Effects on Gene Expression in Rat Liver after Administration of RXR Agonists: UAB30, 4-Methyl-UAB30, and Targretin (Bexarotene)[§]

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ABSTRACT

Examination of three retinoid X receptor (RXR) agonists [Targretin (TRG), UAB30, and 4-methyl-UAB30 (4-Me-UAB30)] showed that all inhibited mammary cancer in rodents and two (TRG and 4-Me-UAB30) strikingly increased serum triglyceride levels. Agents were administered in diets to female Sprague-Dawley rats. Liver RNA was isolated and microarrayed on the Affymetrix GeneChip Rat Exon 1.0 ST array. Statistical tests identified genes that exhibited differential expression and fell into groups, or modules, with differential expression among agonists. Genes in specific modules were changed by one, two, or all three agonists. An interactome analysis assessed the effects on genes that heterodimerize with known nuclear receptors. For proliferator-activated receptor α /RXR-activated genes, the strongest response was TRG > 4-Me-UAB30 > UAB30. Many liver X receptor/RXR-related genes (e.g., *Scd-1* and *Sreb1*, which are associated with increased triglycerides) were highly expressed in TRG and

4-Me-UAB30- but not UAB30-treated livers. Minimal expression changes were associated with retinoic acid receptor or vitamin D receptor heterodimers by any of the agonists. UAB30 unexpectedly and uniquely activated genes associated with the aryl hydrocarbon hydroxylase (Ah) receptor (Cyp1a1, Cyp1a2, Cyp1b1, and *Nqo1*). Based on the Ah receptor activation, UAB30 was tested for its ability to prevent dimethylbenzanthracene (DMBA)-induced mammary cancers, presumably by inhibiting DMBA activation, and was highly effective. Gene expression changes were determined by reverse transcriptase-polymerase chain reaction in rat livers treated with Targretin for 2, 3, 7, and 21 days. These showed similar gene expression changes at all three time points, arguing some steady-state effect. Different patterns of gene expression among the agonists provided insight into molecular differences and allowed one to predict certain physiologic consequences of agonist treatment.

Introduction

Retinoid X receptor (RXR) agonists form heterodimers with the widest range of nuclear receptors, including the peroxisome proliferator activated receptors (PPARs), retinoic acid receptors (RARs), liver X receptors (LXR), thyroxine receptors, constitutive androstenedione receptor (CAR), vitamin D (VDR) receptor, pregnane X receptor (PXR), and farnesoid X receptor (FXR) (Szanto et al., 2004; Sussman and DeLera, 2005). The resulting heterodimers become transcriptional

modulators of a wide variety of genes. There has been significant interest in this class of compounds in cancer because an RXR agonist [Targretin (TRG)] is approved clinically for the treatment of cutaneous T-cell lymphoma (Lansigan and Foss 2010) and has demonstrated clinical efficacy in non-small-cell lung cancer (Dragnev et al., 2011; Kim et al., 2011). In addition, this class of compounds has shown efficacy in a variety of mammary and lung cancer models in rodents (Pereira et al., 2006; Wang et al., 2006b, 2009; Liby, et al., 2007; Zhang et al., 2011), and more recently in humans (Dragnev et al., 2011; Kim et al., 2011). However, the elevation of triglycerides levels by 9-cis-RA, TRG, and various retinoids has been known for many years and is a major concern in the use of these agents, particularly in a cancer-prevention setting (Grubbs et al., 2006; Kolesar et al., 2010). We previously designed selective RXR agonists based on 9-cis-RA that are effective agents with lower toxicity

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ABBREVIATIONS: Ah receptor, aryl hydrocarbon hydroxylase receptor; CAR, constitutive androstenedione receptor; DMBA, dimethylbenzanthracene; Egr1, early growth response 1; FDR, false discovery rate; FXR, farnesoid X receptor; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; RXR, retinoid X receptor; TRG, Targretin; 4-Me-UAB30, 4-methyl-UAB30; VDR, vitamin D receptor.

and with varying ability to inhibit mammary cancer formation (Muccio et al., 1998; Atigadda et al., 2003; Grubbs et al., 2006). One of these analogs (UAB30) did not increase serum triglycerides in rodents (Grubbs et al., 2006) and humans (Kolesar et al., 2010) but was nevertheless effective in preventing mammary cancers in rats.

In the present studies, the ability of three RXR agonists [TRG, UAB30, and 4-Me-UAB30 (4-methyl-UAB30)] to induce gene expression in the liver of treated rats was examined. We aimed to observe the effects of these agonists on genes known to be modulated by agonists for specific receptors, including PPAR α , CAR, LXR, and others. Surprisingly, it was found that although each of these compounds binds to the purified RXR receptor and activates gene expression in an RXR α luciferase construct in cell culture, there was limited overlap in the gene expressions modulated. In addition, the effects of these agents on genes associated with increased triglycerides levels (Scd1 and Srebf1) were evaluated. Agonists that increased triglyceride levels (TRG, 4Me-UAB-30) increased expression of the LXR-modulated genes Scd1 and Srebf1, whereas UAB-30 did not. Interestingly, we found that UAB-30 modulated genes associated with the Ah receptor and blocked initiation of mammary tumors by dimethylbenzanthracene. In addition, we examined the ability of TRG to induce 10 specific genes in livers after 2.3, 7, and 21 days of treatment and found similar results at all three time points. This implies that we had reached some steady state regarding gene expression. Nevertheless, the most surprising result was the limited gene expression overlap of the RXR agonists.

