

Supporting Information

Identification of a new RXR α antagonist targeting the coregulator-binding site

Fan Chen^{a,b}, Jie Liu^a, Mingfeng Huang^a, Mengjie Hu^a, Ying Su^{a,b*}, Xiao-kun Zhang^{a,b*}

^aSchool of Pharmaceutical Sciences, Xiamen University, Xiamen 361005, China; ^bSanford-burnham Medical Research Institute, 10901 N. Torrey Pines Road, La Jolla, California 92037, USA

*To whom correspondence should be addressed.

Contents:

1. Materials and Methods
2. Supplemental Figures

1. Materials and Methods

Reagents

Fermentas TurboFect in vitro transfection reagent, DharmaFECT 1 transfection reagent, goat anti-rabbit and anti-mouse secondary antibody conjugated to horseradish peroxidase from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), anti-mouse/rabbit IgG conjugated with Cy3, anti-mouse/rabbit IgG conjugated with FITC from Chemicon International (Temecula, CA, USA), anti-Akt1 (C-20, sc-1618), anti-Myc (9E10, sc-40), anti-RXR (Δ N 197, sc-774) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-phospho-Akt (Ser473) (D9E, 4060) from Cell Signaling Technology (Boston, MA, USA), anti-poly (ADP-ribose) polymerase (PARP, 556494) from BD Biosciences (San Diego, CA, USA), anti- β -actin antibody from Sigma (St. Louis, MO, USA), anti-PI3 kinase (p85 α ; 04-403), polyvinylidene difluoride membranes from Millipore (Billerica, MA, USA), enhanced chemiluminescence reagents from GE Healthcare (Buckinghamshire, UK) and a cocktail of proteinase inhibitors from Roche (Meylan, France), 9-*cis*-Retinoic Acid, [11, 12-³H] from PerkinElmer (Boston, MA, USA) were used in this study. All other chemicals used were commercial products of analytic grade obtained from Sigma (St. Louis, MO, USA). Compound 23 is ordered from specs.net under catalog number AE-848/34436002) and its purity is >90%. The IUPAC Name for compound **23** is 2-(1,3-benzothiazol-2-ylsulfanyl)-N'-[(E)-(4-methyl-2,7-dioxochromen-8-ylidene)methyl]acetohydrazide

Cell culture and Transient Transfection

MCF-7 breast cancer cells, HepG2 liver cancer cells, A549 lung cancer cells, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). PC-3 prostate cancer cells, were cultured in RPMI 1640 medium containing 10% FBS. Subconfluent cells

with exponential growth were used throughout the experiments. Transient transfection was performed using TurboFect according to the instructions of the manufacturer in regular growth medium.

Virtual screening

To undertake the virtual screening for the identification of compounds that bind to the protein-protein interaction groove on the surface of RXR α , we used the structure of RXR α LBD in complex with CD3254 and a coactivator peptide (PDB code, 3FUG). A chemical library of 200,000 compounds that is commercially available from Specs (www.specs.net) was downloaded to use as the initial screening library. A Pipeline Pilot protocol was used to filter out compounds that failed the Lipinski rules and that are potentially reactive and contain undesired groups. This exercise left us with about 102,000 compounds for docking-based VS. Schrodinger's Ligprep program was used to prepare the compounds for docking, including 3D structure generation, tautomer and protonation enumeration. Docking was performed using the Glide docking package from Schrodinger. 14 compounds were selected for purchase and biological testing after visual evaluation of the first 300 compounds with the best docking score.

