Chemistry & Biology

Sulindac-Derived RXRa Modulators Inhibit Cancer Cell Growth by Binding to a Novel Site

Liqun Chen,^{1,2,4} Zhi-Gang Wang,^{3,4} Alexander E. Aleshin,^{2,4} Fan Chen,^{1,2} Jiebo Chen,^{1,2} Fuquan Jiang,¹

Gulimiran Alitongbieke,¹ Zhiping Zeng,¹ Yue Ma,¹ Mingfeng Huang,¹ Hu Zhou,^{1,2} Gregory Cadwell,² Jian-Feng Zheng,³ Pei-Qiang Huang,³ Robert C. Liddington,² Xiao-kun Zhang,^{1,2,*} and Ying Su^{1,2,*}

¹School of Pharmaceutical Sciences, Xiamen University, Xiamen 361102, China

²Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

³Department of Chemistry and Fujian Provincial Key Laboratory of Chemical Biology, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China

⁴Co-first author

*Correspondence: xzhang@sanfordburnham.org (X.-k.Z.), ysu@sanfordburnham.org (Y.S.) http://dx.doi.org/10.1016/j.chembiol.2014.02.017

SUMMARY

Retinoid X receptor-alpha (RXR α), an intriguing and unique drug target, can serve as an intracellular target mediating the anticancer effects of certain nonsteroidal anti-inflammatory drugs (NSAIDs), including sulindac. We report the synthesis and characterization of two sulindac analogs, K-8008 and K-8012, which exert improved anticancer activities over sulindac in a RXRa-dependent manner. The analogs inhibit the interaction of the N-terminally truncated RXR α (tRXR α) with the p85 α subunit of PI3K, leading to suppression of AKT activation and induction of apoptosis. Crystal structures of the RXRa ligand-binding domain (LBD) with K-8008 or K-8012 reveal that both compounds bind to tetrameric RXRa LBD at a site different from the classical ligand-binding pocket. Thus, these results identify K-8008 and K-8012 as tRXR modulators and define a binding mechanism for regulating the nongenomic action of tRXRa.

INTRODUCTION

Retinoid X receptor alpha (RXR α), a unique member of the nuclear receptor superfamily, regulates a broad spectrum of physiological functions including cell differentiation, growth, and apoptosis (Germain et al., 2006; Szanto et al., 2004). Like other nuclear receptors, RXR α acts as a ligand-dependent transcription factor (Germain et al., 2006; Szanto et al., 2004). Recent accumulating evidence indicates that RXR α also has extranuclear actions. RXR α resides in the cytoplasm at certain stages during development (Dufour and Kim, 1999; Fukunaka et al., 2001) and migrates from the nucleus to the cytoplasm in response to differentiation, apoptosis, and inflammation (Cao et al., 2004; Casas et al., 2003; Zimmerman et al., 2006). RXR α exhibits a modular organization structurally consisting of three main functional domains: an N-terminal region, a DNA-binding domain, and a ligand-binding domain (LBD). The LBD possesses a ligand-binding pocket (LBP) for the binding of small molecule ligands, a transactivation function domain termed AF-2 composed of helix 12 (H12) of the LBD, a coregulator binding surface, and a dimerization surface (Germain et al., 2006; Szanto et al., 2004). The ligand-dependent transcription regulation is predominately mediated through H12 that is highly mobile. Agonist ligand binds to the LBP and helps H12 to adopt the active conformation that forms a surface to facilitate the binding of coactivators and subsequent transactivation. In contrast, in the absence of an agonist ligand or in the presence of an antagonist ligand, H12 adopts an inactive conformation that favors the binding of corepressors to inhibit target gene transcription. Natural RXRα ligand 9-cis-retinoic acid (9-cis-RA) and synthetic ligands have been effective in preventing tumorigenesis in animals, and RXRα has been a drug target for therapeutic applications, especially in the treatment of cancer (Bushue and Wan, 2010; Yen and Lamph, 2006). Targretin, a synthetic RXR-selective retinoid (rexinoid), was approved for treating cutaneous T-cell lymphoma (Dawson and Zhang, 2002). RXRα can bind to DNA and activate transcription of target genes either as a homodimer or a heterodimer with its heterodimerization partners including retinoic acid receptor, vitamin D receptor (VDR), thyroid hormone receptor (TR), and peroxisome-proliferator-activated receptor (Germain et al., 2006; Szanto et al., 2004). In addition to homodimer and heterodimer, RXRa could also self-associate into homotetramers in solution, which rapidly dissociate into active dimers upon binding of a cognate ligand (Chen et al., 1998; Kersten et al., 1995). Tetramer formation of RXRa might serve to sequester the receptor's active species, dimers and monomers, into a transcriptionally inactive tetramer complex (Gampe et al., 2000).

Efforts on discovery of small molecules targeting RXR α for therapeutic application have been primarily focused on the optimization of the molecules that bind to its classical LBP (de Lera et al., 2007; Germain et al., 2006; Szanto et al., 2004). However, various studies have recently identified small-molecule modulators of nuclear receptors that function via unknown sites and undefined mechanisms of action (Buzón et al., 2012; Moore et al., 2010). The recent report of the structure of estrogen receptor- β (ER β) with a second molecule of 4-hydroxytamoxifen bound in its coactivator-binding surface represents a direct example of a second



Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site



CH3

K-80003







estrogen receptor ligand-binding site and provides insight into the possible pharmacological effects of the drug (Wang et al., 2006). Compounds that bind to the surface binding sites of LBD have been demonstrated for other nuclear receptors, including androgen receptor (AR), VDR, and TR (Buzón et al., 2012; Moore et al., 2010). However, compounds that bind to RXR α at the sites other than the classical LBP have not been reported.

We recently showed that certain nonsteroidal anti-inflammatory drugs (NSAIDs), including etodolac (Kolluri et al., 2005) and sulindac (Zhou et al., 2010), could bind to RXRa and modulate its biological activities. Interestingly, sulindac but not 9-cis-RA could inhibit the binding of an N-terminally truncated RXRa protein (tRXR α) to the p85 α regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K), leading to inhibition of the tumor necrosis factor-α (TNF-α)-activated PI3K/protein kinase B (AKT) pathway (Zhou et al., 2010). We also demonstrated, through a designed sulindac analog, K-80003, the feasibility of developing a new generation of RXRa-specific molecules for therapeutic application and mechanistic studies of RXRa (Wang et al., 2013; Zhou et al., 2010). These results identify sulindac and related analogs as unique regulators of tRXRa activity through an undefined binding mechanism. Here we report our synthesis and characterization of K-80003-based analogs, K-8008 and K-8012, which exhibited improved activity in inhibiting the tRXRα-mediated PI3K/AKT signaling pathway. Moreover, our

Figure 1. Chemical Structures and Synthesis (A) The structures of sulindac and its analogs K-80003, K-8008, and K-8012. (B) Synthesis scheme of K-8008 and K-8012.

X-ray crystallographic studies of the LBD of RXR α in complex with K-8008 or K-8012 revealed that both compounds bound to the RXR α LBD in its tetrameric form via a particular site outside of the classical RXR α LBP, providing a different strategy for developing RXR α -based agents for cancer therapy.