Materials and Methods

Animals and Liver Collection for RNA Analysis. The present experiments were similar to our previously published studies examining the efficacy of these agonists in a rat mammary cancer model (Grubbs et al., 2006). In brief, agonists were administered in Teklad mash diet (4%) to female Sprague-Dawley rats obtained from Harlan Sprague-Dawley, Inc. (Madison, WI). The dietary supplements [TRG (150 ppm in diet), 4-Me-UAB30 (200 ppm in diet), and UAB30 (200 ppm in diet)] were obtained from Eisai, Inc. (TRG) or was synthesized at the University of Alabama at Birmingham (4-Me-UAB30, UAB30) (Atigadda et al., 2003). In brief, rats were obtained at 28 days of age and housed in polycarbonate cages (five per cage) in a room lighted 12 hours per day and maintained at 22°C $\pm$ 2°C. The dietary supplements were administered for 7 days. The animals were euthanized by CO₂, and the livers were rapidly excised and frozen in liquid N₂. Until assayed, the livers were kept at -85°C. A second set of rats were administered TRG (150 ppm) only to assess whether the gene changes observed at 7 days were also seen at other times (2.3 days, 7 days, and 21 days). Otherwise animals and livers were generated as performed with the 7-day samples.

RNA Isolation and Microarray Experiment. Total RNA were isolated by Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini Kit and RNase-free DNase Set (Qiagen, Valencia, CA) according to the manufacturers' protocols. One microgram of each RNA sample was processed as prescribed by the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay. The GeneChip WT cDNA Synthesis Kit, cDNA Amplification Kit, and Terminal Labeling Kit (Affymetrix, Inc., Santa Clara, CA) were used for target preparation. A total of 8 μ g of cRNA were input into the second-cycle cDNA reaction. Hybridization cocktails containing 3–4 μ g of fragmented, end-labeled cDNA were prepared and applied to GeneChip Rat Exon 1.0 ST arrays. The arrays were hybridized for 16 h. A wash and stain script (precommercial FS450_0001 script) was applied using the MES_EukGE-WS2v5_450-DEV fluidics station.

Arrays were scanned using the Affymetrix GCS 3000 7G and GeneChip Operating Software v. 1.3 to produce Affymetrix CEL-formatted files which contain quantitative measures for probe hybridization.

Normalization and Probe Filtering. The probe-level data were contained Affymetrix GeneChip Rat Exon 1.0 ST Array probe set CEL files. They were read into the R statistical software using the R/xps package [Stratowa, 2012 (<http://www.bioconductor.org/packages/2.11/bioc/html/xps.html>)]. We performed our analysis on the complete set of probe sets that map to a unique, transcriptionally active locus in the rat genome (which includes probe sets classified as “core,” “extended,” and “full” by the chip manufacturer). This set contains approximately 728,247 exons that target 18,192 unique genes. The data were normalized by the Robust Multichip Average quantile normalization method (Bolstad et al., 2003). To improve the sensitivity of downstream analysis, we further filtered the probe sets, retaining only those with an intensity that falls above or in the extreme top of the observed intensity distribution for the antigenomic or negative control, probes after adjustment for guanine/cytosine base content in at least one of the 20 samples (detection above background P value < 0.05). After this filtering, 421,360 exons targeting 16,838 genes remained. Thus, there is evidence of expression in the exonic regions targeted by the probe sets that remain.

Identifying Transcripts Differentially Expressed. Analysis of variance methods, performed with the R/maanova package (Wu et al., 2002), were used to identify probe sets statistically with differential expression associated with treatment. The factorial experimental design includes one factor (“treatment”) with four levels (4-Me-UAB30, TRG, UAB30, and control) and four samples per factor level, each from a different individual sample. For each probe set g , treatment i , and replicate k , a linear model for the log-transformed expression measure, y_{ijk} , can be formulated as a sum of components that contribute to the overall intensity value:

$$y_{ikg} = \mu_g + \alpha_{ig} + \varepsilon_{ikg} \quad (1)$$

where μ_g is the mean intensity over all 20 samples, α_{ig} is the effect of the factor level corresponding to treatment i ($i = 1, 2, 3, 4, 5$), and ε_{ijk} is the residual error.

To identify transcripts with the highest probability of differential expression between any two experimental groups, we applied the omnibus or general F test. Using R/maanova, we performed a modified general F-test (called Fs), which incorporates shrinkage estimates of residual variance and increases the power to detect differential expression compared with an ordinary F-test (Cui et al., 2005). P values were calculated by permuting model residuals. Using permutation P values rather than tabular P values is more robust to departures from model assumptions such as the underlying statistical distribution. As an adjustment for multiple testing, we used the Benjamin-Hochberg transformation of the P values to estimate false discovery rate (Benjamini and Hochberg, 1995). Transcripts with differences between strains were identified as those with estimated false discovery rates (FDRs) less than 0.01 (i.e., FDR < 0.01). In general, theory suggests that using an FDR threshold of n implies that only about $100 \times n$ out of 100 genes are not actually differential expressions. We also performed F tests for the four contrasts defined by comparing each treatment with the control group and used an analogous approach for detecting differential expression.

Weighted Gene Coexpression Network Analysis. The coexpression patterns of differentially expressed genes (according to our general F-test) were analyzed by weighted gene coexpression network analysis (Ravasz et al., 2002; Zhang and Horvath, 2005). For each pair of probe sets, the Pearson correlation coefficient, r , was transformed to a distance, or dissimilarity metric metric, d , on the unit scale where a correlation of 1 has a dissimilarity of 0 and a correlation of -1 has a dissimilarity of 1. The probe sets were given a weighted connection, d^6 , and then the topographical overlap transformation of these connections was computed using the R/WGCNA package (Langfelder

and Horvath, 2008). For a pair of probe sets, the topographical overlap measures the similarity of their distributions of distances to all other genes. Transcript abundance profiles were hierarchically clustered modules obtained by the hybrid dynamic dendrogram cutting method of the R/dynamicTree Cut package. Modules are referenced by a color index. The expression patterns and module assignments of the treatment genes were displayed via a heat map generated using Java Tree View (Saldanha, 2004).