Plasmid constructions

The pCMV-Myc-RXR α , pCMV-Myc-RXR α -A272W, pCMV-Myc-RXR α -C432W plasmids were obtained by inserting RXR α sequence (with/without mutation) in-frame with an N-terminal Myc epitope coding sequence in the pCMV-Myc vector (Clontech). The pBind-RXR α -LBD, pBind-RXR α -LBD-A272W, pBind-RXR α -LBD-C432W, pBind-RXR α -LBD-F313A, pBind-RXR α -LBD-F313A/A272W, pBind-RXR α -LBD-F313A/C432W, pBind-RXR α -LBD-V298S, pBind-ER α -LBD, pBind-GR α -LBD, pBind-RAR α -LBD, pBind-RAR γ -LBD, pBind-PPAR γ -LBD, pBind-LXR α -LBD and pBind-Nur77 plasmids were obtained by inserting RXR α -LBD (with/without mutation) sequence (amino acids 223–462), ER α -LBD sequence (amino acids 310–547), GR α -LBD sequence (amino acids 531–777), RAR α -LBD sequence (amino acids 186–416), RAR γ -LBD sequence (amino acids 188–418), PPAR γ -LBD sequence (amino acids 237–504), LXR α -LBD sequence (amino acids 167–400), Nur77 sequence in-frame with the Gal4 DBD coding sequence in the pBIND vector (Promega). pBind-receptor expression vector can induce the transcription of the luciferase reporter plasmid pG5-Luc (Promega) when activated by a ligand.

Mammalian one-hybrid assays

MCF-7 or HEK293T cells seeded in 48-well plates were transiently cotransfected with the luciferase reporter plasmid pG5-Luc (40 ng) and the pBind-receptor (40 ng) (see above). Twenty-four hours after transfection, the medium was replaced by medium containing a respective ligand and/or a testing compound. After 18 hours, cells were washed, lysed and assayed by using the Dual-Luciferase Reporter Assay System (Promega). Transfection efficiency was normalized to Renilla luciferase activity.

Reporter assays for NR response elements

HEK293T cells seeded in 48-well plates were transiently cotransfected with one of the luciferase reporter plasmids pGL6-RXRE (100 ng)/pGL6-RARE (100 ng)/pGL6-LXRE (40 ng), one or two NR expressing plasmids like pCMV-Myc-RXR α (40 ng)/pCMV-Myc-RAR α (40 ng)/pCMV-Myc-LXR α (40 ng), and pCMV-Renilla (1 ng) as internal reference. Twenty-four hours after transfection, the medium was replaced by medium containing a respective ligand and/or a testing compound. After 18 hours, cells were washed, lysed and assayed by using the Dual-Luciferase Reporter Assay System (Promega). Transfection efficiency was normalized to Renilla luciferase activity.

Native-PAGE for the separation of RXR homodimer and tetramer

The native-PAGE was carried out by the same method as SDS-PAGE with 5% stacking gel and 8% resolving gel. But SDS was removed from both gel buffer and running buffer.

MTT assays

Confluent cells cultured in 96-well dishes were treated with various concentrations of compounds for 48 hr. The cells were then incubated with 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hr at 37°C. MTT solution was then aspirated, and formazan in cells was instantly dissolved by addition of 150 μ l of DMSO each well. Absorbance was measured at 570 nm.

SPR measurements

The binding kinetics between RXR α -LBD and 23 was analyzed at 25°C on a BIAcore T200 machine with CM5 chips (GE Healthcare). PBSP was used for all measurements. For SPR measurements, RXR α -LBD were purified. We used the blank channel as negative control. About 1,000 response units of RXR α -LBD were immobilized on the chip. When the data collection was finished in each cycle, the sensor surface was regenerated with Glycine-HCl 2.5. A serial of concentrations ranging from 0.5 to 10 μ M were designed for the experiment. Sensograms were fit globally with BIAcore T200 analysis using 1:1 Langmuir binding mode.

siRNA and transfections

siRNAs against RXR α (SASI-HS01-00097639) and control siRNA (SIC001) used were from Sigma. 5 μ L aliquot of 20 μ M siRNA/well was transfected into cells in six-well plates using DharmaFECT 1 transfection reagent, according to the manufacturer's recommendations.

