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INHIBITION OF MAMMARY CARCINOMA CELL GROWTH BY RXR IS MEDIATED BY THE RECEPTOR'S OLIGOMERIC SWITCH

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Abstract

Ligands that activate the nuclear receptor RXR display potent anticarcinogenic activities but the mechanisms by which these compounds inhibit carcinoma cell growth are poorly understood. While RXR can regulate gene expression due to its intrinsic ligand-activated transcription function, this receptor can also regulate transcription by functioning as a ligand-controlled DNA architectural factor. It was thus reported that apo-RXR self-associates into tetramers and that each dimer within these tetramers can separately bind to an RXR response elements. Hence, DNA-binding by RXR tetramers may bring distant genomic regions into close physical proximity. As ligand-binding induces the dissociation of RXR tetramers into dimers, can alter gene expression by modulating DNA architecture. Here we show that inhibition of mammary carcinoma cell growth by RXR ligands stem from the ability of these compounds to regulate the oligomeric state of RXR and is independent of the direct intrinsic transcriptional activity of the receptor. The data suggest that compounds that trigger dissociation of RXR tetramers may comprise a novel class of anti-carcinogenic agents.

Keywords

RXR; tetramer; rexinoid; growth inhibition; DNA architecture

Introduction

Nuclear hormone receptors comprise a family of ligand-activated transcription factors whose activities are regulated by small hydrophobic hormones. Among nuclear receptors, the retinoid X receptor (RXR), which can be activated by the vitamin A metabolite 9-cis-retinoic acid (9cRA), holds a special place.¹ This receptor can bind to DNA and activate transcription as a homodimer, but it also serves as an obligatory common heterodimerization partner for other type II nuclear receptors, e.g. the retinoic acid receptor (RAR), vitamin D receptor (VDR), and peroxisome proliferator activated receptors (PPAR). RXR-containing dimers bind in regulatory regions of their target genes by associating with response elements (RE) comprised

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of two direct repeats of the consensus sequence PuG(G/T)TCA. RXR homodimers bind to an RXRE in which the repeats are separated by 1 basepair (DR-1), and RXR-containing heterodimers associate with other REs whose specificities are determined by variable spacings between the repeats. Activation of DNA-bound dimers by appropriate ligands allows them to recruit transcriptional coactivators to the promoters of target genes and enhance transcriptional rates.² RXR thus functions as a 'master regulator' of multiple signalling pathways that converge at the genome.

Another unique feature of RXR is that the apo-receptor avidly self-associates into homotetramers both in solution and when bound to DNA.^{3–6} RXR tetramers, which comprise the predominant fraction of the apo-receptor, are transcriptionally silent but they rapidly dissociate into active dimers upon binding of a cognate ligand.^{7,8} Ligand-induced tetramer dissociation thus comprises the first step in the activation of the receptor. Interestingly, each dimer within the RXR tetramer can separately associate with an RXRE.³ Consequently, in promoter regions that contain two RXREs and thus bind two RXR dimers in tandem, association of these dimers into tetramers can result in the formation of a DNA loop.⁹ RXR tetramers can thus juxtapose distant DNA sequences onto transcription initiation sites, enabling regulation of gene expression by distant factors. Ligand-binding by the receptor triggers tetramer dissociation, leads to linearization of the DNA loop, negates the activity of the distant factors, and results in changes in gene expression.⁹ Hence, RXR functions as a ligand-controlled DNA architectural factor. As RXR agonists (rexinoids) control both the transcriptional activity of the receptor and its oligomeric state, they can regulate gene expression by two distinct mechanisms, through direct transcriptional activation of RXR, and through RXR-mediated changes in DNA geometry.