Gene-Set Enrichment and Pathway Analysis. Gene-set enrichment and pathway analysis was performed in the GeneGo Metacore software using the Enrichment Workflow and the Interactome Analysis Workflow [Thomson Reuters, 2010 (<http://thomasonreuters.com/content/science/pdf/ls/metacore-cfs-en.pdf>)]. We applied these workflows for various subsets of differentially expressed genes as described in the *Results* section. For each application of the Enrichment Workflow, we reported the GeneGo Pathway Maps, GeneGo Process Networks, GeneGo Diseases, GeneGo Metabolic Networks (Endogenous), and Top GO Processes as Supplemental Tables. For each application of the Interactome Analysis Workflow, we reported the most overconnected proteins by functional category as a supplemental table where the categories are transcription factors, receptors, ligands, kinases, proteases, phosphatases, enzymes, and others. The GeneGo Metacore database was used to identify the known targets of the selected nuclear receptor-related targets, except in the cases of CAR/RXR α and PXR/RXR α . The known targets of these RXR-containing complexes were obtained from the study presented by Tolson and Wang (2010).

Gene ID Conversion. The gene ID conversion tools of the DAVID Bioinformatics Resource (<http://david.abcc.ncifcrf.gov/>) (Dennis et al., 2003) and the RGD (<http://rgd.mcw.edu/>) (Twigger et al., 2007) were used to identify current IDs where needed.

Reverse Transcriptase-Polymerase Chain Reaction of Livers Exposed to Targretin for Varying Time Periods. To determine whether the gene changes observed at 7 days are similarly observed at other times, we treated groups of rats ($n = 5$) with TRG (150 ppm) for 2, 3, 7, and 21 days. Livers were isolated and RNA isolated as already described. Quantitative RT-PCR (reverse transcriptase-polymerase chain reaction) of the isolated livers was performed. For 10 genes that we found to have upregulated expression in the livers of rats treated with TRG relative to controls in the original experiment, we measured the relative expressions by quantitative RT-PCR. Total RNAs were isolated and purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. One microgram of total RNA per sample was converted to cDNA using Script cDNA synthesis kit (Bio-Rad, Hercules, CA). Primers for quantitative RT-PCR analysis were designed by using Primer5 software (Supplemental Table 1). Amplification of each target was performed using a SsoFast EvaGreen Supermix (Bio-Rad) with 500 nM of each primer and 10 ng of cDNA per reaction. Each real-time assay was done in duplicate on a BioRad CFX96 thermal cycler, and data were collected and analyzed with BioRad CFX software (Version 2.1). The internal control gene GAPDH and target genes were amplified with equal efficiencies. The fold change of target gene expression was calculated as $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{\text{treated}} - \Delta CT_{\text{control}}$), and all data are presented as mean $\pm$ S.E. of relative gene expression.

Prevention of Dimethylbenzanthracene-Induced Mammary Tumors. This model examines the ability of an agent to block the initiation of mammary carcinogenesis (positive) in rats. We have published multiple articles using this model and recently showed that 5, 6 benzoflavone was highly effective (Grubbs et al., 1995; Lubet et al., 2011). In brief, female Sprague-Dawley rats were administered UAB30 (200 ppm), 5, 6 benzoflavone (400 ppm), or control diet from 43 days of age until 57 days of age. After 57 days of age, all rats were placed on a control diet. At 50 days of age, rats were given a single intragastric dosing of DMBA (dimethylbenzanthracene; 12 mg). Rats were then monitored for an additional 120 days for the development of mammary cancers.

Results

Microarray Experiment. A microarray experiment was performed employing the Affymetrix GeneChip Rat Exon 1.0 ST Array platform to determine the short-term effects of three RXR agonists on gene expression in rat liver and to relate them to the effects that different RXR agonists have on serum triglyceride levels. The RXR agonists were administered in the diet for 7 days; rats were then killed, and RNA was isolated as described in *Materials and Methods*. The analyses were performed across all the probe sets of this array platform that, based on the chip manufacturer's annotation, map to a unique transcriptionally active locus in the rat genome. This set contains more than 700,000 exons that target more than 18,000 unique genes. A filtering step was used to retain only probe sets that show evidence of target gene expression in at least one of the 20 samples, after which about 400,000 exons remained.

Differential Expression Is Less for UAB30 than for Other Agonists. To assess the statistical significance of variation in between-treatment-group liver gene expression, an empirical Bayes-like general F-test was applied to identify genes that were variable between treatment groups (Cui et al., 2005). Greater differential expression than expected by chance (Supplemental Fig. 1) was found. For example, there were 777 exons of 185 unique genes that were differentially expressed based on highly stringent permutation-based P values after adjustment by a false-discovery-rate-based multiple-test correction (i.e., $FDR < 0.005$). The pairwise contrasts between the control group and each of the three treatment groups were also tested. The distribution of significance scores (permutation-based P values) show that, estrogen receptor compared with controls, there is greater differential expression for 4-Me-UAB30 and TRG than for UAB30 (Supplemental Fig. 1, b–d). Specifically, at $FDR < 0.01$, 544 (4-Me-UAB30), 298 (TRG), and 14 (UAB30) exons of 143 (4-Me-UAB30), 76 (TRG), 5 (UAB30) unique genes were differentially expressed. Using the same stringent differential expression criterion among the genes selected by the general F-test criterion that was used, the number of genes differentially expressed between controls and UAB30 was very small (five genes). However, when the FDR criterion was relaxed to $FDR < 0.25$, it was found that 145 exons for 25 unique genes were differentially expressed for UAB30. Of these 25, 15 genes were represented in the set of genes selected by the empirical Bayes general F-test criterion. Using this less stringent criterion, we observed 497 genes whose expression was modulated by 4-Me-UAB30 and 210 genes whose expression was modulated by TRG. Thus, there is evidence for differential expression between UAB30 and controls, but the number of genes and the quantity of differential expression are small relative to the differential expression between the other two RXR agonists and controls.

