatments.

K: Graph showing relative Adfp mRNA levels (% of cyclophilin) over time with Bex and Vehicle treatments.

Figure 2
Figure 5