Rexinoids display potent anticarcinogenic activities and are increasingly considered and are being used as preventive and therapeutic agents in cancer. It has been reported that rexinoids suppress tumor formation in several experimental models of breast cancer, and that they are efficacious in prevention of skin carcinogenesis.^{10–13} Rexinoids have been approved for treatment of human patients with refractory advanced-stage cutaneous T-cell lymphoma^{14,15} and were shown to display clinical benefits in patients with breast cancer.¹⁶ It has been suggested that inhibition of carcinoma cell growth by rexinoids is exerted through inhibition of AP1-mediated transcription. It was also proposed that RXR triggers a G1 cell cycle arrest by inducing the expression of growth-inhibitory factors, e.g. RAR β , and by suppressing the expression of growth-stimulating proteins, such as cyclin D1 and COX-2.¹⁷ However, information on target genes that mediate tumor suppression by rexinoids is scarce, and the molecular mechanisms that underlie this activity remain poorly understood. Investigation of this issue has been hampered by the multi-faceted nature of the transcriptional activities of RXR which may be mediated by RXR homodimers or by one or more of the various RXR-containing heterodimers. The recent findings that rexinoids can also regulate gene expression by controlling the oligomeric state of RXR and, consequently, DNA geometry add an additional level of complexity.

Here, we undertook to dissect between anti-proliferative activities of RXR that are exerted by the receptor's direct transcriptional activity *vs.* activities that stem from the function of the receptor as a DNA architectural factor. We show that the direct transcriptional activity of RXR is not required for rexinoid-induced upregulation of genes involved in cell cycle control and apoptosis, or for rexinoid-triggered inhibition of MCF-7 mammary carcinoma cell growth. Instead, we demonstrate that these activities originate exclusively from the ability of rexinoids to trigger an RXR tetramer/dimer transition. Strikingly, an antagonist that can control the oligomeric switch of RXR displayed anti-proliferative activities similar to those of RXR agonists. The observations suggest that important biological activities of RXR, including its

anticarcinogenic activities, are exerted through the ability of the receptor to regulate DNA geometry rather than from its classical function as a direct transcription factor.

Results

The ligand-controlled switch of the RXR oligomeric state regulates gene expression through a mechanism independent of the receptor's direct transcriptional activity

RXR tetramers dissociate into dimers upon binding of RXR agonists.^{3,5-7} This response can be demonstrated by electrophoretic mobility-shift assays (EMSA) showing that, in the absence of ligand, RXR associates with DNA as a tetramer, and that the tetramer-DNA complex is transformed into a dimer-DNA complex in the presence of RXR agonists such as 9cRA or the synthetic ligand SR11345 (Fig. 1a). These observations demonstrate that an ability to induce tetramer dissociation is a common feature of activating RXR ligands. Retinoids thus regulate gene expression by classical RXR-mediated direct transcriptional activation, but they may also modulate transcriptional rates by triggering alterations in DNA geometry which result from the ligand-regulated change in the receptor's oligomeric state. To dissect between these activities, we utilized an RXR mutant lacking its C-terminal helix 12 (RXRΔH12). This mutant properly binds to DNA, forms tetramers, and undergoes an oligomeric switch upon ligand binding.⁹ It is thus intact in its ability to mediate ligand-responsive DNA looping. However, RXRΔH12 is unable to associate with coactivators and is transcriptionally silent.¹⁸ Hence, the mutant is expected to display WT activity in regard to RXR-mediated regulation of DNA architecture, but to function as a dominant negative protein in regard to direct transcriptional activation. To examine this premise, transactivation assays were carried out in MCF-7 mammary carcinoma cells. Cells were co-transfected with a luciferase reporter driven by an RXRE, an expression vector for RXRα, and increasing amounts of an expression vector for RXRΔH12. As shown in Fig. 1b, treatment of the cells with the synthetic agonist SR11345 activated the reporter, attesting to the presence and functionality of RXR. Ectopic overexpression of RXRΔH12 completely abolished the activation, demonstrating that the mutant functions as a dominant negative to inhibit the transcriptional activity of the receptor.