RESULTS

٦N

ŃН

K-8008 and K-8012 Are Antagonists of RXR $\!\alpha$

In an effort to identify improved sulindac analogs for cancer therapy, we designed and synthesized a series of analogs around K-80003 (Wang et al., 2013; Zhou et al., 2010). The analogs shown in Figure 1 were initially evaluated by the reporter assay using a chloramphenicol acetyltransferase (CAT) reporter containing TREpal that is known to bind to RXR α homodimer (Zhang et al., 1992). 9-*cis*-RA strongly induced the TREpal reporter activity, which was inhibited by BI-1003, a known RXR α antagonist (Lu et al., 2009). K-8008 and K-8012 also ex-

hibited an inhibitory effect on 9-*cis*-RA-induced TREpal reporter activity in a concentration-dependent manner (Figure 2A), although they did not show any agonist activity at the concentrations used (Figure 2B). The antagonist effect of K-8008 and K-8012 was much better than sulindac and comparable to K-80003 (Figure 2A). We also used the Gal4-RXR α -LBD chimera and Gal4 reporter system to evaluate the inhibitory effect of K-8008 and K-8012 on 9-*cis*-RA-induced reporter activity. Cotransfection of Gal4-RXR α -LBD strongly activated the Gal4 reporter in the presence of 9-*cis*-RA, which was inhibited by BI-1003 as well as K-8008 and K-8012 (Figure 2C). Doseresponse experiments showed that the IC₅₀ values for K-8008 and K-8012 to inhibit 9-*cis*-RA-induced Gal4-RXR α -LBD transactivation were about 13.2 and 9.2 μ M, respectively (Figure 2D). Thus, K-8008 and K-8012 are antagonists of RXR α .

K-8008 and K-8012 Induce Apoptosis and Inhibit AKT Activation by Preventing tRXRα from Binding to p85α

We next evaluated K-8008 and K-8012 for their effect on the growth of cancer cells. Compared to sulindac, K-8008 and K-8012 were much more effective in inhibiting the growth of various cancer cells, including A549 lung cancer (Figure 3A), PC3 prostate cancer, and ZR-75-1 and MB231 breast cancer cells (Figure S1 available online). One unique property of sulindac and analogs is their ability to inhibit TNF- α -induced AKT activation

Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site



Figure 2. Antagonist Effect of Sulindac Analogs

(A and B) Inhibition of RXR α transactivation. (TREpal)₂-tk-CAT and RXR α were transiently transfected into CV-1 cells. Cells were treated with or without 9-*cis*-RA (10⁻⁷ M) in the presence or absence of the indicated concentration of sulindac and its analogs. CAT activity was determined. Bl-1003 (1 μ M) was used for comparison. 1, 2, 3, and 4 stand for sulindac, K-80003, K-8008, and K-8012, respectively.

(C) Inhibition of 9-*cis*-RA-induced Gal4 reporter activity. pBind-RXR α -LBD and pG5luc were transiently transfected into HCT-116 cells. Cells were treated with or without 9-*cis*-RA (10⁻⁷ M) in the presence or absence of BI-1003 (1 μ M), K-8008 (50 μ M), and K-8012 (50 μ M). Luciferase (LUC) activity was determined.

(D) Dose-dependent effect of K-8008 and K-8012. HCT-116 cells transfected with pBind-RXR α -LBD and pG5luc were treated with the indicated concentrations of K-8008 and K-8012 in the presence or absence of 9-*cis*-RA (10⁻⁷ M). All reporter activity is expressed as means \pm SD from three independent experiments.

(Zhou et al., 2010). Thus, A549 lung cancer cells were treated with TNF- α in the absence or presence of K-8008 or K-8012. Treatment of cells with TNF- α enhanced AKT activation as revealed by western blotting (Figure 3B). However, when cells were cotreated with either K-8008 or K-8012, the TNF- α -induced AKT activation was suppressed in a dose-dependent manner (Figure 3B). Similar results were obtained in other cancer cell lines (Figure S2).

TNF- α is a multifunctional cytokine that controls diverse cellular events such as cell survival and death (Balkwill, 2009; Wang and Lin, 2008). Inhibition of TNF- α -induced AKT activation by sulindac and analogs in cancer cells led to a shift of TNF- α signaling from survival to death (Zhou et al., 2010). We therefore evaluated the effect of K-8008 and K-8012 alone or in combination with TNF- α on the cleavage of poly(ADP-ribose) polymerase (PARP), an indication of apoptosis in cancer cells (Lazebnik et al., 1994). Treatment of A549 cells with TNF- α did not have effect on PARP cleavage, whereas treatment with sulindac or analogs slightly induced PARP cleavage. Combination of sulindac or analogs with TNF- α , however, caused a significant induction of PARP cleavage (Figure 3C and Figure S3). Thus, K-8008 and K-8012, like sulindac, could convert TNF- α signaling from survival to death in cancer cells.

The growth inhibitory effect of K-8008 and K-8012 and the induction of apoptosis by K-8008 occurred at low micromolar concentrations, suggesting that they might exert their anticancer effects through RXR α binding. To address the issues, cancer cells were transfected with RXR α small interfering RNA (siRNA) and evaluated for the effect of K-8008 on inducing PARP cleavage and inhibiting AKT activation. Knocking down RXR α expression by RXR α siRNA transfection significantly diminished the effect of K-8008 on inducing PARP cleavage (Figure 3D) and inhibiting TNF- α -induced AKT activation (Figure 3E). To address

the role of tRXR α , RXR α - Δ 80, a RXR α mutant lacking its N-terminal 80 amino acids and mimicking tRXR α (Wang et al., 2013; Zhou et al., 2010), was transfected into HeLa cells. Transfection of RXR α - Δ 80 but not the full-length RXR α enhanced the effect of K-8008 on inducing PARP cleavage in the presence of TNF- α (Figure 3F). Together, these results demonstrate that tRXR α plays a crucial role in mediating the biological effects of K-8008.

We next determined whether K-8008 could affect tRXRa interaction with $p85\alpha$, an interaction known to activate AKT (Zhou et al., 2010). HeLa cells were transfected with Myc-tagged RXR α - Δ 80 and Flag-tagged p85 α expression vectors and treated with or without TNF-a and/or K-8008. Coimmunoprecipitation assays using anti-Myc antibody showed that Flag-p85a was coimmunoprecipitated together with Myc-RXRa-A80 in cells treated with TNF- α (Figure 3G). However, when cells were cotreated with K-8008, TNF-a-induced interaction of Myc-RXR α - Δ 80 with Flag-p85 α was almost completely inhibited. We also examined the effect of K-8008 on interaction of endogenous tRXRa with p85a in A549 cells. Cell lysates prepared from A549 cells treated with TNF- α in the presence or absence of K-8008 were analyzed by coimmunoprecipitation using $\Delta 197$ anti-RXR α antibody that recognizes both tRXR α and RXR α (Zhou et al., 2010). Figure 3H shows that treatment of cells with TNF-a promoted the interaction of endogenous tRXRa with p85a, consistent with previous findings (Zhou et al., 2010). When cells were cotreated with K-8008, the interaction was largely inhibited. Such an effect of K-8008 on inhibiting TNF-a-induced p85a interaction with tRXRa was also observed in other cancer cell lines, including PC3 and HepG2 cells (Figure S4). Together, these results demonstrate that K-8008 can induce TNF-a-dependent apoptosis by suppressing the tRXRa-mediated activation of AKT through its inhibition of tRXR α interaction with p85 α .



Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site



Figure 3. Biological Effects of K-8008 and K-8012

(A) Growth inhibition by sulindac and its analogs. A549 lung cancer cells were treated with various concentrations of the indicated compounds. Cell viability was measured by the MTT colorimetric assay. The values represent averages (±SD) from five independent experiments. 1, 2, 3, and 4 stand for sulindac, K-80003, K-8008, and K-8012, respectively.

(B) Inhibition of TNF- α -induced AKT activation. A549 cells were pretreated with sulindac or analogs for 1 hr before being exposed to TNF- α (10 ng/ml) for 30 min. Phosphorylated AKT and total AKT were analyzed by immunoblotting.

