

NSAID Sulindac and Its Analog Bind RXR α and Inhibit RXR α -Dependent AKT Signaling

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SUMMARY

Nonsteroidal anti-inflammatory drugs (NSAIDs) exert their anticancer effects through cyclooxygenase-2 (COX-2)-dependent and independent mechanisms. Here, we report that Sulindac, an NSAID, induces apoptosis by binding to retinoid X receptor- α (RXR α). We identified an N-terminally truncated RXR α (tRXR α) in several cancer cell lines and primary tumors, which interacted with the p85 α subunit of phosphatidylinositol-3-OH kinase (PI3K). Tumor necrosis factor- α (TNF α) promoted tRXR α interaction with the p85 α , activating PI3K/AKT signaling. When combined with TNF α , Sulindac inhibited TNF α -induced tRXR α /p85 α interaction, leading to activation of the death receptor-mediated apoptotic pathway. We designed and synthesized a Sulindac analog K-80003, which has increased affinity to RXR α but lacks COX inhibitory activity. K-80003 displayed enhanced efficacy in inhibiting tRXR α -dependent AKT activation and tRXR α tumor growth in animals.

INTRODUCTION

Sulindac sulfide (Sulindac hereafter) (Haanen, 2001) is one of the early nonsteroidal anti-inflammatory drugs (NSAIDs) known to inhibit the activities of cyclooxygenases (COXs), of which COX-1 is constitutively expressed whereas COX-2 is induced by mitogenic and inflammatory stimuli. The discovery that regular use of aspirin, an NSAID, reduces the incidence of colon cancer has provided the impetus to develop NSAIDs for cancer prevention and treatment (Grosch et al., 2006; Thun et al., 2002). Sulindac has received extensive attention because of its potent induction of apoptosis and inhibition of cancer cell growth (Haanen, 2001; Yamamoto et al., 1999; Zhang et al., 2000). NSAIDs are believed to exert their anticancer effects through inhibition

of COX-2, which is often overexpressed in human premalignant and malignant tissues and plays a role in carcinogenesis. Compelling evidence, however, also indicates that NSAIDs can function through COX-2-independent mechanisms (Grosch et al., 2006; Yamamoto et al., 1999; Zhang et al., 2000). For example, cells lacking COX-1, COX-2, or both show comparable sensitivity to NSAID-induced apoptosis, whereas NSAIDs that do not inhibit COX-2 also induce apoptosis and inhibit carcinogenesis. Recent evidence that COX-2 inhibition is associated with increased cardiovascular risk (Fitzgerald, 2004) underscores the importance in the identification of non-COX-2 targets, which may lead to strategies for developing improved anticancer drugs. Although several non-COX-2 targets for NSAIDs have been reported (Grosch et al., 2006), more efforts to identify

Significance

Our results demonstrate that RXR α mediates NSAID Sulindac-induced apoptosis. We provide evidence that tRXR α detected in cancer cells acts nongenomically to activate the PI3K/AKT pathway and promote cancer cell growth and survival, which is inhibited by Sulindac. Our finding that Sulindac and TNF α synergistically induce apoptosis through activation of TNF α -dependent apoptotic pathway offers therapeutic strategies to sensitize cancer cells to the killing effect of this cytokine. Our characterizations of RXR-selective Sulindac analog K-80003 demonstrate that Sulindac's anticancer effects can be dissociated from its COX inhibition and identify a RXR α -based lead for cancer therapy. These findings should appeal to a broad audience of clinicians, cancer biologists, molecular biologists, and drug designers.

additional targets and characterize their mechanism of action are needed in order to develop improved target-based drugs for cancer therapy.