We then set out to identify genes in MCF-7 cells that respond to an RXR ligand solely through the ability of the receptor to function as a DNA architectural factor. To exclude direct transcriptional responses, cells were transfected with RXRΔH12, grown for 24 hr. to allow accumulation of the mutant, and then treated with vehicle or SR11345 for 4 hr. RNA was extracted and Affymetrix expression array analyses were carried out to identify genes whose expression levels are altered in response to the ligand. The analyses showed that, under these conditions, ligand treatment resulted in upregulation of 225 genes and in decreased expression of 86 genes. Upregulated genes included various genes involved in regulation of cell cycle progression and in apoptotic responses, and several genes known to mediate growth arrest in response to DNA-damage (e.g. Table 1). Among these, we focused on the cell cycle regulator B-cell translocation gene, member 2 (Btg2) and on the apoptotic protein caspase 9. In agreement with the data of the Affymetrix expression array, quantitative RT-PCR (Q-PCR) analyses verified that, in MCF-7 cells ectopically expressing RXRΔH12, treatment with SR11345 significantly upregulated the expression of caspase 9 and Btg2 (Fig. 1c, d). In accordance with the reports that Btg2 functions by downregulating the expression of cyclin D1^{19,20}, SR11345 treatment also resulted in a 3 fold decrease in cyclin D1 mRNA (Fig. 1e). These observations suggest that growth inhibitory activities of retinoids may be mediated, at least in part, by the ability of the receptor to regulate DNA geometry and not through its direct transcriptional activity.

An RXR antagonist that triggers tetramer dissociation mimics agonists in evoking transcriptional response

To further examine the ability of RXR to regulate gene expression through changes in its oligomeric state, we used the RXR antagonist PA452.²¹ Transcriptional activation assays that were carried out using MCF-7 cells transfected with an RXRE-driven luciferase reporter confirmed that this compound functions as a potent RXR antagonist in these cells (Fig 2a). Surprisingly, EMSA revealed that, similarly to RXR agonists, PA452 triggers dissociation of RXR tetramers (Fig. 2b). To further verify this activity, DNA cyclization assays were carried out. In these, an oligonucleotide containing two RXRE separated by 250 bp was utilized. The DNA sequence of the oligonucleotide was based on the promoter of the retinol binding protein (RBP) gene, which contains two retinoid elements.²² Two modifications were made. In order to ensure strong binding of RXR, a consensus RXRE was used in place of the native retinoid elements of the RBP promoter. Additionally, the spacing between the RXREs was increased to 250 bp. The restriction site *avaI* was added at both ends to yield an oligonucleotide of a total length of 382 bp. The fragment was labeled with ³²P, incubated with RXR, and mixtures were treated with DNA ligase to catalyze ligation of the DNA ends to form circular DNA. The notion behind this experiment is that, as binding of tetrameric RXR induces the DNA to loop, addition of the protein will place the ends of a fragment closer together, and ligation will be facilitated (Fig. 2c, top). Ligand-binding, which induces tetramer dissociation, will negate the loop and thus decrease circularization efficiency. Following ligation, mixtures were treated with proteinase K to degrade proteins, and with exonuclease III to degrade linear DNA. Remaining circular species were then resolved by electrophoresis and visualized. As shown in Fig 2c, circular DNA was observed in the presence but not the absence of RXR, and their abundance was decreased in the presence of 9cRA. Hence, RXR enhanced DNA-cyclization, and the activity was inhibited upon ligand-triggered tetramer dissociation. The RXR antagonist PA452 also inhibited RXR-mediated circularization (Fig. 2c), indicating that, similarly to 9cRA, the antagonist induced dissociation RXR tetramers.