(C) Induction of apoptosis by sulindac and analogs in the presence of TNF-α. Cells cultured in medium with 1% FBS were treated with TNF-α (10 ng/ml) and/or compound (40 μM) for 4 hr and analyzed for PARP cleavage by immunoblotting.

(D) RXRα siRNA transfection inhibits the apoptotic effect of K-8008. HeLa cells transfected with control or RXRα siRNA for 48 hr were treated with K-8008 (40 µM) and/or TNF-α (10 ng/ml) for 6 hr and analyzed by immunoblotting.

(E) RXR α siRNA transfection antagonizes the inhibitory effect of K-8008 on AKT activation. HeLa cells transfected with control or RXR α siRNA for 48 hr were treated with K-8008 (40 μ M) for 1.5 hr before being exposed to TNF- α (10 ng/ml) for 30 min and analyzed by immunoblotting.

(F) Myc-RXR α - Δ 80 transfection enhances the apoptotic effect of K-8008. HeLa cells transfected with Myc-RXR α or Myc-RXR α - Δ 80 were treated with K-8008 (20 μ M) and/or TNF- α for 12 hr. PARP cleavage and transfected RXR α expression were analyzed by immunoblotting.

Chemistry & Biology

Sulindac Analogs Bind to an RXR α Site





To further evaluate the anticancer effect of K-8008, mice with HepG2 tumor xenografts were treated with 20 mg/kg K-8008 or K-80003. Administration of K-8008 inhibited the growth of HepG2 tumor in a time-dependent manner (Figure 4A), resulting in a 61.23% reduction of tumor weight after a 12-day treatment (Figures 4B and 4C), which was comparable with the inhibitory effect of K-80003 (54.84% reduction). Consistent with our in vitro observation, examination of three tumors treated with or without K-8008 showed reduction of AKT activation by K-8008 (Figure 4D). Moreover, TUNEL staining revealed induction of apoptosis by K-8008 (Figure 4E). Significantly, administration of either K-80003 or K-8008 did not show any apparent toxic effects such as loss of body weight (Figure 4F).

Although sulindac and analogs can bind tRXR α and induce tRXR α -dependent apoptosis of cancer cells, how they bind to tRXR α to regulate tRXR α functions remains undefined. According to current understanding of the mechanism by which ligands regulate the transcriptional activity of nuclear receptors, K-8008

Figure 4. Inhibition of HepG2 Tumor Growth in Animals

(A–C) Nude mice with HepG2 heptoma xenografts were intraperitoneally injected daily with vehicle, K-8008 (20 mg/kg), or K-80003 (20 mg/kg) for 12 days. Tumors were removed and measured. Tumor sizes and weights in control and K-8003-and K-8008-treated mice were compared. In (B), one of three similar experiments is shown. Error bars represent SEM.

(D) Lysates prepared from three tumors treated with vehicle or K-8008 were analyzed by western blotting assay for p-AKT expression.

(E) H&E staining and TUNEL assay. Tumor sections were stained for H&E or TUNEL by immunohistochemistry. Increased apoptotic tumor cells were observed in tumor from K-8008-treated mice.

(F) K-8008 does not exhibit apparent toxicity. Body weight was measured every 3 days. Each point represents the mean \pm standard deviation of six mice. The differences between the compound-treated group and control group are not significant (p > 5%). One of three similar experiments is shown. Error bars represent SEM.

and K-8012 might bind to the canonical binding site, the LBP of RXR α , acting as conventional antagonists. Thus, we evaluated their binding to the LBP of RXR α using the classical radioligand competition binding assay (Zhou et al., 2010). Unlike 9-*cis*-RA and K-80003 that com-

peted well with $[{}^{3}H]9$ -*cis*-RA for binding to the LBP of RXR α , K-8008 and K-8012 failed to replace $[{}^{3}H]9$ -*cis*-RA for its binding to the RXR α LBP (Figure 5A).

Results of the [³H]9-cis-RA binding competition assay demonstrated that K-8008 and K-8012 did not bind to the canonical binding site, suggesting a different binding mechanism. Other than the classical LBP, recent structural and functional studies have revealed the existence of distinct small molecule binding sites on the surface of the LBD of nuclear receptors (Buzón et al., 2012; Moore et al., 2010). One of the potential alternative sites in which small molecules could bind and function as antagonists is the coregulator-binding site in the LBD. Antagonistic properties displayed by ligands through binding to the coregulator site have been reported for several members of the nuclear receptor family. For example, small molecules mimicking the structure of the coactivator LXXLL *a*-helical motif have been developed for modulating the activities of AR, ER, and VDR (Mita et al., 2010; Rodriguez et al., 2004; Arnold et al., 2005; Moore et al., 2010). We therefore tested whether K-8008 and K-8012 could bind to an alternative surface binding site by using the time-resolved fluorescence resonance energy transfer

⁽G) K-8008 inhibits the interaction of transfected tRXR α and p85 α . HeLa cells transfected with Myc-RXR α - Δ 80 and/or Flag-p85 α for 24 hr were treated with vehicle or 20 μ M K-8008 in the presence of absence of 10 ng/ml TNF- α for 1 hr. Cell lysates were immunoprecipitated (IP) using anti-Myc antibody and analyzed by western blotting (WB) analysis using the indicated antibody.

⁽H) K-8008 inhibits the interaction of endogenous tRXR α and p85 α . A549 cells were pretreated with vehicle or 40 μ M K-8008 for 1 hr before being exposed to 10 ng/ml TNF- α for 30 min. Cell lysates were immunoprecipitated with Δ N197 anti-RXR α antibody and analyzed by western blotting. See also Figure S1–S4.

Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site







K-8008 and K-8012 Bind to a Tetrameric Structure of the RXR α LBD

To gain direct and structural understanding of the binding of K-8008 or K-8012 to RXR α , we performed crystallographic studies of these ligands bound to the RXR α LBD. Crystals of protein/ligand complexes were obtained using a cocrystallization method. The structures of RXR α LBD in complex with K-8008 and K-8012 were determined to the resolution of 2.0 and 2.2 Å, respectively. Both protein/ligand complexes crystallized as tetrameric oligomers in the space group of P2₁ with similar unit cell parameters, and the molecular replacement method was used to obtain the initial phasing by using the published RXR α structure, Protein Data Bank (PDB) code 1G1U. Statistics of structure refinement and data collection are summarized in Table S1.

The crystal structure of the RXR α LBD in complex with the K-8008 exists as noncrystallographic homotetramer similar to the reported apo homotetramer (Gampe et al., 2000), in which two homodimers pack in a bottom-to-bottom manner (Figure 6A

Figure 5. Unique Binding of K-8008 and K-8012 to $\text{RXR}\alpha$

(A) K-8008 and K-8012 fail to compete with the binding of 9-*cis*-RA to RXRα. RXRα-LBD protein was incubated with [³H]9-*cis*-RA in the presence or absence of sulindac analogs K-80003, K-8008, K-8012, or unlabeled 9-*cis*-RA. Bound [³H]9-*cis*-RA was quantitated by liquid scintillation counting. One of three similar experiments is shown. Error bars represent SEM. 1, 2, 3, and 4 stand for sulindac, K-80003, K-8008, and K-8012, respectively. (B) K-8008 and K-8012 reduce 9-*cis*-RA-induced FRET signal. GST-RXRα-LBD was incubated with K-8008 or K-8012 in the presence or absence of 9-*cis*-RA (10⁻⁸ M). BI-1003 (1 µM) was used as a control. The values represent averages (±SD) from three independent experiments.