Genes Fall into Coexpression Network Modules that are Biologically Enriched. The genes detected as differentially expressed by the general F-test, in general, may exhibit multiple patterns of coexpression across the samples. Weighted gene coexpression network analysis was used to cluster those genes into modules with correlated patterns of variation (Fig. 1). Eight modules containing up to 62 genes were found. We refer to the modules using color indices (genes per module: blue, 62; brown, 29; green, 27; magenta, 8; pink, > 3;

Fig. 1. Heat map with coexpression module assignments from clustering of variable genes. A heat map is shown for the differentially expressed genes (based on the general F-test). Samples are hierarchically clustered based on Euclidean distance, and exons are hierarchically clustered using the topographical overlap dissimilarity distance between probe sets, which is a correlation-based measure used for clustering. Treatment groups exhibited substantial separation in the sample dendrogram. Expression values are centered and scaled within each exon (i.e., row). Low expression is indicated by white, and high expression is indicated by black. Coexpression modules define highly connected sets of correlated expression profiles as indicated by color indices in the vertical margins of the maps. The modules are characterized in Supplemental Table 2.

red, 20; turquoise, 29; yellow 34). Three modules in which TRG-treated rats were upregulated were enriched with target genes for PPAR α /RXR α (green, yellow, brown). One of those modules (brown) also exhibited upregulation with 4-Me-UAB30 and was enriched with target genes known to be activated by the PPAR α /RXR α heterodimer complex (six genes, $P < 1E-07$, e.g., Acaa1, Ehhadh Mdh1), LXR α /RXR α (three genes, $P < 1E-04$, Scd1, Acaca, Cd36), and TR α /RXR α (three genes, $P < 1E-04$, e.g., Slc2a5, Mdh1). There was also some enrichment ($P < 0.002$) for CAR/RXR α (two genes), PPAR γ /RXR α (three genes), nuclear SREBP1 (five genes), MLX (two genes including Thrsp), and c-Myc (10 genes). The key gene of the nuclear SREBP1 pathway, Srebf1, also had a pattern of expression that highly correlated with the pattern of this module (Fig. 1). UAB30 showed relatively little or no response for genes of this module. Another module (turquoise) exhibited downregulation for 4-Me-UAB30 and, to a lesser extent, TRG. This module was enriched for target genes of FXR and FXR-regulated cholesterol and bile acids cellular transport pathways (Supplemental Table 2). Downregulation of this pathway is associated with higher triglycerides levels in rats (Evans et al., 2009). Again, UAB30 showed relatively

little or no response for genes of that module. Thus, two modules (brown and turquoise) had common patterns of expression in both TRG- and 4-Me-UAB30-treated rats that are associated with higher triglycerides levels.

Two modules (yellow and green) exhibited increased expression after TRG treatment, whereas expression of these genes was slightly reduced by 4-Me-UAB30 (green). Both these modules were enriched with genes that have been previously shown to be activated by PPAR α /RXR α or by PPAR α (not necessarily dimerized with RXR α). Thus, there appears to be a greater PPAR α -related transcriptional activation for TRG than for the other RXR-agonists.

The largest module (blue, 60 genes) contained several genes that are activated by PPAR γ and RXR α (Cyp26a1, Cyp26b1, Cyp26c1, Orm1, Jak3) or PPAR γ (not necessarily dimerized with RXR α [Egr1 (early growth response 1), Akt 1]). The genes Cyp26a1, Cyp26b1, Cyp 26c1 as well as Cyp2c13, Cyp4a8 are cytochrome P450 genes, all of which are involved in retinol metabolism. Ugt 2a3, also in this module, is similarly involved in retinol metabolism. The Egr1 protein interacts with many other proteins. In the context of this experiment, these annotations suggested that there was greater PPAR γ /RXR α activity for 4-Me-UAB30-treated rats than for the other RXR agonists. The expression for one of these genes (Egr1) was also increased by UAB30, albeit to a lesser extent than by 4-Me-UAB30.

Expression Patterns for Genes Regulated by RXR-Containing Complexes Are Diverse. The interactome analysis of the gene modules revealed that several modules were highly enriched with targets of RXR-containing complexes. However, because a highly stringent criterion for determining the differentially expressed genes was used, the analysis does not indicate whether genes that are regulated by a particular RXR complex are all affected similarly or if a particular RXR agonist affects particular targets. To address this, the MetaCore database [Thomson Reuters, 2010 (<http://thomsonreuters.com/content/science/pdf/lis/metacore-cfs-en.pdf>)] was queried separately for each of 31 transcription factors that are RXR-containing complexes or are components of these complexes. We identified genes annotated in the rat liver to be under transcriptional regulation or whose expression was otherwise influenced by the transcription factors (Supplemental Table 3). Then, for each gene set, the expression patterns observed for all exons of those genes were examined. It was observed that no set showed a uniform response across all genes. However, the target genes of a number of proteins or protein complexes included subsets of genes with similar patterns with relative uniformity within experimental treatment group. For example, the strongest signal observed among the genes annotated to be activated by LXR α /RXR α was a group of genes that exhibited higher expression for TRG and 4-Me-UAB30 than for the other groups (Fig. 2; Table 1), including brown module genes such as Acaca and Cd36. Among genes annotated to be activated by PPAR α /RXR α was a group that exhibited higher expression for TRG than for the other three groups (e.g., yellow or green module genes Acadm, Gpd1, Me1, and Ephx1). There were, however, some genes that also showed high expression in the 4-Me-UAB30 samples (Fig. 3; Table 2) (such as Ech1 and Echdc1). Several genes with higher expression in 4-Me-UAB30-treated rats were activated by PPAR γ /RXR α or PPAR γ (e.g., Lrp1, Aldh2, blue module genes, Cyp26a1,

Fig. 2. Genes activated by LXR α /RXR α . A heat map is shown for the exons of genes activated by LXR α /RXR α in the rat liver. Samples are hierarchically clustered based on Euclidean distance, and exons are hierarchically clustered using the topographical overlap dissimilarity distance between probe sets, which is a correlation-based measure used for clustering. Low expression is indicated by black, and high expression is indicated by white. Increased expression was observed for 4-Me-UAB30 and TRG on a large block of exons. Expression values are centered and scaled within each exon (i.e., row).