Western blotting

Cell lysates were prepared by lysing cells with lysis buffer containing 25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH 7.4 with a cocktail of proteinase inhibitors on ice for 30 min. Equal amounts of the lysates were electrophoresed on an SDS-PAGE gel (8 or 12%) and transferred onto polyvinylidene difluoride membranes, which were then blocked with 5% nonfat milk in TBST [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20] for 1 hr, incubated with various primary antibodies overnight at 4°C and incubated with secondary antibodies for 1 hr at room temperature. Immunoreactive products were detected by chemiluminescence with an enhanced chemiluminescence system.

Co-immunoprecipitation (Co-IP) assays

For Co-IP assay, cells grown in 10-cm dishes were transfected with various plasmids for 24 hr. After transfection, Cell lysates were incubated with the appropriate antibody for 2 hr, and subsequently incubated with protein A-Sepharose beads for 2 hr. The protein-antibody complexes recovered on beads were subjected to western blotting using appropriate antibodies after separation by SDS-polyacrylamide gel electrophoresis.

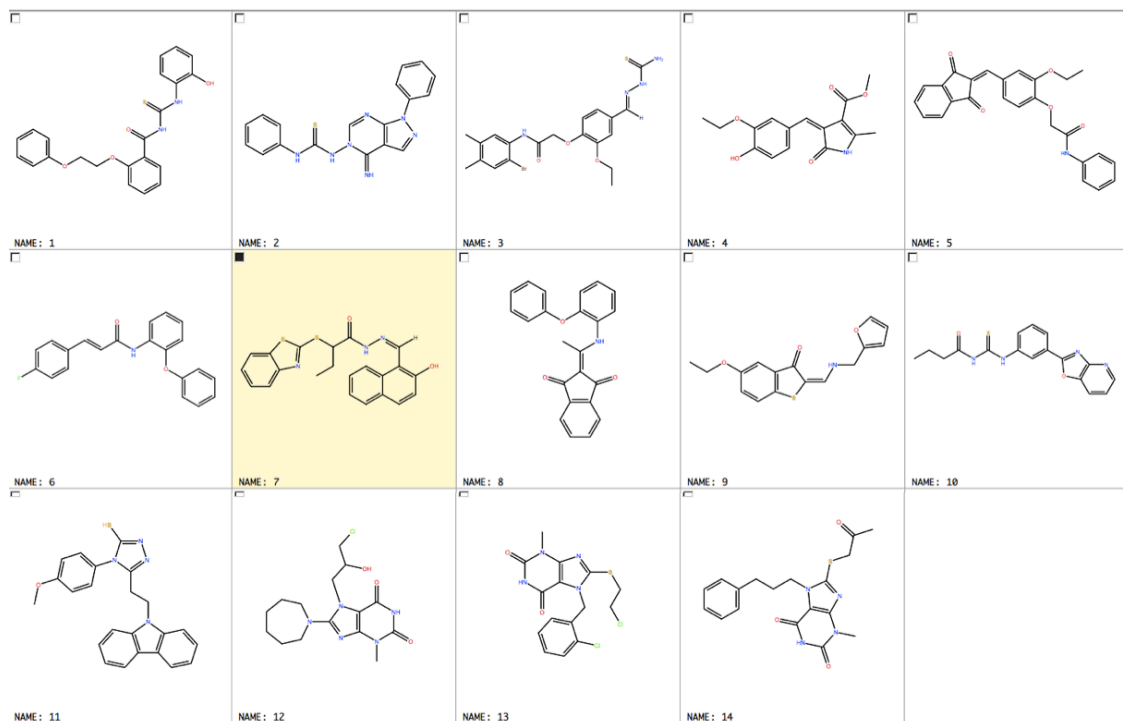
Ligand-binding competition assays

The His-tagged human RXR α -LBD (223-462) or cell lysates prepared from HEK-293T cells transfected with Myc-RXR α or mutant was incubated in tubes with unlabeled 9-*cis*-RA or different concentrations of compounds in 200 μ L binding buffer [0.15 M KCl, 10 mM Tris-HCl (pH7.4), 8% glycerol, 0.5% CHAPS] at 4°C for 1 hr. [³H]-9-*cis*-RA was added to the tubes to a final concentration of 7.5 nM and final volume of 300 μ L then incubated overnight at 4°C. The RXR α -LBD was captured by nickel-coated beads, Myc-RXR was captured by hydroxylapatite. Bound [³H]-9-*cis*-RA was quantitated by liquid scintillation counting.