(C) Dose-dependent effect of K-8008 and K-8012 on 9-*cis*-RA-induced FRET signal. GST-RXR α -LBD was incubated with K-8008 or K-8012 in the presence of 9-*cis*-RA (10⁻⁸ M). The values represent averages (±SD) from three independent experiments.

and Figure S5A). Superposition of our crystal structure with the published apo structure (PDB code 1G1U) shows that the corresponding monomers have almost identical folds with small shifts found in the orientation of H12 in the

monomer where a K-8008 molecule is bound (Figure 6B). N-terminal residues, from 223 to 260, were found to be disordered and undetermined in the complex structures, though residues 231-260 were defined in the apo structure. In a tetramer, two modulator molecules were found to bind to one homotetramer, with a binding stoichiometric ratio of 1:2 between ligand and protein, because one ligand molecule binds only to one monomer within a dimer (Figure 6A). Observation of the same stoichiometric ratio between ligand and protein has been reported for another RXRα-LBD/antagonist (Gampe et al., 2000). K-8008 binds to a region that is close to the dimer-dimer interface, making interaction primarily with one monomer of the dimer and some interaction with one monomer of the other dimer. The structure of RXR α LBD in complex with K-8012 is very similar to that of RXR α LBD in complex with K-8008 (Figure S5B). Therefore we describe and discuss only the protein/K-8008 complex structure here.

Both K-8008 and K-8012 bind to a hydrophobic region of LBD near the entry and the edge of the cognate LBP. This region does not overlap with the binding region of 9-*cis*-RA (Figure 6B), which explains why both compounds failed to compete with the binding of 9-*cis*-RA (Figure 5A). To get a sense of how far the K-8008 binding region is away from the LBP, we calculated the distance between the centroid of the bound 9-*cis*-RA and the centroid of the bound K-8008 molecule using the tools available in Maestro. In the superimposed structures of the K-8008-bound LBD structure with the 9-*cis*-RA-bound LBD structure, the two centroids are about 7 Å apart (Figure S6A). The K-8008 hydrophobic binding region is made of side chains primarily from one monomer: Ala271 and Ala272 from H3; Trp305 and Leu309 from H5; Leu326 and Leu330 from the

Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site





Figure 6. Crystal Structure of the RXR LBD in Complex with K-8008

(A) The tetramer structure of RXR α LBD in complex with K-8008. The two bound K-8008 molecules are shown as magenta sticks surrounded by an electron density mesh (see Figure 6D legend for details). The two biological dimers (A1-B1 and A2-B2) are shown as green/cyan and yellow/brown pairs, respectively. The N and C termini of four subunits are marked by the corresponding residue numbers.

(B) Superposition of the RXR α LBD monomers from the K-8008-binding structure (brown ribbons) and the apo protein structure (purple ribbons, from PDB entry 1G1U). K-8008 is shown as sticks (carbon/nitrogen atoms are in magenta/ blue). The classic ligand binding site is also marked by a VDW ball model (in cyan/red) of 9-*cis*-RA taken from a superimposed PDB entry 1FBY.

(C) The hydrophobicity of the K-8008 binding site presented as a surface fragment on top of the RXR α LBD monomer (brown ribbon). The hydrophobic side chains that contribute to the region are shown in green, and K-8008 is shown in the same fashion as in Figure 6B. For clarity, residues contributing to this region are not labeled.

(D) The protein side chains (in sticks) that make VDW interaction with K-8008. The displayed region is an enlargement of the black box in Figure 6A. The view is slightly rotated, and fragments of the green subunit that do not interact with K-8008 are removed. The protein surface is shown as semitransparent envelope. The Fo-Fc electron density is shown around the ligands as a black mesh. It was calculated at a $3-\sigma$ level with omitted ligand atoms. The positive end of the H11 helix dipole is highlighted in orange.

(E) Side chains around K-8008 that make significant changes in comparison with the apo protein (PDB code 1G1U). Side chains of the apo protein are shown in green sticks, and the protein/K-8008 complex is shown in orange sticks. The complex structure is shown in brown ribbon, and the apo structure is in purple. K-8008 is presented in the same fashion as in Figure 6B.

See also Figures S5 and S6.

beta-turn; Leu433 from H10; Leu436 from L10-11; Phe437, Phe438, lle442, and Gly443 from H11 of chain B2; and Leu436 from L10-11 of chain A1 (Figure 6C). With respect to the monomer of RXR α LBD, this region is located on the surface of the RXR α monomer molecule. However, in the tetramer structure, this region is buried. K-8008 makes both hydrophobic interaction and polar interaction with the protein. The negatively charged tetrazole of the ligand sits on the top of the N-terminal end of H11 with a distance of 3 Å from the backbone N of residue Phe438 (Figure S6B), making charge-diploe interaction with H11 (Figure 6B). At 4.0 Å cutoff, the lipophilic part of the ligand makes van der Waals (VDW) contacts with side chains of Ile268, Ala271, Trp305, Leu436, Phe438, Phe439, and Ile442 from chain B2 and Leu436 from chain A1 (Figures 6A and 6D). The overall structure of the K-8008-bound RXR α LBD is identical to the apo structure of RXR α LBD tetramer. Binding of K-8008 does not induce much significant change in the surrounding side chains except for the side chains of Phe439 and Leu309. The side chain of Phe439 swings out to make room for the ligand to bind, and the side chain of Leu309 rearranges to make better VDW contact with the ligand (Figure 6E). Compared to the structure of the RXR α LBD bound to 9-*cis*-RA (Egea et al., 2000), K-8008 binding does not result in change in the shape of the LBP, whereas 9-*cis*-RA binding induces a substantial change in the shape of the LBP that includes the movement of H3 and H11 and the reorientation of H12 (Figure 7A). 9-*cis*-RA binding results in the formation of the coactivator-binding site; however, K-8008 binding does not promote the formation of the

Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site





Figure 7. Structural Comparison and Mutagenesis Analysis of the K-8008 Binding Site

(A) Structural superposition of the protein/K-8008 complex and the protein/9-cis-RA complex. The 9-cis-RA-bound structure (PDB code 1FBY) is in pink cartoon, and 9-cis-RA is in cyan (C atoms) and red (O atoms) sticks. The K-8008-bound structure is in light orange cartoon, and K-8008 is in gray (C atoms) and blue (N atoms) sticks. Side chain Arg316 is displayed for distance comparison between distances to -COOH and to tetrazole.

(B-D) Mutational analysis of the K-8008 binding site. The LBD of RXRa or mutants cloned into pBind vector and pG5luc were transiently cotransfected into HCT-116 cells. Cells were treated with or without 9-cis-RA (10⁻⁷ M) in the presence or absence of BI-1003 (1 µM) or K-8008 (50 µM). Luciferase (LUC) activity was determined.

See also Figure S6. The reporter activity is expressed as mean ±SD from three independent experiments

coactivator-binding site. Instead it leaves the receptor protein in an autorepression state. Thus, K-8008 acts as an antagonist. Similar to 9-cis-RA ligand, K-8008 is made of largely a lipophilic body linked to a charged group. The carboxylate group of 9-cis-RA acts as an anchor via forming charge-charge interaction with Arg316; however, the bioisostere tetrazole of the carboxylate in K-8008 makes no interaction with Arg316. The tetrazole group in the RXR α LBD/K-8008 structure is about 15 Å away from Arg316. Both 9-cis-RA and K-8008 make extensive hydrophobic interactions with the protein. Some of the residues contributing to the hydrophobic interactions between ligand (9-cis-RA or K-8008) and the protein are shared, such as Ile268, Ala271, and Leu326, but because of the LBP shape change induced by the binding of 9-cis-RA, these shared residues are located in different regions of the structures of RXRa LBD/K-8008 and RXRα LBD/9-cis-RA (Figure S6C).