Cyp26b1, Cyp26c1, Orm1, Jak3, Egr1, and Akt 1) (Fig. 1). Among genes annotated to be activated by PXR/RXR α , Sult1b1, and various glutathione S-transferases (e.g., Gsta3, Gsta3, Gstp1, Gstm4) were upregulated by 4-Me-UAB30 (Fig. 4; Table 3). Ces6, Ces21, Cyp3a2, and Cyp3a23 were upregulated by TRG (Fig. 4; Table 3). Akrlc14, Ugt1a1, Ugt1a6, and other glutathione S-transferases (Gstm1, Gstm3, Gstm6l) were upregulated by UAB30 (Fig. 4; Table 3). There was increased expression among genes annotated to be activated by FXR/RXR α in the TRG samples (Abcb11, Ppargc1a, Slc27a5) Supplemental Table 4, Supplemental Figure 4). By contrast, none of the studied RXR-agonists increased genes annotated to be activated by RAR α /RXR α (Supplemental Fig. 2),

nor did they significantly activate genes annotated to be activated by VDR/RXR α (Supplemental Fig. 3).

Enrichment for Genes Specifically Altered by UAB30 Is Different. One small module of genes selected by the general F-test criterion (pink) exhibited a strong response for UAB30 but not for the other agonists. The genes of this module were contained in the set of genes differentially expressed between UAB30 and controls using the slightly more relaxed criterion FDR < 0.25. The genes with increased expression in UAB30 (Fig. 4; Table 4) had overrepresentation of targets not for RXR-containing complexes, e.g., CAR-RXR complex (Supplemental Figure 5) but rather AHR, NRF2, and HNF1 α (Table 4). The genes with increased expression in UAB30 included at least five genes whose protein products are associated with Ah receptor (aryl hydrocarbon receptor) activation [Cyp1a1, Cyp1a2, and Cyp1b1, Ugt1a6 and Nqo1 (NADH dehydrogenase, quinone1)]. Two of the three known rat orthologs to the four human genes primarily involved in UAB30 metabolism (Gorman et al., 2007) were upregulated (LOC293989: FC = 1.8, P = 0.001, Cyp2c79: FC = 1.3, P = 0.03) in UAB30 samples, whereas the other was not (Ugt1a9: FC = -1.05, P = 0.86). However, another UDP-glucuronosyltransferase (Ugt1a6) was up over 3-fold in the UAB30 samples. In toto, the pathways and the interactions perturbed by UAB30 were substantially different from those for the other RXR agonists and do not include categories commonly associated with triglyceride metabolism. (Fig.5)

Comparison with Similar Studies Performed with RT-PCR. Based on our previous data with TRG (Wang, et al., 2006a), we had performed RT-PCR for a more limited number of genes in livers treated with TRG or UAB30. The results of the present arrays were compared with the prior quantitative RT-PCR findings. A substantial concordance was observed. All fold change directions were the same between the two experiments for these genes (Supplemental Table 4). Furthermore, for eight genes for which PCR was performed and for which significant differential expression was observed, a high degree of correlation in fold change (r = 0.73, P = 0.03; Supplemental Fig. 6) was found. We performed a second RT-PCR experiment with TRG to determine whether the gene expression changes observed at 7 days in the microarray experiment were similarly observed in livers of animals treated for different time periods (2 1/3, 7, and 21 days). This experiment examined 10 genes that showed increased gene expression in the initial 7-day microarray experiment on independent samples. In the follow-up quantitative RT-PCR experiment, we found the average fold changes for all the TRG-treated time-point-defined groups were positive for each gene (Table 5). The fold increases ranged from 1.4-fold to

TABLE 1

Genes activated by liver X receptors (LXR α)/retinoid X receptors (RXR α)

For selected genes known to be activated by LXR α /RXR α , a fold change (FC) is shown for each treatment group relative to the control group. These are the fold changes for the exon of that gene with the smallest permutation P value when an empirical Bayes overall F-test is applied. The corresponding unadjusted and false discovery rate (FDR)-adjusted permutation P values are shown.

Description	Gene Symbol	FC MeUAB030 Control	FC Targretin (TRG) Control	FC UAB030 Control	Permutation P Value	FDR-Adjusted P value
Up: 4Me and TRG	Acaca	2.44	2.87	1.13	0.00E+00	0.00E+00
Up: 4Me and TRG	Cd36	2.09	2.01	1.47	0.00E+00	0.00E+00
Up: 4Me and TRG	Nr0b2	2.42	1.87	-1.04	5.27E-05	1.58E-02
Up: 4Me and TRG	Scd1	2.17	2.89	1.24	2.27E-07	3.72E-04
Up: 4Me and TRG	Srebf1	1.55	2.65	-1.06	1.53E-04	3.17E-02

Fig. 3. Genes activated by PPAR α /RXR α . A heat map is shown for the exons of genes activated by PPAR α /RXR α in the rat liver. Samples are hierarchically clustered based on Euclidean distance, and exons are hierarchically clustered using the topographical overlap dissimilarity distance between probe sets, which is a correlation-based measure used for clustering. The most dominant signal is increased expression for the TRG group. There are also some genes with increased expression in the 4-Me-UAB30 treated group.

9.6-fold (2.3 days: 1.8- to 7.7-fold increase; 7 days: 1.4- to 7.0-fold increase; 21 days: 1.8- to 9.6-fold increase) (Table 5). The difference compared with controls after 21 days is significant for each gene ($P < 0.05$). The difference compared with controls after 2.5 days and 7 days is significant for all genes at $P < 0.15$ and for most genes at $P < 0.05$ (2.5-day group: 9/10; 7-day group: 6/10) (Table 5). In summary, these genes invariably show substantial upregulation at each of three time points ranging from less than 3 days to 3 weeks,

and almost all observed increases are statistically significant, even with just five individual rats per group.

Effects of UAB30 and 5,6 Benzoflavone on DMBA-Induced Rat Mammary Tumors. Sprague-Dawley rats were administered UAB30 (200 ppm) or 5,6 benzoflavone (400 ppm) in the diet beginning at age 43 days and continuing until 57 days of age. At 50 days of age, rats were administered a single oral dose of DMBA. Treatment with either UAB30 or 5, 6 benzoflavone (a known Ah receptor agonist) reduced the incidence of rat mammary tumors by more than 85% (Fig. 6).