Retinoid X receptor- α (RXR α), a member of the nuclear receptor superfamily, plays a role in many biological processes including carcinogenesis (Altucci and Gronemeyer, 2001; Dawson and Zhang, 2002). 9-*cis*-Retinoic acid (9-*cis*-RA), several polyunsaturated fatty acids, and the NSAID Etodolac (Kolluri et al., 2005) can bind to RXR α to regulate different biological functions. Targretin, a synthetic RXR ligand, is currently used for treating cutaneous T cell lymphoma (Dawson and Zhang, 2002), demonstrating the suitability of targeting RXR α for cancer therapy. Consistently, the oncogenic potential of RXR α has been demonstrated. Genetic disruption of RXR α enhances tumorigenesis (Huang et al., 2002; Li et al., 2001), and RXR binding to PML/RAR is essential for the development of acute promyelocytic leukemia (Zeisig et al., 2007; Zhu et al., 2007). In addition, the RXR α protein level is often reduced in cancer cells and tumor tissues (Picard et al., 1999; Takiyama et al., 2004; Zhong et al., 2003), which is in part due to limited proteolytic processing of RXR α by calpain or cathepsin (Matsushima-Nishiwaki et al., 1996; Nagaya et al., 1998; Nomura et al., 1999; Takiyama et al., 2004; Zhong et al., 2003). However, the biological function of the resulting truncated RXR α (tRXR α) proteins remains unknown.

The mechanisms by which RXR α regulates diverse biological functions remain to be fully determined and are expected to be complex (Kastner et al., 1995; Mangelsdorf and Evans, 1995). Like other nuclear receptors, RXR α is known to regulate the transcription of target genes by binding to DNA response elements. Accumulating evidence, however, indicates that RXR α may also have extranuclear actions. RXR α resides in the cytoplasm in certain cell types and at different stages during development (Fukunaka et al., 2001). It migrates from the nucleus to the cytoplasm in response to differentiation (Katagiri et al., 2000), apoptosis (Cao et al., 2004), and inflammation (Ghose et al., 2004; Zimmerman et al., 2006). Interestingly, tRXR α resulted from limited proteolytic cleavage in tumor cells is also cytoplasmic (Zhong et al., 2003). Whether and how it acts in the cytoplasm to modulate carcinogenesis is currently unknown.

In this study, we examined whether tRXR α serves as an intracellular target mediating the apoptotic effect of Sulindac. In addition, we investigated the mechanism by which cytoplasmic tRXR α acts to promote tumor growth. Furthermore, we explored the possibility to dissociate Sulindac's anticancer effects from its COX inhibition activity.

RESULTS

Sulindac Binds to RXR α

We previously reported that R-Etodolac binds RXR α and induces a RXR α -dependent apoptosis of cancer cells in vitro and in animals (Kolluri et al., 2005). During the course of identifying other NSAIDs as potential RXR α ligands, we found that Sulindac bound to RXR α , but not RAR (see Figure S1A available online), with an IC₅₀ of 80 μ M (Figure 1A), which is in its concentration range that induces apoptosis (Yamamoto et al., 1999; Zhang et al., 2000). High-performance liquid chromatography

(HPLC) analysis showed a direct binding of Sulindac to RXR α protein but not other nuclear receptors such as RAR and Nur77 in cells (Figure 1B and Figure S1B). The binding was also illustrated by altered sensitivity of RXR α ligand-binding domain (LBD) or full-length (fl)-RXR α protein to chymotrypsin digestion by Sulindac in vitro (Figure 1C). Furthermore, we took advantage of the presence of fluorine atom in Sulindac and examined ¹⁹F nuclear magnetic resonance (NMR) spectra. Figure 1D shows that the signal intensity of the fluorine spectrum of Sulindac was strongly suppressed by RXR α LBD but not by Nur77 protein, demonstrating a direct and specific binding. Sulindac binding inhibited transactivation of RXR α homodimers (Figure 1E) and certain heterodimers (Figure 1F and Figure S1C) in the reporter assays, demonstrating that Sulindac is a RXR α transactivation antagonist.