BI1003

BI1003

To further support and cross-validate the identification of this binding site on RXRa, we designed a couple of mutants that could potentially impact the binding of K-8008 but not 9-cis-RA. Comparison of the binding nature of the K-8008 molecule to that of the 9-cis-RA reveals that Leu433 is close to the tetrazole group and that replacing Leu433 with Glu could weaken the binding of K-8008 because of the repulsive interaction between the deprotonated Glu and the negatively charged tetrazole. However, Leu433Glu is not likely to impact the binding of 9-cis-RA as in the 9-cis-RA-bound RXRa structure (PDB code 1FBY); Leu433 is not close to the ligand and is also partially solvent-exposed. This prediction is supported by our reporter assays showing that 9-cis-RA could similarly activate the wildtype RXR α (Figure 7B) and the Leu433Glu RXR α mutant, RXRα-L433E (Figure 7C). By contrast, K-8008 inhibited 9-cis-RA-induced transactivation of RXRa but not RXRa-L433E. Structural comparison also suggests that mutating both Phe438 and Phe439 into Ala could affect the binding of K-8008 but not 9-cis-RA. Indeed, simultaneous substitution of Phe438 and Phe439 with Ala resulted in a mutant, RXRα-F438,9A, whose transactivation could be strongly induced by 9-cis-RA, but K-8008 failed to inhibit the induced transcriptional activity (Figure 7D). Together, the mutagenesis studies confirm the K-8008 binding site identified by the crystal structure.

DISCUSSION

BI1003

We report here our identification of two sulindac analogs, K-8008 and K-8012, which showed potent tRXR inhibitory effects through a unique binding mechanism. Our results demonstrated that K-8008 and K-8012 were more effective than sulindac in inhibiting RXRα transactivation (Figure 2A). Sulindac binds to RXR α with an IC₅₀ of 82.9 μ M based on the classical ligand competition assays (Zhou et al., 2010). K-8008 and K-8012 could antagonize 9-cis-RA-induced transactivation and inhibit coactivator peptide binding to RXRa with IC50 value of around 10 µM. Consistently, K-8008 and K-8012 showed improved activity than sulindac in inhibiting AKT activation and inducing apoptosis. About 100 µM of sulindac is normally used to achieve its anticancer effects (Weggen et al., 2001; Yamamoto et al., 1999; Zhang et al., 2000), whereas 10-50 µM of K-8008 and K-8012 were able to inhibit AKT activation and induce apoptosis of cancer cells. Furthermore, K-8008 showed potent inhibitory effect on the growth of tumor cells in animals without apparent toxicity. Similar to sulindac, inhibition of AKT activation and induction of apoptosis by K-8008 and K-8012 were RXR α dependent, likely because of their inhibition of the interaction between tRXR α and p85 α .

K-8008 and K-8012 were synthesized as part of the structureactivity relationship studies of sulindac, by replacing the carboxylate group in K-80003 with its bioisotere tetrazole (Herr, 2002). Based on the principle of bioisosteric replacement (Matta et al., 2010), we anticipated that tetrazole group acted like the carboxylate group, a common motif found in most of the cognate RXR ligands, which interacts with Arg316 in the LBP, and therefore both K-8008 and K-8012 would compete 9-cis-RA for

Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site

binding. To our surprise, both compounds, unlike sulindac and K-80003, failed to compete with 9-cis-RA for binding to the LBP, demonstrating that they exert their antagonist effect through a different binding mechanism from sulindac and K-80003. Our structure analysis confirmed that the tetrazole group of K-8008 and K-8012 binds to a region away from Arg316, and it anchors to the RXRa protein by sitting atop the N terminus of helix 11, forming the charge-helix dipole interaction. This charge-dipole interaction may also function to stabilize the orientation and conformation of H11 because in most of the cases ligand binding to the cognate LBP induces the conformation change and reorientation of H11(Egea et al., 2000; Sato et al., 2010). Tetrazole is a nonclassical isotere of -COOH moiety (Herr, 2002; Matta et al., 2010). It has a similar PKa to -COOH but has a different steric characteristics and different number of atoms from -COOH. Spatially tetrazole moiety is bulkier than -COOH, and it seems that some spatial clash could prevent the tetrazole group of K-8008 from making an anchoring chargecharge interaction with Arg316. As a result, K-8008 and K-8012 bind to a novel region. The structural results show that the compounds bind to a region that does not overlap with the 9-cis-RA binding space, offering a structural explanation for the inability of K-8008 and K-8012 to compete with 9-*cis*-RA for RXRα binding. Furthermore, our mutagenesis data (Figures 7B–7D) support the existence of this K-8008 binding site.

Our crystal structures revealed that K-8008 and K-8012 bind to a RXRa LBD tetramer structure through a novel hydrophobic region that is located on the surface of a monomer and near the dimer-dimer interface in the tetramer. Unlike the binding of other published ligands, the binding of K-8008 does not change the shape of the apo RXRa LBP. In addition, K-8008 interacts with monomers of each dimer in the tetramer, contributing to the dimer-dimer interaction. Taken together, K-8008 or K-8012 binding may help to stabilize the tetramer. Stabilizing the tetrameric state of RXRa through ligand binding may have important implications for the regulation of the nongenomic biological activities of RXRa. Previous studies have demonstrated that tetramer formation of RXRa serves as a mechanism to suppress its transactivation (Gampe et al., 2000; Kersten et al., 1998). The fact that inhibition of tRXRa interaction with p85a by K-8008 and K-8012 was associated with their binding to the RXRa tetramer raises an intriguing question that the formation of RXRa homotetramer may also represent a mechanism to suppress its interaction with cytoplasmic p85a and activation of tRXRa-dependent PI3K/AKT signaling.

Unlike classical nuclear receptor antagonists that bind to the RXR α LBP with high affinity, weak antagonism is commonly observed for antagonists that target the receptor surface binding sites (Caboni et al., 2012; Gunther et al., 2008; Hwang et al., 2009; Rodriguez et al., 2004). Similarly, K-8008 and K-8012 bind to a surface hydrophobic site and display weak antagonist effects. However, the therapeutic relevance of targeting the RXR α through this binding site is evidenced by our observation that both K-8008 and K-8012 could inhibit tRXR α activities in cancer cells in vitro and tumor growth in animals (Figure 4). In fact, the existence and therapeutic relevance of the novel sites other than the LBP have been described for several nuclear receptors, including estrogen receptor, androgen receptor, and vitamin D receptor (Caboni et al., 2012; Kojetin et al., 2011). Furthermore, in the case of sulindac and analogs, weak antagonism may be clinically relevant because conventional administration of sulindac can result in about 10-15 μ M sulindac in the serum of patients (Davies and Watson, 1997; Yamamoto et al., 1999). Up to approximately 50 μ M of sulindac could be detected in the plasma of humans depending on dose and schedule (Davies and Watson, 1997), and sulindac can be concentrated in epithelial cells at concentrations that are at least 20-fold higher than those seen in the serum (Duggan et al., 1980; Yamamoto et al., 1999). Consistently, we observed that administration of K-8008 at the dose that effectively inhibited the growth of tumor cells did not show any apparent toxicity to animals (Figure 4). Thus, although showing a relatively weak binding to RXR α , these compounds could be clinically relevant.