The observations that PA452 triggers the RXR oligomeric switch raise the possibility that, while this compound is an antagonist in regard to the direct transcriptional activity of the receptor, it may function as an agonist in regard to the alternate RXR activity. To examine this possibility, MCF-7 cells were treated with the agonist SR11345 or the antagonist PA452, and the expression levels of Btg2 and caspase 9 were determined. The data indicated that both ligands upregulated the expression of Btg2 and caspase 9 mRNA (Fig. 2d) as well as caspase 9 protein (Fig. 2e). Additional experiments revealed that this activity is cell-specific. Neither ligand affected Btg2 or caspase 9 expression in the prostate cancer RWPE1 cells, the breast cancer MDA-MB231 cells, or the hepatocyte cell line HepG2 (not shown). However, similarly to their activities in MCF-7 cells, both the agonist and the antagonist increased the expression of these genes in HL-60 cells (Fig. 2f). Examination of the basal expression levels of Btg2 and caspase 9 revealed that both genes are highly repressed in the cell lines that failed to respond to RXR (Fig. 2g, 2h). Silencing of Btg2 and caspase 9 in RWPE1, HepG2, and MDA-MB231 cells may provide a rationale for their irresponsiveness to retinoids.

It was previously reported that caspase 9 and Btg2 comprise direct target genes for RAR-RXR heterodimers.^{20,23} As shown in Figure 3a, both the RXR agonist SR11345 and the RAR-selective ligand TTNPB increased the expression of mRNA for these genes, and the effect was additive when the two ligands were used in combination. It could be argued that the effect of the RXR agonist was mediated through RAR-RXR heterodimers and not through the ability of RXR to regulate DNA geometry. If this was the case, upregulation of these genes by an RXR ligand would be blunted in the presence of an antagonist for RA.²⁴ As shown in Fig. 3b, the pan-RAR antagonist LE540²⁵ had little effect on the ability of either the RXR agonist or the RXR antagonist to upregulate either Btg2 or caspase 9. The data thus indicate that retinoid

receptors and their ligands regulate the expression of Btg2 and caspase 9 by two distinct mechanisms, i.e. through direct transcriptional activation of RAR, and through RXR-mediated alterations in DNA geometry.

An RXR antagonist that triggers tetramer dissociation mimics agonists in inhibiting carcinoma cell growth

The observations that both an agonist and the RXR antagonist PA452 upregulate the expression of the cell cycle regulator Btg2 and the apoptotic protein caspase 9 raise the possibility that both of these compounds may exert antiproliferative activities. MTT cell proliferations assays that were carried out to examine this possibility revealed that, indeed, both the agonist and the antagonist significantly inhibited MCF-7 cell growth (Fig. 4a). To gain insight into the mechanism that underlie the inhibitory activity, the effects of the ligands on cell cycle progression and apoptosis were monitored. MCF-7 cells were treated with either SR11345 or PA452 (1 μ M) for 5 days, stained with propidium iodide, and analyzed by flow cytometry (Fig. 4b). The data showed that treatment with either the agonist or the antagonist induced the cells to undergo apoptosis, reflected by a marked increase in the fraction of cells in the sub-G₁ population. While not statistically significant, the data suggest that treatment with the ligands may have also resulted in an arrest at the G₁ to S transition.

Ligand-controlled tetramer-dimer transition is critical for RXR-mediated regulation of caspase 9 and Btg2

The importance of ligand-induced tetramer dissociation for the anti-proliferative activity of rexinoids was further examined by monitoring the effects on gene expression of RXR mutants that are defective in their oligomerization behavior. One of these, RXR-R321A, forms tetramers with wild-type affinity but these tetramers do not dissociate upon ligand binding.^{7, 9} It can thus be predicted that, in the presence of this mutant, responses to an RXR ligand will be impaired. The other mutant, RXR-F318A, does not form tetramers at all and is constitutively dimeric.^{7,9} Expression of this mutant can thus be predicted to lead to a constitutively high expression of caspase 9 and Btg2. To examine these predictions, MCF-7 cells were transfected with expression vectors for WT-RXR, RXR-R321A, or RXR-F318A. Cells were treated with the RXR agonist SR11345, or the antagonist PA452, and levels of Btg2 and caspase 9 mRNA were measured. As predicted, the expression levels of both Btg2 (Fig. 5a, 5c) and caspase 9 (Fig. 5b, 5d) were constitutively low in the presence of R321A, and constitutively high in the presence of F318A.