Discussion

The effects on gene expression of three known RXR agonists were determined. 4-Methyl-UAB30 and UAB30 were designed based on empirical structure-activity relationships and computer modeling. We have found in transactivation assays (Muccio et al., 1998) that although TRG and 4-Me-UAB30 have similar binding affinities (~ 25 nM), the affinity for UAB30 is roughly 120 nM. None of the agents significantly bound to RAR at 2000 nM (Muccio et al., 1998). Previously, we showed that these agents prevented ER $^{+}$ mammary cancer (Grubbs et al., 2006). TRG and 4-Me-UAB30 reduced ER $^{+}$ mammary cancers by $\sim 80\%$, whereas UAB30 inhibited roughly 55–65%. However, TRG and 4-Me-UAB30 increased serum triglycerides by 300–400%, whereas UAB30 had minimal effects on triglycerides (Grubbs et al., 2006). We expected rexinoids to alter the interactome involving the PPAR α /RXR α complex since a number of these genes associated with peroxisome proliferation were highly induced by TRG and 9cisRA (Wang et al., 2006a). Also, activation of PPAR α was associated with hepatocytomegaly and liver tumor promotion in rodents (Viswakarma et al., 2010), and we observed hepatocytomegaly with both TRG and 4-Me-UAB30. TRG increased a wide variety of PPAR α -related genes, whereas 4-Me-UAB30 and UAB30 modulated a more select group (Fig. 3; Table 2). The large differences in gene expression between UAB30 and 4-Me-UAB30 were surprising since they differ by a single methyl group. Among the limited number of genes altered by UAB30, at least five genes were associated with the activation of the Ah receptor [Cyp1a1, Cyp1a2, Cyp1b1, Ugt1a6, and Nqo1 (quinone reductase 1)]

TABLE 2

Genes activated by peroxisome proliferator-activated receptors (PPARs) α /retinoid X receptor (RXRs) α

For selected genes known to be activated by PPAR α /RXR α , a fold change (FC) is shown for each treatment group relative to the control group. These are the fold changes for the exon of that gene with the smallest permutation P value when an empirical Bayes overall F-test is applied. The corresponding unadjusted and false discovery rate (FDR)-adjusted permutation P values are shown.

Description	Symbol	FC MeUAB030 Control	FC Targretin (TRG) Control	FC UAB030 Control	Permutation P value	FDR-Adjusted P value
Up: Trg	Abcd3	-1.17	2.14	1.00	5.71E-07	6.77E-04
Up: Trg	Acadm	-1.19	1.39	-1.01	9.26E-05	2.31E-02
Up: Trg	Acx1	-1.10	1.80	1.12	6.30E-06	3.92E-03
Up: Trg	Acs11	-1.07	1.43	1.12	1.56E-05	7.08E-03
Dn: 4Me	Acs14	-1.63	1.09	-1.08	1.08E-04	2.54E-02
Up: Trg	Acs15	1.13	1.92	-1.18	1.14E-07	2.66E-04
Up: 4Me	Cyp4b1	2.00	1.00	1.68	1.09E-02	2.88E-01
Dn: 4Me & UAB	Cyp7a1	-4.10	1.28	-5.11	9.08E-07	9.59E-04
Up: 4Me & Trg	Ech1	2.24	2.21	1.69	4.99E-05	1.55E-02
Up: 4Me & Trg	Echdc1	2.47	2.17	1.41	1.90E-04	3.63E-02
Up: Trg	Ephx1	-1.22	1.74	1.15	1.70E-06	1.44E-03
Up: 4Me & Trg	Ephx2	1.97	1.85	1.28	5.27E-03	2.14E-01
Up: Trg	Gpd1	1.00	2.06	1.03	1.65E-06	1.44E-03
Up: Trg Dn: 4Me	Gys2	-1.45	1.32	-1.07	2.79E-05	1.04E-02
Up: Trg	Me1	1.03	4.93	1.37	0.00E+00	0.00E+00
Dn: 4Me	Scp2	-1.33	1.05	-1.03	1.24E-03	1.06E-01

Fig. 4. Genes activated by PXR/RXR α . A heat map is shown for the exons of genes activated by PXR/RXR α in the rat liver. Samples are hierarchically clustered based on Euclidean distance, and exons are hierarchically clustered using the topographical overlap dissimilarity distance between probe sets, which is a correlation-based measure used for clustering. There are distinct sets of genes with increased expression for TRG and 4-Me-UAB30.

(Table 4). We showed that these genes were highly induced by the Ah receptor agonist 5,6-benzoflavone (Lubet et al., 2009). UAB30 clearly activated Ah receptor related genes (Table 4), and our prior findings showed that compounds that activate this receptor inhibit DMBA-induced mammary cancer and aflatoxin-induced liver cancer (Grubbs et al., 1995; Lubet et al., 2011). Therefore, we tested UAB30 (200 ppm) in the DMBA-

induced mammary model, which reduced cancers by >80%, as much as a suboptimal dose (400 ppm) of 5,6 benzoflavone (Fig. 6). Thus, the array data alone allowed us to predict a significant biologic outcome based strictly on the gene expression data. At least two additional questions might arise regarding the results with DMBA. First, is activation of the Ah receptor the only way to alter DMBA metabolism and DMBA-induced

TABLE 3

Genes activated by pregnane X receptor (PXR)/ retinoid X receptor (RXR) α

For selected genes known to be activated by PXR/RXR α , a fold change (FC) is shown for each treatment group relative to the control group. These are the fold changes for the exon of that gene with the smallest permutation P value when an empirical Bayes overall F-test is applied. The corresponding unadjusted and false discovery rate (FDR)-adjusted permutation P values are shown.