2008; Mizwicki et al., 2004; Moore et al., 2010; Sun et al.,

SIGNIFICANCE

RXRa represents an intriguing target for pharmacologic intervention. Unfortunately, the development of RXRabased drugs has been hampered by the side effects associated with targeting its cognate LBP. Thus, discovery of new strategies for targeting RXRa is significant. We report here our identification of two sulindac-derived analogs that exert their anticancer effects by binding to a site of RXRa, which is different from the classical LBP. These molecules could serve as chemical probes for studying the role of tRXRa in cancer. Furthermore, our results provide an opportunity to specifically target this surface site and thus may circumvent side effects associated with binding to the classical RXRa LBP. The development of tRXRa-selective inhibitors targeting a novel binding site may support a departure from the traditional approach of targeting the LBP and lead to a new paradigm targeting a functionally important surface site, which may lead to more effective and specific therapeutics.

EXPERIMENTAL PROCEDURES

Compound Synthesis

K-8008 and K-8012 were synthesized using scheme of Figure 1B. See Supplemental Information for details.

Cell Culture and Transfection

PC3 prostate cancer, ZR-75-1 breast cancer, and HeLa cervical cancer cells were grown in RPMI 1640. CV-1 African green monkey kidney, HCT-116 colon cancer, and A549 lung cancer cells were cultured in DMEM containing 10% fetal bovine serum. The cells were maintained at 5% CO₂ at 37°C. Subconfluent cells with exponential growth were used throughout the experiments. Cell transfections were carried out by using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Myc-RXR α -p80 and Flag-p85 α expression vectors as well as RXR α siRNA were described (Zhou et al., 2010). RXR α -L433E and RXR α -F438,9A mutants were constructed by standard procedure, and their LBDs were cloned into pBind expression vector (Wang et al., 2013).

CAT Assay

(TREpal)₂-tk-CAT (100 ng), β -galactosidase (100 ng), and RXR α (20 ng) were transiently transfected into CV-1 cells (Zhang et al., 1992). Cells were then treated with or without 9-*cis*-RA (10⁻⁷ M) in the presence or absence of increasing concentrations of compounds for an additional 24 hr. Cells were harvested and assayed for CAT and β -galactosidase (β -gal) activity. To



Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site

normalize for transfection efficiency, CAT activities were corrected to $\beta\mbox{-}gal$ activities.

Mammalian One Hybrid

HCT-116 cells seeded in 24-well plates were transiently transfected with 50 ng of pG5luc, 25 ng of pBind-RXR α -LBD, or mutant. Twenty-four hours after transfection, the medium was replaced by medium containing the sulindac analogs and/or 9-*cis*-RA. Cells were washed, lysed, and assayed by using the Dual-Luciferase Reporter Assay System (Promega). Transfection efficiency was normalized to *Renilla* luciferase activity.

Protein Expression and Purification

The human RXR α LBD (residues Thr223 to Thr462) was cloned as an N-terminal histidine-tagged fusion protein in pET15b expression vector and overproduced in *Escherichia coli* BL21 strain. Briefly, cells were harvested and sonicated, and the extract was incubated with the His60 Ni Superflow resin. The protein-resin complexes were washed and eluted. The eluent was collected and concentrated to 5 mg/ml for subsequent trails (Bourguet et al., 1995; Peet et al., 1998). For crystallization experiment, the His tag was cleaved by bovine thrombin (Sigma) and removed on the Resource-Q column (GE Healthcare), using 0.1–1.0 M NaCl gradient and the Tris-Cl buffer (pH 8.0). The additional purification was done by the gel filtration on a Superdex 200 2660 column (GE

Ligand-Binding Competition Assay

The His-tagged human RXR α -LBD(223–462) was incubated in tubes with unlabeled 9-*cis*-RA or different concentrations of compounds in 200 μ l of binding buffer (0.15 M KCl, 10 mM Tris+HCl [pH 7.4], 8% glycerol, and 0.5% CHAPS detergent) at 4°C for 1 hr. [³H]9-*cis*-RA was added to the tubes to final concentration of 7.5 nM and final volume of 300 μ l and incubated overnight at 4°C. The RXR α -LBD was captured by nickel-coated beads. Bound [³H]9-*cis*-RA was quantitated by liquid scintillation counting.

TR-FRET Retinoic X Receptor a Coactivator Assay

Invitrogen's LanthsScreen TR-FRET RXR α Coactivator Assay was conducted according to the manufacturer's protocol. The TR-FRET ratio was calculated by dividing the emission signal at 520 nm by the emission signal at 495 nm.

MTT Assay

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.

Western Blotting

Cells were lysed, and equal amounts of the lysates were electrophoresed on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% skimmed milk in TBST (50 mM Tris-HCI [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) for 1 hr and then incubated with primary antibodies and secondary antibodies and detected using the ECL system (Thermo). The dilutions of the primary antibodies were anti-RXRa (pN197; Santa Cruz) in 1:1,000, anti-PARP (H-250; Santa Cruz) in 1:3,000, anti-p85 α (Millipore) in 1:1,000, anti-p-AKT (D9E; Cell Signaling Technology) in 1:1,000, anti-AKT1/2/3 (H-136; Santa Cruz) in 1:1,000, anti-Flag (F1804; Sigma).

Coimmunoprecipitation Assay

Cells were harvested and lysed in buffer containing 50 mM HEPES-NaOH (pH 7.5), 2.5 mM EDTA, 100 mM NaCl, 0.5% NP40, and 10% glycerol, with 1 mM dithiothreitol and proteinase inhibitor cocktail. Immunoprecipitation was performed as described (Zhou et al., 2010).

HepG2 Xenografts

Nude mice (BALB/c, SPF grade, 16–18 g, 4–5 weeks old) were housed at 28° C in a laminar flow under sterilized conditions. Mice were injected sub-

cutaneously with 100 μ l of HepG2 cells (2 \times 10⁻⁶). For drug treatment, mice (n = 6) were treated intraperitoneally after 7 days of transplantation with K-8008 (20 mg/kg), K-80003 (20 mg/kg), or vehicle (Tween 80) once a day. Body weight and tumor size were measured every 3 days. Mice were scarified after 12-day drug treatment, and the tumors were removed for various assessments. All experimentations and animal usage were performed and approved by the Animal Care and Use Committee of Xiamen University.

Histology and Apoptosis Analysis

Paraffin wax-embedded tumors were cut into $5-\mu$ m-thick sections. These sections were deparaffinized and stained with hematoxylin and eosin (H&E) according to the standard protocol. Tumor sections of HepG2 xenografts were also stained with TUNEL for assessing spontaneous apoptosis according to the manufacturer's instructions (In situCell Death Detection Kit; Roche). The images were taken under a fluorescent microscope (Carl Zeiss).

Crystallization and Structure Solution of the RXR LBD/Ligand Complexes

The initial crystallization conditions were determined using the sitting-drop vapor-diffusion method and the crystallization screens Index and PEG-Ion (Hampton research). Other crystallization chemicals were from Hampton Research and Sigma. The data were collected from crystals grown in sitting drops of the 96-well Intelli-Plates (ARI) by the vapor-diffusion method. 0.2 μI of the protein/ligand complex containing 0.37 mM of RXR LBD, 0.5-0.7 mM of a ligand, 100 mM NaCl, and 20 mM Tris-Cl buffer (pH 8.0) were mixed with 0.2 μl of the well solution (20% PEG3330 and 0.2 M magnesium formate for the K-8008 complex or 0.2 M sodium acetate for the K-8012 complex) and incubated at 20°C. The first crystals appeared in 5-10 days and grew within same amount of time into 0.2 × 0.2 × 0.05 mm plates. The crystals were flash-frozen against the well solution containing 20% PEG400 as a cryoprotectant. The diffraction data were collected from the cryo-cooled crystals (at 100 K) at Beamline BL11-2 of SSRL and processed using the program suites XDS (Kabsch, 2010) and ccp4i (Collaborative Computational Project, Number 4, 1994).