To examine the importance of the oligomeric switch of RXR for its pro-apoptotic activity, three mutants were used: the constitutively tetrameric RXR-R321A, the constitutively dimeric RXR-F318A, and RXR Δ H12, a mutant which is transcriptionally silent but is intact in its oligomeric behavior. Cells were transfected with vectors harboring cDNA for WT-RXR or the corresponding mutants, treated with SR11345 or PA452 (1 μ M, 5 days), and analyzed by flow cytometry to assess the fraction of cells undergoing apoptosis (Fig. 6). Like the WT receptor, RXR Δ H12 mediated apoptosis triggered by both the agonist and the antagonist (Fig. 6a). In accordance with the constitutively tetrameric state of RXR-R321A, this mutant failed to mediate ligand-induced apoptosis (Fig. 6b). Correspondingly, cells over-expressing the constitutively dimeric mutant, RXR-F318A, showed increased apoptosis even in the absence of ligand (Fig. 6c).

Discussion

The studies described in this manuscript aimed to clarify whether the anticarcinogenic activities of rexinoids stem from enhancement of the direct transcriptional activity of RXR or from modulation of DNA geometry due to the ligand-controlled oligomeric switch of the receptor.

The data show that RXR Δ H12, a mutant devoid of direct transcriptional activity but intact in regard to its oligomeric behavior, displays a wild-type ability to mediate rexinoid-induced upregulation of genes involved in cell cycle progression and apoptosis in mammary carcinoma cells. Furthermore, the data demonstrate that an RXR antagonist that induces tetramer dissociation mimics RXR agonists in its ability to upregulate gene expression and inhibit carcinoma cell growth. While the exact nature of DNA regions that are connected to regulate the expression of specific genes through the tetramer/dimer switch of RXR remains to be clarified, the observations indicate that important biological activities of rexinoids, including their anti-proliferative activities, originate solely from their ability to trigger dissociation of RXR tetramers. Hence, compounds that induce dissociation of RXR tetramers, regardless of their ability to activate the receptor, may comprise a novel class of anti-carcinogenic agents.

Materials and Methods

Reagents

9cRA was purchased from Sigma Chemical Co. RXR transcriptional agonist (*E*)-4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-2-methylpropenyl]benzoic acid (SR11345) was synthesized by the route described for its 3-desmethyl isomer.²⁹ 2-[(3-*n*-Hexyloxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)(methyl)amino]pyrimidine-5-carboxylic acid (PA452)³⁰ and 4-(13*H*-10,11,12,12-tetrahydro-10,10,13,13,15-pentamethyldinaphtho[2,3-*b*][1,2-*e*]diazepin-7-yl)benzoic acid (LE540)³¹ were kindly provided by Dr. Hiroyuki Kagechicka (Tokyo Medical and Dental School, Tokyo, Japan). 4-[(*E*)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) was purchased from Biomol. Antibodies against caspase 9 and β -tubulin were obtained from Abcam (Cambridge, MA) and Sigma Chemical Co., respectively. Anti-mouse and anti-rabbit immunoglobulin horseradish peroxidase-conjugated antibodies were from BioRad.

Vectors

Recombinant histidine-tagged RXR α lacking the amino terminal A/B domain (RXR α Δ AB), and mammalian expression vectors for RXR, RXR Δ H12, RXR-F318A, and RXR-R321A were previously described.^{3,7-9}

Cells

COS-7 and MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). HL-60 cells were cultured in RPMI supplemented with 10% FBS.