Sulindac Induces RXR α -Dependent Apoptosis

To determine the role of RXR α in Sulindac-induced apoptosis, we examined its death effect in F9 cells and F9 cells lacking RXR α (F9-RXR α ^{-/-}). Sulindac induced extensive apoptosis in F9 cells, but had little effect in F9-RXR α ^{-/-} cells (Figures 2A–2C). Moreover, the apoptotic effect of Sulindac was reduced in cells with diminished RXR α level (Figures 2D and 2E), whereas it was enhanced in cells with ectopically expressed RXR α in RXR α -negative CV-1 cells (Figure 2F). To address the role of Sulindac binding to RXR α , we constructed the RXR α /F313S/R316E mutant in which Phe³¹³ and Arg³¹⁶ essential for maintaining the functional integrity of RXR α ligand-binding pocket (LBP) (Bourguet et al., 2000) were substituted with Ser and Glu, respectively. The mutant failed to respond to ligand-induced homodimer or heterodimer transactivation (Figure S2) and showed reduced apoptotic responses to Sulindac (Figure 2G). Thus, RXR α is involved in Sulindac-induced apoptosis.

Bax, a proapoptotic Bcl-2 family member, is required for the apoptotic effect of Sulindac (Zhang et al., 2000). We therefore determined if RXR α was involved in activation of Bax by Sulindac. Sulindac induced cleavage of PARP (Figure 2H) and apoptosis (Figure 2I) in HCT116 colon cancer cells, but not in HCT116 cells lacking Bax (Bax^{-/-}). The fact that HCT116 cells are deficient of COX-2 (Sheng et al., 1997) demonstrates that Sulindac-induced apoptosis can be COX-2 independent. Immunoblotting assays showed that Bax underwent extensive oligomerization on mitochondria in response to Sulindac, which was abrogated by RXR α siRNA (Figure 2J). In addition, immunostaining using anti-Bax antibody (Bax/ Δ 21) and a Bax conformation-sensitive antibody Bax/6A7 (Nechushtan et al., 1999) demonstrated that Sulindac-induced Bax conformational change and mitochondrial targeting were impaired by RXR α siRNA (Figure 2K). Together, these results demonstrate that RXR α can act as an intracellular target mediating the apoptotic effect of Sulindac.

Sulindac Inhibits RXR α -Dependent AKT Activation by TNF α

Activation of phosphatidylinositol-3-OH kinase (PI3K) and its downstream effector, AKT, regulates the biological function of substrates such as Bax (Cantley, 2002). We therefore investigated whether Sulindac activated Bax through inhibition of AKT activation and found that Sulindac potently suppressed