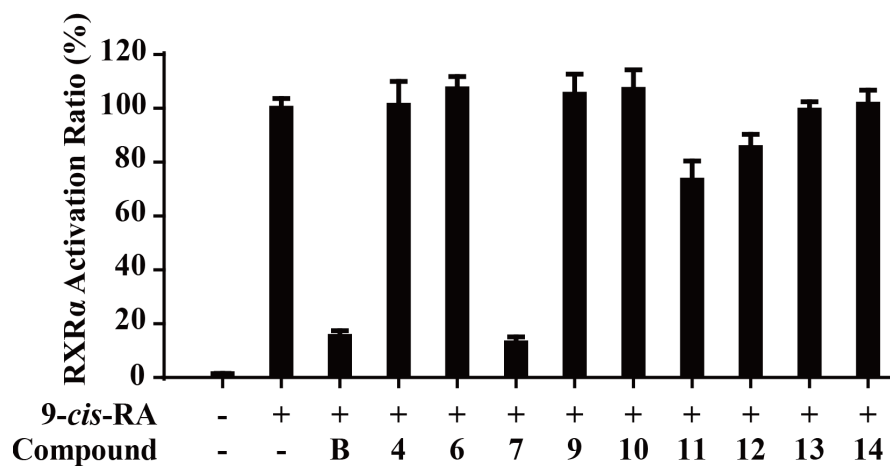
Statistical analysis

Data were expressed as mean \pm SD. Each assay was repeated in triplicate in three independent experiments. Statistical significance of differences between groups was analyzed by using Student's t test or ANOVA analysis. $p < 0.05$ was considered significant.