Affymetrix expression array

MCF-7 cells were transfected with an expression vector for RXR H12. 48 hours post transfection, cells were treated with vehicle or SR11345 (2.5 μ M) for 4 hr. Total RNA was prepared from triplicate cell cultures using RNeasy (Qiagen, Valencia, CA). Sample processing and analyses, including cDNA synthesis, cRNA synthesis and labeling, and array applications were performed at the Gene Expression Analysis facility of Case Western Reserve University. RNA quality was assessed prior to cDNA synthesis. 5 μ g RNA from each sample was used to generate cDNA, and 1 μ g product was used in an *in vitro* transcription reaction containing biotinylated UTP and CTP. 20 μ g full-length cRNA was fragmented and analyzed on Affymetrix U133A/B high-density oligonucleotide array. Arrays were hybridized, stained, and washed using the GeneChip Fluidics Station 450. Detection and quantitation of target hybridization were performed with a GeneArray Scanner 3000 (Affymetrix, Santa Clara, CA). Iobion's Gene traffic was used to perform Robust Multi-Chip Analysis and clustering genes with similar activity by summary function. An unpaired *t* test giving *p* values of <0.1 were defined as significantly changed.

Transactivation assays

COS-7 cells (1×10^5) were plated in 6-well plates in DMEM supplemented with 2.5% charcoal-treated FBS. Cells were transfected using Fugene6 (Roche) with vectors harboring RXR α (50 ng), a luciferase reporter driven by a DR-1 (0.5 μ , and pCH110 (β -galactosidase tinternal control, 0.25 μ g). pCDNA3 was used as empty vector. 24 hr. post-transfection, cells were treated with vehicle or ligand (1 μ M) and incubated for 16 to 20 hr. Luciferase expression was assayed using the luciferase assay (Promega) and corrected for transfection efficiency by the activity of β -galactosidase. Transactivation assays of MCF-7 cells with the RXR mutants, RXR Δ H12, RXR-F318A or RXR-R321A were performed using Superfect.

Electrophoretic mobility-shift assays (EMSA)

EMSA were carried out as previously described.³

DNA circularization assays

DNA circularization assays were carried out as previously described.⁹ Briefly, a 370 bp DNA fragment containing two RXREs spaced by 250 bp pairs was excised from the plasmid using *Ava*I restriction enzyme. DNA fragments were purified by gel extraction kit (Qiagen) and radio-labeled by Klenow filling. Labeled DNA was purified using a Qiagen column. Recombinant RXR Δ AB (400 nM) was incubated with or without ligand (4°C, 15 min.) Labeled DNA (100 nM) was added and mixtures incubated at room temperature (20 min.). DNA ligase was added, mixtures incubated for 30 min., treated with exonuclease III (15 min. 37°C). Proteins were then digested with proteinase K (20 min. 37°C). Circularized DNA was resolved by electrophoresis on non-denaturing 5% acrylamide gels and visualized by autoradiography.

Fluorescence activated cell sorting (FACS)

MCF-7 cells were seeded in 60 mm plates in DMEM supplemented with 10% charcoal-treated FBS. Cells were transfected with expression vectors for RXR, RXR Δ H12, RXR-F318A or RXR-R321A (6 μ g) using Superfect and grown overnight. Ligand (1 μ M) was added and replenished every 48 hr. for 5 days. Cells were collected, washed with PBS, fixed in 70% methanol (5 hr.) and stained with propidium iodide (5 μ g/ml, overnight, 4°C). Samples were analyzed at the Case Western Reserve University cell sorting facility, using a Becton Dickinson LSRII cell sorter.

Western blotting

Cells were lysed in a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 1 mM PMSF, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin and 2 μ g/ml pepstatin A. Protein concentrations were determined by the Bradford assay and 50 μ g/lane cell lysate was resolved by 10% SDS-PAGE and probed by Western blots using appropriate antibodies.

Quantitative Real-time PCR

Total RNA was extracted using RNeasy (Qiagen, Valencia, CA). 1 μ g mRNA was reverse transcribed into cDNA using the qScript cDNA from Quanta Biosciences (Gaithersburg, MD). Quantitative real-time PCR (Q-PCR) analyses were performed in quadruplicates using the PerfeCTa qPCR supermix (Quanta Biosciences) and TaqMan chemistry and Assays on Demand probes (Applied Biosystems) for caspase 9 (Hs00154260-m1) and Btg2 (Hs00198887-m1). As internal control, 18s rRNA (4319413E-0710034) was used. Detection and data analysis were carried out on an ABI StepOne Plus Real-Time PCR System.