The structures were solved by the molecular replacement program Phaser (McCoy et al., 2007) using PDB entry 1G1U as an initial model. The model rebuilding and refinement were done with Coot (Emsley and Cowtan, 2004) and the program suite Phenix (Adams et al., 2010). The initial models and parameter files for the ligands were prepared by eLBOW of Phenix. The data collection and refinement statistics are presented in Table S1.

Data Analyses

Data are expressed as means \pm SD from three or more experiments. Statistical analysis was performed using Student's t test. Differences were considered statistically significant with p < 0.05.

ACCESSION NUMBERS

The coordinates for the crystal structures of RXR α LBD in complex with K-8008 or K-8012 were deposited with the Protein Data Bank under accession codes 4N8R and 4N5G, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.02.017.

AUTHOR CONTRIBUTIONS

X.-K.Z., Y.S., P.Q.H., and R.C.L. supervised the project; L.C, Z.G.W., A.E.A, P.Q.H., X.-K.Z., and Y.S. designed the study and experiments; L.C., F.C., J.C., F.J., G.A., Y.M., and M.H. conducted the biological and animal experiments with input from H.Z. and Z.P.Z.; A.E.A. carried out the crystallization, diffraction data collection, and structure determination with the help of G.C. on protein purification. Z.G.W. and J.F.Z. synthesized the compounds; L.C., r.

Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site



Z.G.W., A.E.A., P.Q.H., X.-K.Z., and Y.S. analyzed the data; and X.-K.Z. and Y.S. wrote the manuscript with input from all authors.

ACKNOWLEDGMENTS

This work was supported by grants from the U.S. Army Medical Research and Material Command (W81XWH-11-1-0677), the National Institutes of Health (CA140980, GM089927, and CA179379), the National Natural Science Foundation of China (NSFC-91129302 and NSFC-21332007), the Ministry of Education of China (IRT1037), the Science and Technology Bureau of Xiamen (3502Z20133008), and the National Basic Research Program (973 Program) of the Ministry of Science and Technology, China (2010CB833200).

Received: October 21, 2013 Revised: January 22, 2014 Accepted: February 19, 2014 Published: April 3, 2014

REFERENCES

Adams, P.D., Afonine, P.V., Bunkóczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., et al. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. *66*, 213–221.

Arnold, L.A., Estébanez-Perpiñá, E., Togashi, M., Jouravel, N., Shelat, A., McReynolds, A.C., Mar, E., Nguyen, P., Baxter, J.D., Fletterick, R.J., et al. (2005). Discovery of small molecule inhibitors of the interaction of the thyroid hormone receptor with transcriptional coregulators. J. Biol. Chem. *280*, 43048–43055.

Balkwill, F. (2009). Tumour necrosis factor and cancer. Nat. Rev. Cancer 9, 361–371.

Bourguet, W., Ruff, M., Bonnier, D., Granger, F., Boeglin, M., Chambon, P., Moras, D., and Gronemeyer, H. (1995). Purification, functional characterization, and crystallization of the ligand binding domain of the retinoid X receptor. Protein Expr. Purif. 6, 604–608.

Bushue, N., and Wan, Y.-J.Y. (2010). Retinoid pathway and cancer therapeutics. Adv. Drug Deliv. Rev. 62, 1285–1298.

Buzón, V., Carbó, L.R., Estruch, S.B., Fletterick, R.J., and Estébanez-Perpiñá, E. (2012). A conserved surface on the ligand binding domain of nuclear receptors for allosteric control. Mol. Cell. Endocrinol. *348*, 394–402.

Caboni, L., Kinsella, G.K., Blanco, F., Fayne, D., Jagoe, W.N., Carr, M., Williams, D.C., Meegan, M.J., and Lloyd, D.G. (2012). "True" antiandrogens-selective non-ligand-binding pocket disruptors of androgen receptorcoactivator interactions: novel tools for prostate cancer. J. Med. Chem. 55, 1635–1644.

Cao, X., Liu, W., Lin, F., Li, H., Kolluri, S.K., Lin, B., Han, Y.-H., Dawson, M.I., and Zhang, X.K. (2004). Retinoid X receptor regulates Nur77/TR3-dependent apoptosis [corrected] by modulating its nuclear export and mitochondrial targeting. Mol. Cell. Biol. *24*, 9705–9725.

Casas, F., Daury, L., Grandemange, S., Busson, M., Seyer, P., Hatier, R., Carazo, A., Cabello, G., and Wrutniak-Cabello, C. (2003). Endocrine regulation of mitochondrial activity: involvement of truncated RXRalpha and c-Erb Aalpha1 proteins. FASEB J. *17*, 426–436.

Chen, Z.-P., Iyer, J., Bourguet, W., Held, P., Mioskowski, C., Lebeau, L., Noy, N., Chambon, P., and Gronemeyer, H. (1998). Ligand- and DNA-induced dissociation of RXR tetramers. J. Mol. Biol. *275*, 55–65.

Collaborative Computational Project, Number 4 (1994). The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. *50*, 760–763.

Davies, N.M., and Watson, M.S. (1997). Clinical pharmacokinetics of sulindac. A dynamic old drug. Clin. Pharmacokinet. 32, 437–459.

Dawson, M.I., and Zhang, X.K. (2002). Discovery and design of retinoic acid receptor and retinoid X receptor class- and subtype-selective synthetic analogs of all-trans-retinoic acid and 9-cis-retinoic acid. Curr. Med. Chem. 9, 623–637. de Lera, A.R., Bourguet, W., Altucci, L., and Gronemeyer, H. (2007). Design of selective nuclear receptor modulators: RAR and RXR as a case study. Nat. Rev. Drug Discov. 6, 811–820.

Dufour, J.M., and Kim, K.H. (1999). Cellular and subcellular localization of six retinoid receptors in rat testis during postnatal development: identification of potential heterodimeric receptors. Biol. Reprod. *61*, 1300–1308.

Duggan, D.E., Hooke, K.F., and Hwang, S.S. (1980). Kinetics of the tissue distributions of sulindac and metabolites. Relevance to sites and rates of bioactivation. Drug Metab. Dispos. 8, 241–246.

Egea, P.F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P., and Moras, D. (2000). Crystal structure of the human RXRalpha ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. EMBO J. *19*, 2592–2601.

Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. *60*, 2126–2132.

Fukunaka, K., Saito, T., Wataba, K., Ashihara, K., Ito, E., and Kudo, R. (2001). Changes in expression and subcellular localization of nuclear retinoic acid receptors in human endometrial epithelium during the menstrual cycle. Mol. Hum. Reprod. 7, 437–446.

Gampe, R.T., Jr., Montana, V.G., Lambert, M.H., Wisely, G.B., Milburn, M.V., and Xu, H.E. (2000). Structural basis for autorepression of retinoid X receptor by tetramer formation and the AF-2 helix. Genes Dev. *14*, 2229–2241.

Germain, P., Chambon, P., Eichele, G., Evans, R.M., Lazar, M.A., Leid, M., De Lera, A.R., Lotan, R., Mangelsdorf, D.J., and Gronemeyer, H. (2006). International Union of Pharmacology. LXIII. Retinoid X receptors. Pharmacol. Rev. 58, 760–772.

Gunther, J.R., Moore, T.W., Collins, M.L., and Katzenellenbogen, J.A. (2008). Amphipathic benzenes are designed inhibitors of the estrogen receptor α /steroid receptor coactivator interaction. ACS Chem. Biol. *3*, 282–286.