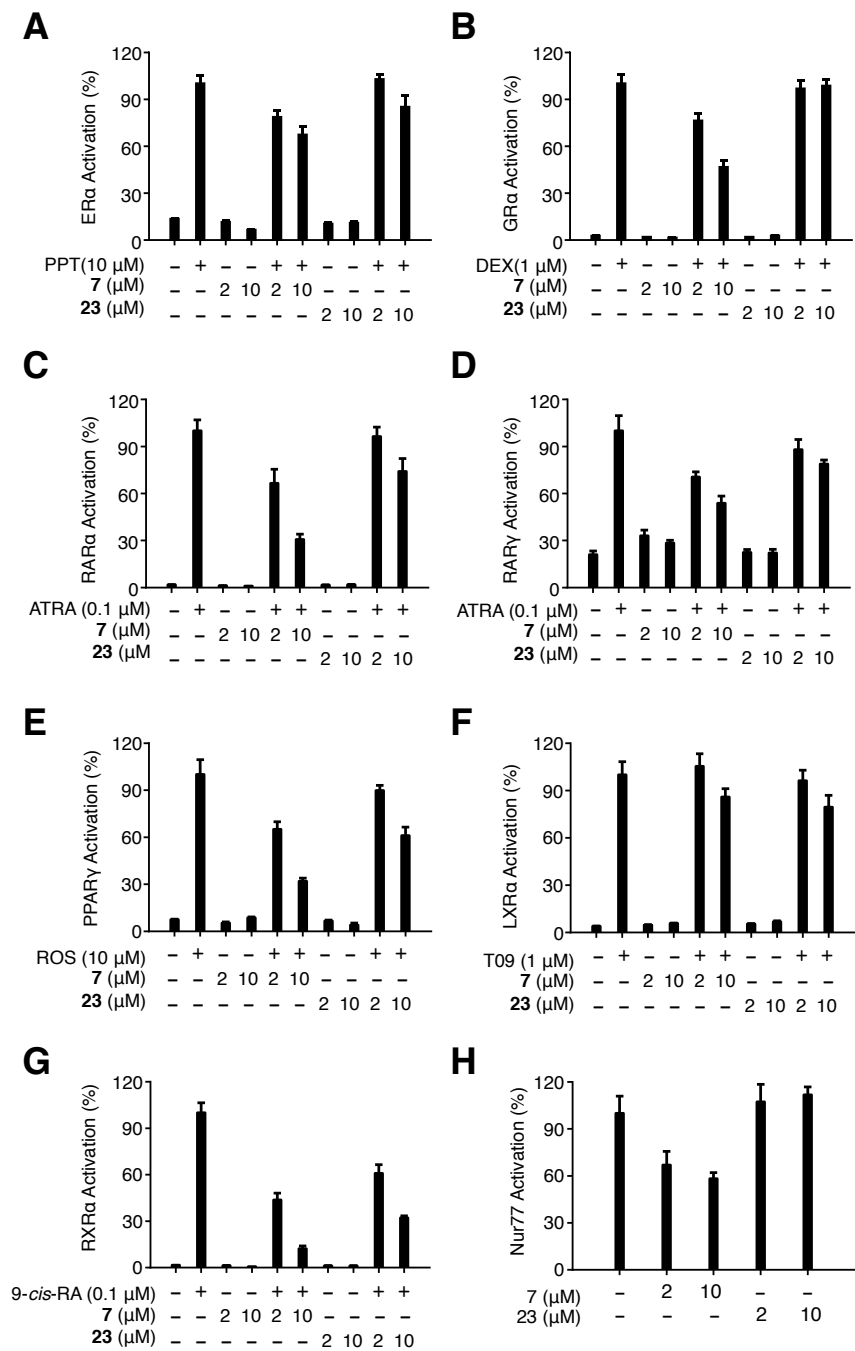
2. Supplemental Figures



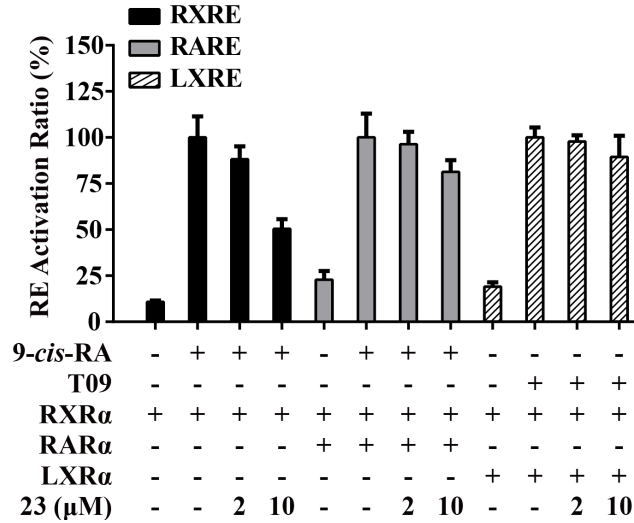
Supplemental Figure 1A. Chemical structures of the 14 selected compounds. Compound 7 is highlighted.