MTT Assays

MCF-7 cells (5000 cells) were seeded in 96-well plates in the presence or absence of the indicated ligands for 5 days. Ligands were replenished every 48 hr. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent, 50 μ l of 1 mg/ml) was added directly to the cells and left at 37°C for 4 hr. or until crystals formed. Media was carefully removed, crystals suspended in 150 μ l of 40 mM HCL in isopropanol. Absorbance was measured at 550 nm.

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Abbreviations

RXR	retinoid X receptor
PPAR	peroxisome proliferator activated receptors
9cisRA	9-cis retinoic acid
RAR	retinoic acid receptor
VDR	vitamin D receptor
RE	response element
EMSA	electrophoretic mobility shift assays
RBP	retinol binding protein
RXRE	retinoid X DNA response element

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Figure 1. Reginoids induce dissociation of RXR tetramers and control gene expression in MCF-7 cells expressing RXRΔH12

(a) EMSA using recombinant RXRΔAB and an oligonucleotide containing an RXRE. Protein and ³²P-labeled DNA were incubated in the absence of ligands or with the RXR ligands 9cRA or SR11345 (SR). Mixtures were resolved by non-denaturing 5% PAGE and visualized by autoradiography. (b) RXRΔH12 functions as a dominant negative to inhibit the transcriptional activity of WT-RXR. Transactivation assays were carried out in COS-7 cells transfected with a luciferase reporter driven by an RXRE, an expression vector for RXRα, and increasing amounts of a vector harboring RXRΔH12 cDNA. Cells were treated with the RXR agonist SR11345 (SR) for 16 hr. prior to cell lysis and analysis. Data are mean±S.E.M (n=3). (c-e) MCF-7 cells ectopically over-expressing RXRΔH12 were treated with vehicle or SR11345 for 4 hr. RNA was extracted and the levels of caspase 9 (c), Btg2 (d) and cyclin D1 (e) mRNA were measured by Q-PCR.

Figure 2. An RXR antagonist induces tetramer dissociation and upregulates Btg2 and caspase 9 expression

(a) PA452 is a potent RXR antagonist. Transactivation assays were carried out using COS-7 cells co-transfected with a luciferase reporter driven by an RXRE and a plasmid expressing β -galactosidase. 24 hr. post transfection, cells were treated with the RXR agonist SR11345 (SR, 1 μ M) and increasing concentration of PA452 for 20 hr., prior to lysis and analysis. Luciferase activity was normalized to β -galactosidase. Data are mean $\pm$ S.E.M (n=3). (b) PA452 induces dissociation of RXR tetramers. EMSA using recombinant RXR Δ AB and a 32 P-labeled oligonucleotide containing an RXRE. Protein and DNA were incubated in the absence of ligands or in the presence of 9cRA or PA452. Mixtures were resolved by non-denaturing 5% PAGE and visualized by autoradiography. (c) 9cRA and PA452 inhibit DNA cyclization by RXR. Top: a scheme showing RXR tetramers bound to DNA containing two tandem RXREs. Bottom, cyclization assays. An oligonucleotide containing two RXREs separated by 250 bp was subjected to cyclization in the presence or absence of denoted ligands (see Materials and Methods). (d-g) PA452 upregulated the expression of caspase 9 and Btg2. MCF-7 cells were serum-starved for 24 hr. prior to the addition SR11345 (SR) or PA452 (PA). Cells were incubated for 4.5 hr., and the expression of mRNA for Btg2 (d, left panel), and caspase 9 (d, right panel) measured by Q-PCR. Values are mean $\pm$ S.E.M (n=3). (e) MCF-7 cells were treated with SR or PA for 24 hr. prior to cell lysis. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies recognizing full-length and cleaved caspase 9. β -tubulin served as a loading control. (f) HL-60 cells were treated with SR11345 or PA452 (1 μ M) for 4 hr. and expression of caspase 9 (left panel) and Btg2 (right panel) measured by Q-PCR. Data are mean $\pm$ S.D. (n=3). (g) (h) RNA was extracted from MCF-7, RWPE1, HepG2, MDA-MB231 and HL-60 cells and expression level of mRNA for caspase 9 (g) and Btg2 (h) measured by Q-PCR and normalized to expression level in MCF-7 cells. Data are mean $\pm$ S.D. of 2 independent experiments, each carried out in triplicates