Description	Symbol	FC MeUAB030 Control	FC Targretin (TRG) Control	FC UAB030 Control	Permutation P value	FDR-Adjusted P value
Up: 4Me	Gsta4	1.73	1.35	1.15	7E-02	5E-01
Up: 4Me	Gstp1	1.76	1.02	1.03	1E-02	3E-01
Up: 4Me	Sult1b1	1.60	1.02	1.17	7E-03	2E-01
Up: Trg	Ces2l	2.16	4.33	1.37	2E-06	3E-03
Up: Trg	Cyp3a23	1.76	3.26	1.56	2E-07	3E-04
Up: UAB30	Gstm3	-1.61	1.1	1.29	1E-04	5E-02

initiation? In fact, there appear to be two clearly documented mechanisms for accomplishing this goal: induction of the Ah receptor, which alters various cytochromes and certain phase II enzymes, and induction of the antioxidant response element, which highly induces a wide variety of phase II drug-metabolizing enzymes. We and others have discussed these two major alternative pathways. In fact, none of the three RXR agonists significantly induced the ARE-related genes [GST Pi, aldo keto reductase 3A7 (aflatoxin)]. Only UAB30 induced the Ah-related genes. We previously tested TRG for the ability to inhibit DMBA metabolism and found it to be ineffective (unpublished data). We have not tested 4-Me-UAB30. The induction of Ah receptor-mediated genes by UAB30 raises multiple questions. First, will UAB30 block initiation by other carcinogens (e.g., aflatoxin and aromatic amines that are blocked by 5,6-benzoflavone (discussed in Lubet et al., 2009). Second, how UAB30 activates the Ah receptor is unclear. One would not expect UAB30 to be an effective Ah receptor agonist since binding to the receptor requires a highly planar molecule and the low-energy conformation is L-shaped to fit into the RXR binding pocket (Muccio, unpublished data). Furthermore, 4-Me-UAB30, which differs by a single methyl group, only minimally affected these genes. We have shown that UAB30 inhibits DMBA initiation of tumorigenesis and TRG does not. We previously demonstrated that all three agents inhibit the progression stage of tumor development in ER⁺ rat tumors and the progression stage of ER⁻ tumors in MMTV-Neu/P53 transgenic mice (unpublished data). This clear overlap of

response to progression in both ER⁺ and ER⁻ rodent models leads us to expect a more similar gene response than we observed.

TRG and 4-Me-UAB30 altered expression of a wide number of genes associated with specific nuclear receptor-RXR heterodimers [e.g., PPAR α /RXR α , PXR/RXR α , LXR α /RXR α (Figs. 2–4; Tables 1–3)] but had limited effect on other RXR heterodimers (e.g., vitamin D receptor/RXR, VDR/RXR, RAR/RXR) (Supplemental Figs. 2 and 3). Thus, the RXR agonists do not stimulate Tran activation of genes for VDR/RXR and RAR/RXR. These heterodimers require the presence of VDR or RAR agonists, respectively (nonpermissive or conditional permissive). In contrast, genes modulated by the heterodimers PPAR α /RXR α respond to either PPAR α agonists or to RXR agonists (Wang et al., 2006a). The combinations of agonists for both nuclear receptors often lead to synergistic gene activation (permissive). This study also revealed different responses to the different RXR agonists even among genes that are annotated to be activated by the same nuclear receptor heterodimer (e.g., PPAR α /RXR α , LXR α /RXR α) (Figs. 2–4; Tables 1–3). Some of the diversity may be due to the complexities of transcriptional control. Coregulators (coactivators/corepressors) may be affected by the presence of specific RXR agonists and conformation changes in the protein heterodimer complex.

Finally, we determined whether gene expression changes in the liver might help explain the differential effects on triglycerides levels (TRG and 4-Me-UAB30 increased;

TABLE 4

Genes differentially expressed between UAB30 and controls [false discovery rate (FDR) < 0.25]

For selected genes upregulated on UAB30 relative to controls, a fold change (FC) is shown for each treatment group relative to the control group. These are the fold changes for the exon of that gene with the smallest permutation P value when an empirical Bayes overall F-test is applied. The corresponding unadjusted and FDR-adjusted permutation P values are shown. Note: Two other genes that satisfied the criteria for increased expression by UAB30 and whose homologs were targets of AHR in *Homo sapiens* were Per1 and Bhlhe40.

Description	Symbol	FC MeUAB030 Control	FC Targretin Control	FC UAB030 Control	Permutation P Value	FDR-Adjusted P value
Up: UAB	Cyp1a1 α	1.22	1.97	54.57	0.00E+00	0.00E+00
Up: UAB Dn: 4Me	Cyp1a2 α	-1.76	-1.10	2.13	0.00E+00	0.00E+00
Up: UAB	Cyp1b1 α	1.19	1.17	3.04	1.15E-05	5.82E-03
Up: All	Cyp2b1 γ	3.46	11.33	4.13	2.70E-05	1.01E-02
Up: 4Me and UAB	Egr1 δ, β	2.80	-1.06	2.99	1.70E-07	3.38E-04
Up: All	Gsta5 δ	2.28	2.92	2.75	1.23E-05	6.06E-03
Up: UAB Dn: 4Me	Gstm3	-1.61	1.10	1.29	1.75E-04	3.46E-02
Up: 4Me & UAB	Gstp1, β	1.86	1.05	1.37	1.71E-01	6.93E-01
Up: 4Me & UAB	Nqo1 α, β	2.64	1.08	2.23	1.70E-07	3.38E-04
Up: UAB Dn: 4Me	Ugt1a6 α, β, δ	-1.83	1.35	3.21	5.68E-08	1.47E-04
Up: Trg & UAB	Zbtb16 α, δ	1.01	2.40	4.32	6.23E-05	

 α Mediated by Ah receptor. β Mediated by NRF2. γ Mediated by CAR. δ Mediated by HNF1 α .

Fig. 5. Genes differentially expressed between UAB30 and controls (FDR < 0.25). A heat map is shown for the exons of differentially expressed genes between UAB30 and controls (FDR < 0.25). Samples are hierarchically clustered based on Euclidean distance, and exons are hierarchically clustered using the topographical overlap dissimilarity distance between probe sets, which is a correlation-based measure used for clustering.