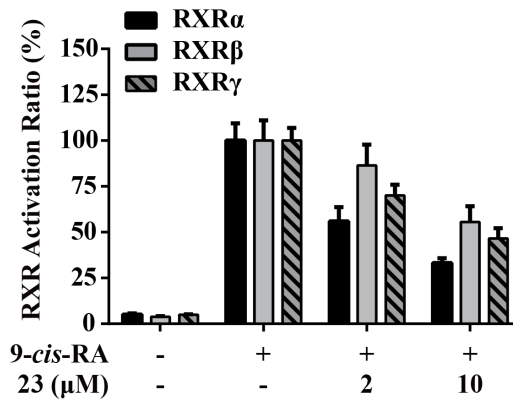
Supplemental Figure 1B. Identification of RXR α -selective antagonist 7. MCF-7 cells transfected with the reporter plasmid pG5-Luc and pBind-RXR α -LBD were treated with 9-cis-RA (10^{-7} M) alone or together with a compound ($10 \mu\text{M}$ for compounds from Specs and $1 \mu\text{M}$ for BI1003 as “B”) for 18 hr. Cells were lysed and reporter activities measured by using the Dual-Luciferase Reporter Assay System. Transfection efficiency was normalized to Renilla luciferase activity. Data for 5 (Compounds 1, 2, 3, 5 and 8) of the 14 compounds are not available due to solubility issues.



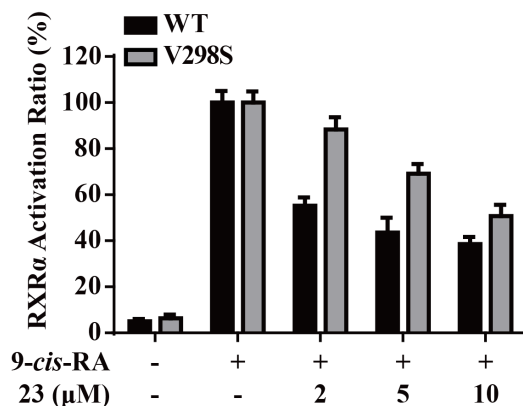
Supplemental Figure 2. Activation of nuclear receptors by compounds 7 and 23. MCF-7 cells cotransfected with pG5-Luc and pBind-ER α -LBD, pBind-GR α -LBD, pBind-RAR α -LBD, pBind-RAR γ -LBD, pBind-PPAR γ -LBD, pBind-LXR α -LBD, pBind-RXR α -LBD and pBind-Nur77 were treated respectively with their specific agonists (as indicated), in the presence or absence of compounds for 18 hr. Reporter activities were measured as described above.



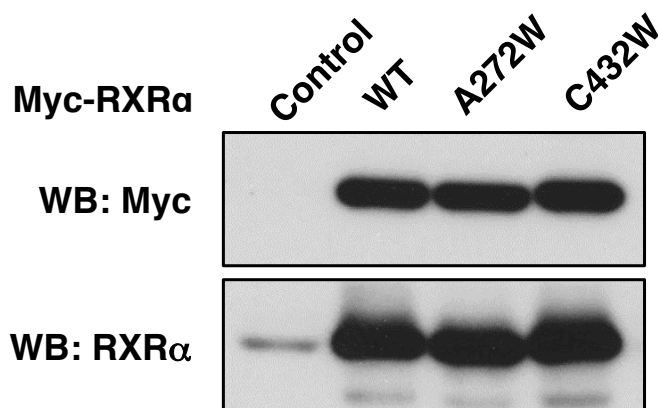
Supplemental Figure 3. The antagonist effect of 23 on three NR response elements. HEK293T cells cotransfected with one of the luciferase reporter plasmids pGL6-RXRE (100 ng)/pGL6-RARE (100 ng)/pGL6-LXRE (40 ng), one or two NR expressing plasmids like pCMV-Myc-RXRα (40 ng)/pCMV-Myc-RARα (40 ng)/pCMV-Myc-LXRα (40 ng), and pCMV-Renilla (1 ng) as internal reference. Twenty-four hours after transfection, the medium was replaced by medium containing a respective ligand and/or a testing compound. After 18 hours, cells were washed, lysed and assayed by using the Dual-Luciferase Reporter Assay System. Transfection efficiency was normalized to Renilla luciferase activity.