Figure 3. Upregulation of Btg2 and caspase 9 by RXR ligands is not affected by an RAR antagonist MCF-7 cells grown in a serum free medium were treated with the indicated ligands (1 μ M) for 4.5 hr. Ligands used: the RXR agonist SR11345 (SR), the RAR agonist TTNPB (T), the pan-RAR antagonist LE540 (LE), and the RXR antagonist PA452 (PA). Expression levels of mRNA for Btg2 and caspase 9 were measured by Q-PCR and normalized to 18S RNA. Data are mean $\pm$ S.D (n=3).

Figure 4. An RXR agonist and an RXR antagonist inhibit cell proliferation by inducing apoptosis and a G1 cell cycle arrest

(a) MCF-7 cells were treated with the indicated concentrations of SR11345 or PA452 for 5 days. Cell proliferation was assessed by MTT assays. Data are means $\pm$ S.D (n= 3). (b) MCF-7 cells, maintained in a DMEM medium containing 1% charcoal-treated FBS, were treated with the indicated ligands (1 μ M) for 5 days. Ligands were replenished every 48 hr. Cells were then harvested, washed with PBS, fixed in 70% methanol, and stained with propidium iodide. Distribution of cells between different populations was analyzed by FACS. Experiments were repeated three times with similar results.

Figure 5. Expression of Btg2 and caspase 9 is regulated by the tetramer/dimer switch of RXR
MCF-7 cells were transfected with expression vectors for WT-RXR, the constitutively tetrameric RXR-R321A, or the constitutively dimeric RXR-F318A. Following an overnight incubations, cells were treated with SR11345 or PA452 (1 μ M) for 4 hr. RNA was extracted and expression levels of Btg2 mRNA (a, c) and caspase 9 (b, d) were measured by Q-PCR. 18S RNA was used for normalization. Data are mean $\pm$ S.D. (n=3).

Figure 6. Induction of apoptosis by RXR ligands is mediated through the receptor's tetramer/dimer switch

MCF-7 cells were transfected with expression vectors for WT- RXR, RXR-ΔH12, RXR-R321A or RXR-F318A and treated with denoted ligands (1 μM) for 5 days. Cells were harvested, fixed with 70% methanol, stained with propidium iodide and analyzed by FACS. The experiment was repeated twice with similar results.

Table 1

Some genes whose expression is upregulated by rexinoids through the ligand-controlled tetramer/dimer switch of RXR

gene	proposed function	fold increase Affymetrix array	fold increase Q-PCR
BTG family, member 2 (Btg2)	regulation of the G ₁ /S transition	2.8	2.3±0.5
caspase 9	apoptosis-related cysteine peptidase	2.8	6.0±1.6
TR3/Nur77/NGFI-B	proapoptotic orphan nuclear receptor	4.5	5.1±1.3
Tumor necrosis factor (TNF superfamily, member 2)	regulation of multiple processes including cell proliferation, differentiation and apoptosis	3.1	3.1±0.3
Growth arrest and DNA-damage-inducible, 45beta (GADD45b)	inhibition of cell growth	3.7	ND
Growth arrest and DNA-damage-inducible, 45alpha (GADD45a)	apoptosis-associated, inhibition of cell growth	2.7	ND
Growth arrest and DNA-damage-inducible, 34 (GADD34)	apoptosis-associated, inhibition of cell growth	2.8	ND
Growth arrest and DNA-damage-inducible 45gamma (GADD45g)	apoptosis, differentiation	2.4	ND