Herr, R.J. (2002). 5-Substituted-1H-tetrazoles as carboxylic acid isosteres: medicinal chemistry and synthetic methods. Bioorg. Med. Chem. *10*, 3379–3393.

Hwang, J.Y., Arnold, L.A., Zhu, F., Kosinski, A., Mangano, T.J., Setola, V., Roth, B.L., and Guy, R.K. (2009). Improvement of pharmacological properties of irreversible thyroid receptor coactivator binding inhibitors. J. Med. Chem. *52*, 3892–3901.

Kabsch, W. (2010). Xds. Acta Crystallogr. D Biol. Crystallogr. 66, 125–132.

Kersten, S., Kelleher, D., Chambon, P., Gronemeyer, H., and Noy, N. (1995). Retinoid X receptor alpha forms tetramers in solution. Proc. Natl. Acad. Sci. USA *92*, 8645–8649.

Kersten, S., Dong, D., Lee, W., Reczek, P.R., and Noy, N. (1998). Autosilencing by the retinoid X receptor. J. Mol. Biol. 284, 21–32.

Kojetin, D.J., Burris, T.P., Jensen, E.V., and Khan, S.A. (2008). Implications of the binding of tamoxifen to the coactivator recognition site of the estrogen receptor. Endocr. Relat. Cancer *15*, 851–870.

Kolluri, S.K., Corr, M., James, S.Y., Bernasconi, M., Lu, D., Liu, W., Cottam, H.B., Leoni, L.M., Carson, D.A., and Zhang, X.K. (2005). The R-enantiomer of the nonsteroidal antiinflammatory drug etodolac binds retinoid X receptor and induces tumor-selective apoptosis. Proc. Natl. Acad. Sci. USA *102*, 2525–2530.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G., and Earnshaw, W.C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature *371*, 346–347.

Lu, J., Dawson, M.I., Hu, Q.Y., Xia, Z., Dambacher, J.D., Ye, M., Zhang, X.-K., and Li, E. (2009). The effect of antagonists on the conformational exchange of the retinoid X receptor alpha ligand-binding domain. Magn. Reson. Chem. 47, 1071–1080.

Matta, C.F., Arabi, A.A., and Weaver, D.F. (2010). The bioisosteric similarity of the tetrazole and carboxylate anions: clues from the topologies of the electrostatic potential and of the electron density. Eur. J. Med. Chem. 45, 1868–1872.

McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J. (2007). Phaser crystallographic software. J. Appl. Crystallogr. *40*, 658–674.

Mita, Y., Dodo, K., Noguchi-Yachide, T., Miyachi, H., Makishima, M., Hashimoto, Y., and Ishikawa, M. (2010). LXXLL peptide mimetics as inhibitors



Chemistry & Biology

Sulindac Analogs Bind to an RXRa Site

of the interaction of vitamin D receptor with coactivators. Bioorg. Med. Chem. Lett. 20, 1712–1717.

Mizwicki, M.T., Keidel, D., Bula, C.M., Bishop, J.E., Zanello, L.P., Wurtz, J.M., Moras, D., and Norman, A.W. (2004). Identification of an alternative ligandbinding pocket in the nuclear vitamin D receptor and its functional importance in 1alpha,25(OH)2-vitamin D3 signaling. Proc. Natl. Acad. Sci. USA *101*, 12876–12881.

Moore, T.W., Mayne, C.G., and Katzenellenbogen, J.A. (2010). Minireview: Not picking pockets: nuclear receptor alternate-site modulators (NRAMs). Mol. Endocrinol. 24, 683–695.

Peet, D.J., Doyle, D.F., Corey, D.R., and Mangelsdorf, D.J. (1998). Engineering novel specificities for ligand-activated transcription in the nuclear hormone receptor RXR. Chem. Biol. 5, 13–21.

Rodriguez, A.L., Tamrazi, A., Collins, M.L., and Katzenellenbogen, J.A. (2004). Design, synthesis, and in vitro biological evaluation of small molecule inhibitors of estrogen receptor α coactivator binding. J. Med. Chem. 47, 600–611.

Sato, Y., Ramalanjaona, N., Huet, T., Potier, N., Osz, J., Antony, P., Peluso-Iltis, C., Poussin-Courmontagne, P., Ennifar, E., Mély, Y., et al. (2010). The "Phantom Effect" of the rexinoid LG100754: structural and functional insights. PLoS One 5, e15119.

Sun, A., Moore, T.W., Gunther, J.R., Kim, M.S., Rhoden, E., Du, Y., Fu, H., Snyder, J.P., and Katzenellenbogen, J.A. (2011). Discovering small-molecule estrogen receptor a/coactivator binding inhibitors: high-throughput screening, ligand development, and models for enhanced potency. ChemMedChem *6*, 654–666.

Szanto, A., Narkar, V., Shen, Q., Uray, I.P., Davies, P.J.A., and Nagy, L. (2004). Retinoid X receptors: X-ploring their (patho)physiological functions. Cell Death Differ. *11* (*Suppl 2*), S126–S143.

Wang, X., and Lin, Y. (2008). Tumor necrosis factor and cancer, buddies or foes? Acta Pharmacol. Sin. 29, 1275–1288.

Wang, Y., Chirgadze, N.Y., Briggs, S.L., Khan, S., Jensen, E.V., and Burris, T.P. (2006). A second binding site for hydroxytamoxifen within the coactivatorbinding groove of estrogen receptor β . Proc. Natl. Acad. Sci. USA *103*, 9908–9911.

Wang, Z.-G., Chen, L., Chen, J., Zheng, J.-F., Gao, W., Zeng, Z., Zhou, H., Zhang, X.K., Huang, P.-Q., and Su, Y. (2013). Synthesis and SAR study of modulators inhibiting tRXR α -dependent AKT activation. Eur. J. Med. Chem. 62, 632–648.

Weggen, S., Eriksen, J.L., Das, P., Sagi, S.A., Wang, R., Pietrzik, C.U., Findlay, K.A., Smith, T.E., Murphy, M.P., Bulter, T., et al. (2001). A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature *414*, 212–216.

Yamamoto, Y., Yin, M.-J., Lin, K.-M., and Gaynor, R.B. (1999). Sulindac inhibits activation of the NF-kappaB pathway. J. Biol. Chem. 274, 27307–27314.

Yen, W.C., and Lamph, W.W. (2006). A selective retinoid X receptor agonist bexarotene (LGD1069, Targretin) prevents and overcomes multidrug resistance in advanced prostate cancer. Prostate 66, 305–316.

Zhang, X.K., Lehmann, J., Hoffmann, B., Dawson, M.I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. (1992). Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. Nature 358, 587–591.

Zhang, L., Yu, J., Park, B.H., Kinzler, K.W., and Vogelstein, B. (2000). Role of BAX in the apoptotic response to anticancer agents. Science 290, 989–992.

Zhou, H., Liu, W., Su, Y., Wei, Z., Liu, J., Kolluri, S.K., Wu, H., Cao, Y., Chen, J., Wu, Y., et al. (2010). NSAID sulindac and its analog bind RXRalpha and inhibit RXRalpha-dependent AKT signaling. Cancer Cell *17*, 560–573.

Zimmerman, T.L., Thevananther, S., Ghose, R., Burns, A.R., and Karpen, S.J. (2006). Nuclear export of retinoid X receptor alpha in response to interleukin-1beta-mediated cell signaling: roles for JNK and SER260. J. Biol. Chem. *281*, 15434–15440.