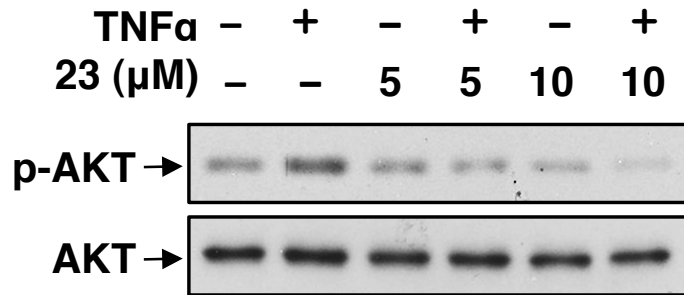
Supplemental Figure 4. The antagonist effect of 23 on RXR (α, β and γ) transactivation. HEK293T cells cotransfected with pG5-Luc and pBind-RXRα-LBD, pBind-RXRβ-LBD, pBind-RXRγ-LBD were treated respectively with 9-cis-RA (10⁻⁷M) alone or together with 23 for 18 hr. Cells were lysed and reporter activities measured by using the Dual-Luciferase Reporter Assay System. Transfection efficiency was normalized to Renilla luciferase activity.



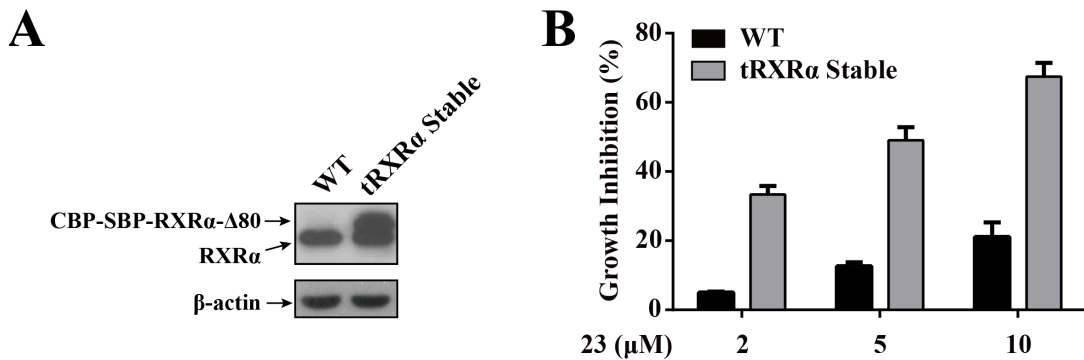
Supplemental Figure 5. Mutation of Val298 impairs the antagonist effect of 23. HEK293T cells cotransfected with the reporter plasmid pG5-Luc and pBind-RXRα-LBD or pBind-RXRα-LBD-V298S were treated with 9-cis-RA (10^{-7} M) alone or together with 23 for 18 hr. Cells were lysed and reporter activities measured by using the Dual-Luciferase Reporter Assay System. Transfection efficiency was normalized to Renilla luciferase activity.



Supplemental Figure 6. Expression of RXRα and mutant proteins in HEK-293T cells. HEK293T cells grown in 10-cm dishes were transfected with various plasmids for 24 hr. Lysates prepared were analyzed by Western blotting for RXRα and mutant expression.



Supplemental Figure 7. Inhibition of AKT activation by 23 in PC-3 prostate cancer cells. PC-3 cell line were pre-treated with 23 for 24 hr before exposed to TNF α (20 ng/ml) for additional 30 min. Lysates prepared were analyzed by Western blotting for AKT activation.



Supplemental Figure 8. Growth inhibitory effect of 23 can be significantly enhanced by tRXR α . Confluent MCF-7 (WT and tRXR α stable) cells cultured in 96-well dishes were treated with various concentrations of compounds for 48 hr. The cells were then incubated with 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hr at 37°C. MTT solution was then aspirated, and formazan in cells was instantly dissolved by addition of 150 μ l of DMSO each well. Absorbance was measured at 570 nm. (A) Expression of CBP-SBP-RXR α - Δ 80 in tRXR α stable MCF-7 cell line. (B) Growth inhibitory effect of 23 on two different MCF-7 cell lines (WT and tRXR α stable).