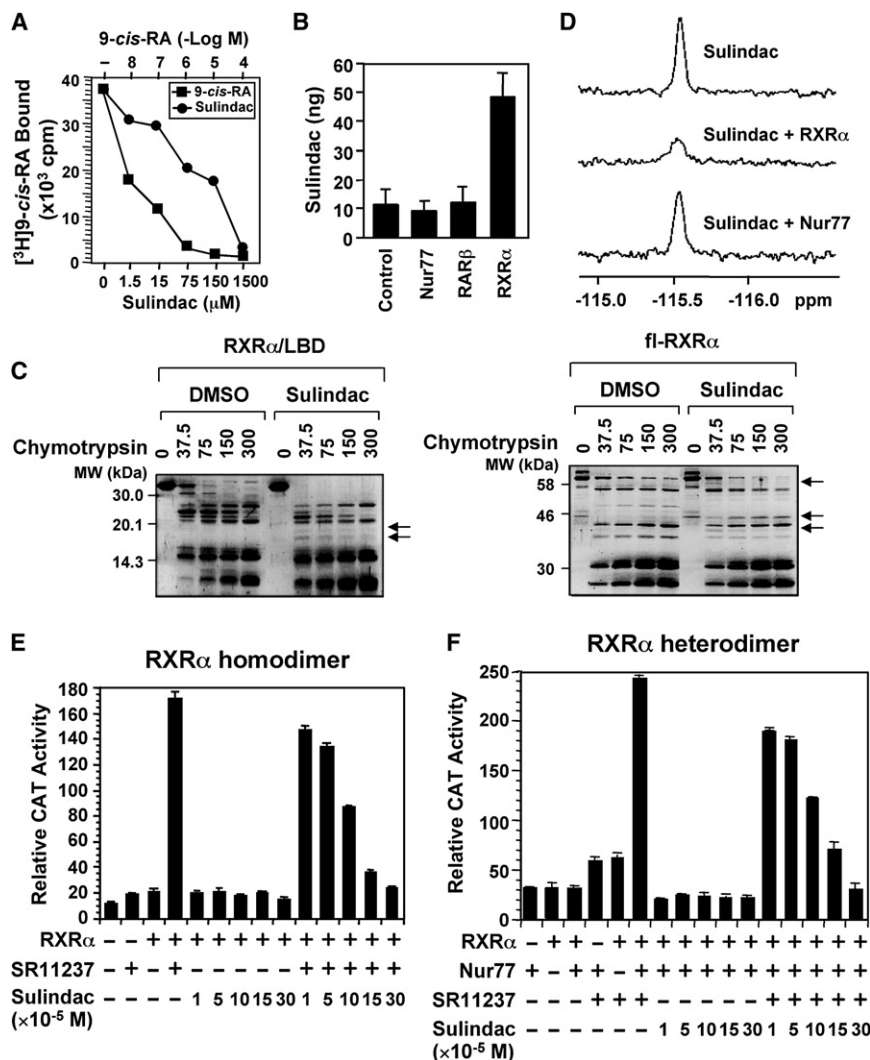


Figure 1. Sulindac Binds to RXR α

(A) Sulindac binding to RXR α in vitro. RXR α LBD protein was incubated with [³H]9-cis-RA in the presence or absence of Sulindac or unlabeled 9-cis-RA. Bound [³H]9-cis-RA was quantitated by liquid scintillation counting.

(B) Sulindac binding to RXR α in cells. HEK293 cells stably expressing RXR α , Nur77 or RAR β were treated with 100 μ M Sulindac for 3 hr. The same amount of purified RXR α , Nur77, or RAR β protein was subjected to HPLC analysis for the presence of Sulindac.

(C) Altered sensitivity of RXR α LBD or GST-RXR α to chymotrypsin (μ g/ml) by Sulindac (100 μ M).

(D) Comparison of ¹⁹F NMR spectra of Sulindac (100 μ M) in the absence and presence of 10 μ M RXR α LBD or Nur77 protein.

(E and F) Sulindac inhibits transactivation of RXR α homodimers and heterodimers. (TREpal)₂-tk-CAT (Zhang et al., 1992a) (E) or β RARE-tk-CAT (Zhang et al., 1992b) (F), Nur77 and/or RXR α were transiently transfected into CV-1 cells. Cells were treated with or without SR11237 (10⁻⁶ M) in the presence or absence of Sulindac. CAT activity was determined. One of three to five similar experiments is shown. Error bars represent SEM. See also Figure S1.

hibited AKT activation when used together with TNF α (Figure 3D), raising an intriguing possibility that TNF α can sensitize cancer cells to Sulindac by converting AKT activation from a RXR α -independent to a RXR α -dependent manner.

TNF α -Induced Interaction of tRXR α with p85 α and its Inhibition by Sulindac

Our observations that RXR α was required for AKT activation by TNF α and retinoic

acid prompted us to examine whether RXR α interacted with p85 α . Our initial intensive attempts by coimmunoprecipitation assays using anti-RXR α antibody (D20) against sequences in the N terminus of RXR α (Figure 3E) failed to detect a clear interaction, although the antibody effectively immunoprecipitated the RXR α protein (Figure S3E). Because tRXR α proteins produced through limited proteolytic cleavage in cancer cells (Figure 3F) were cytoplasmic (Nagaya et al., 1998; Zhong et al., 2003), we asked whether the cytoplasmic tRXR α was responsible for binding to p85 α . For this purpose, we used another anti-RXR α antibody (Δ N197) that recognizes the RXR α LBD (Figure 3E). Indeed, p85 α was readily coimmunoprecipitated by the Δ N197 antibody in a TNF- α (Figure 3E) or RA (Figures S3D and S3E) dependent manner. Coimmunoprecipitation of p85 α was accompanied with immunoprecipitation of tRXR α , which was not detected by the D20 RXR α antibody (Figure S3E), indicating its lack of N-terminal sequences. Using the Δ N197 antibody, we also observed that interaction of p85 α with tRXR α in the presence of TNF- α (Figure 3E) or 9-cis-RA (Figure S3D) was inhibited by Sulindac. These results suggested that tRXR α might bind to p85 α , leading to AKT activation.