UAB30 had minimal effect). Previous studies with RXR agonists showed that after TRG treatment, LXR α /RXR α modulates certain genes, including *Scd1* and *Srebf1*, that are associated with increased triglycerides. In the current study, these genes were increased by both TRG and 4-Me-UAB30 but were minimally affected by UAB30. Llalloyer et al. (2009) argued that the TRG-increased serum triglycerides in mice were due to activation of the LXR α /RXR α heterodimers by increasing levels of *Scd1* and *Srebf1*. Our study supports this finding. In addition to *Scd1* and *Srebf1*, other potential triglyceride-related genes were induced by TRG and 4-Me-UAB30 but not UAB30. LXR α /RXR α (brown module) controls the expression of *Acaca*, *Acaa1*, and *Cd36*.

Acaca and *CD 36*, all directly involved in the metabolism of triglycerides. The *Srebf1* protein, SREBP1, regulates transcription of the brown module genes *Pdk1*, *Mdh1*, and *Idh3B* (Dennis et al., 2003). Other brown module genes that were increased both by TRG and 4-Me-UAB30 were *Acsm3*, *Acsm5*, *Ces6*, *Crot*, and *Decr2*. These genes are involved in lipid and fatty acid metabolism, whereas brown module genes *Dlat*, *Pdhhb*, and *Mdh1* are involved in acetyl-CoA metabolism (Dennis et al., 2003). Fatty acid metabolism and acetyl-CoA metabolism are closely related to triglyceride metabolism, and the brown module genes *Pdlim1*, *Acsm3*, *Acsm5*, *Cyp2c22*, *Nupr1*, *Scd1*, and *Vwce* are all found within triglyceride quantitative trait loci regions of the rat genome on chromosome 1 (Twigger et al., 2007). Thus, there appears to be a wide variety of genes beyond *Scd1* and *Srebf1* that may contribute to the triglyceride effects.

One question that often arises is how meaningful are the results observed by arrays. This has really multiple components, including the following: Are the RT-PCR results reproducible, and are they consistent over time? The first study was performed in some overall view by comparing a series of RT-PCR determinations on a different set of similarly treated samples; results were obtained by array analysis, and we observed strong overall agreement (Supplementary Table 4). The second question was whether results obtained at 7 days of treatment were similar at other treatment times. As shown in Table 5 for 10 genes, expression changes observed at 7 days were similarly observed after 2.3 days or 21 days of treatment. Although absolute numbers may have varied, the same gene changes were seen and the magnitude of the changes was similar.

In conclusion, three RXR-selective agonists modulated gene expression differently in rat liver, even among those genes annotated to be activated by the same RXR heterodimer. One of the clearest implications of this study is that each RXR-agonist must be fully characterized for their gene expression profiles to understand their physiologic effects fully. In our study, a pattern of increased expression was observed for LXR α /RXR α complex target genes related to increased triglycerides levels for two RXR-selective agonists (4-Me-UAB30 and TRG), but not for UAB30, which did not increase triglycerides. However, we identified many additional genes that are associated with this triglyceride effect, which is

TABLE 5

Fold change (FC) 9FC0 and *P* values for Targretin time-series reverse transcriptase (RT)-polymerase chain reaction (PCR) experiment

The FC and *P* values for the follow up Targretin time-series RT-PCR experiment are shown. The fold changes are positive for all genes at all time points. The difference compared with controls after 21 days is significant for all genes (*P* < 0.05). The difference compared with controls after 2.5 days and 7 days is significant for all genes at *P* < 0.15 and for most genes at *P* < 0.05 (2.3 days: 9/10; 7 days: 6/10).

Gene Symbol	2.3 Days Vs. Controls		7 Days Vs. Controls		21 Days Vs. Controls	
	Fold Change	<i>P</i> value	Fold Change	<i>P</i> Value	Fold Change	<i>P</i> value
<i>Abcd3</i>	2.45	3.0E-03	1.45	1.0E-01	2.53	2.4E-03
<i>Acaca</i>	2.60	1.1E-02	4.62	4.8E-03	6.76	4.4E-04
<i>Acox1</i>	2.55	9.4E-03	1.63	9.5E-02	3.34	3.3E-03
<i>Ech1</i>	5.56	4.5E-05	1.85	2.5E-02	3.87	2.1E-04
<i>Ehhadh</i>	7.74	1.5E-04	3.90	2.9E-03	9.34	8.4E-05
<i>Ethel1</i>	2.59	1.3E-02	2.70	1.1E-02	4.62	6.8E-04
<i>Fasn</i>	2.40	1.4E-02	6.99	2.2E-03	9.41	1.6E-04
<i>Por</i>	1.84	1.4E-01	1.47	1.1E-01	1.80	3.6E-02
<i>Scd1</i>	3.10	1.4E-02	6.27	1.1E-03	9.56	1.1E-04
<i>Srebf1</i>	1.98	4.1E-02	1.79	1.0E-01	2.80	1.4E-02

Fig. 6. Effect of UAB30 on mammary tumorigenesis. UAB30 and the positive control 5,6 benzoflavone were administered to female Sprague-Dawley rats for 1 week before and 1 week after DMBA treatment at 50 days of age. The study was terminated 120 days after the rats received the carcinogen.

a dose-limiting toxicity of many retinoids. The reduced triglyceride effect for UAB30, which was reflected in minimal gene expression changes, makes it a promising drug for cancer prevention, which requires chronic administration of drugs to high risk, but otherwise healthy, populations. These studies showed that UAB30 activated Ah receptor genes, which predicted the efficacy of this agent in the DMBA mammary cancer model. This was presumably due to inhibition of DMBA initiation and is an additional chemoprevention mechanism for this specific agent. Thus, the gene data alone allow one to make predictions about significant physiologic effects. Also, it was found that observing gene changes at 7 days was reflective of similar changes at 2.3 and 21 days, implying a steady state.

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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Grubbs, Muccio, Lubet, Bland.

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