AKT activation in HCT116 and other cancer cell lines (Figure 3A). Transfection of RXR α siRNA significantly reduced AKT activation (Figure 3B), similar to the effect of Sulindac, raising the possibility that Sulindac might inhibit RXR α -mediated AKT activation. Although Sulindac failed to inhibit AKT activation induced by epidermal growth factor (Figure S3A), it potently inhibited AKT activation induced by retinoic acid in a RXR α -dependent manner (Figures S3B and S3C).

TNF α could also activate PI3K/AKT signaling (Ozes et al., 1999; Pincheira et al., 2008). We thus examined whether RXR α played a role in AKT activation by TNF α . Treatment of A549 lung cancer cells with TNF α led to strong AKT activation, which was potently inhibited by Sulindac (Figure 3C). Transfection of RXR α siRNA, which inhibited not only the expression of the 54 kDa fl-RXR α but also a 44 kDa tRXR α , significantly impaired the ability of TNF α to activate AKT (Figure 3C), demonstrating that RXR α was critical for AKT activation by TNF α . Although Sulindac showed little inhibitory effect on AKT activation in cancer cells with high basal AKT activation, such as ZR-75-1 breast cancer and PC3 prostate cancer cells, it effectively in-

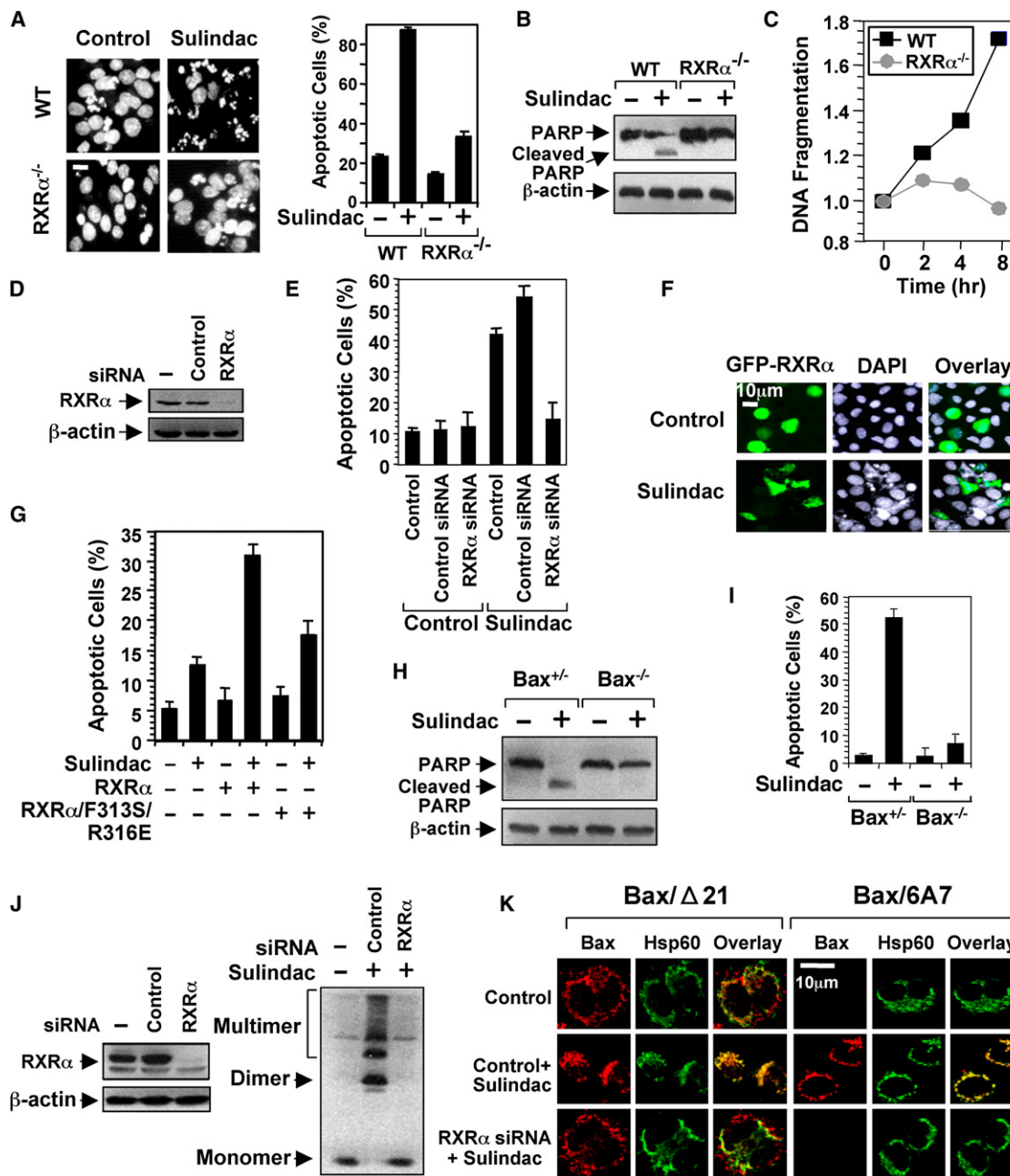


Figure 2. Sulindac Induces RXR α -Dependent Apoptosis and Bax Activation

(A–C) The apoptotic effects of Sulindac in F9 or F9 cells lacking RXR α (F9 RXR $\alpha^{-/-}$). Cells treated with Sulindac (75 μ M) for 24 hr were analyzed by DAPI staining (A), PARP cleavage (B), and DNA fragmentation (C). The scale bar represents 10 μ m.

(D and E) RXR α siRNA inhibits apoptosis induction by Sulindac. H460 lung cancer cells transfected with control or RXR α siRNA were treated with Sulindac (75 μ M) for 24 hr and analyzed by DAPI staining for apoptosis.

(F) Transfection of RXR α enhances the apoptotic effect of Sulindac. CV-1 cells transfected with GFP-RXR α were treated with Sulindac (75 μ M) for 24 hr and analyzed by DAPI staining. GFP-RXR α -transfected cells underwent extensive nuclear fragmentation and condensation.

(G) Disruption of the RXR α LBP impairs the apoptotic effect of Sulindac. CV-1 cells transfected with GFP-RXR α or GFP-RXR α /F313S/R316E were treated with Sulindac (75 μ M) for 24 hr and analyzed by DAPI staining. Apoptosis scored in receptor-transfected cells. See also Figure S2.

(H and I) Role of Bax in apoptosis induction by Sulindac. HCT116 cells or HCT116 cells lacking Bax (Bax $^{-/-}$) were treated with or without Sulindac (75 μ M) for 24 hr. Apoptosis determined by PARP cleavage (H) and DAPI staining (I).

(J and K) RXR α siRNA inhibits Sulindac-induced Bax activation. Knocking down RXR α in HCT116 cells by RXR α siRNA revealed by immunoblotting. HCT116 cells transfected with or without RXR α siRNA or control siRNA for 48 hr were treated with Sulindac for 6 hr, and analyzed for Bax oligomerization (J) and Bax conformational change and mitochondrial targeting by immunostaining/confocal microscopy using Bax/ Δ 21, Bax/6A7, or anti-Hsp60 antibody (K). About 60% of cells showed Bax conformational change presented. One of three to five similar experiments is shown. Error bars represent SEM.

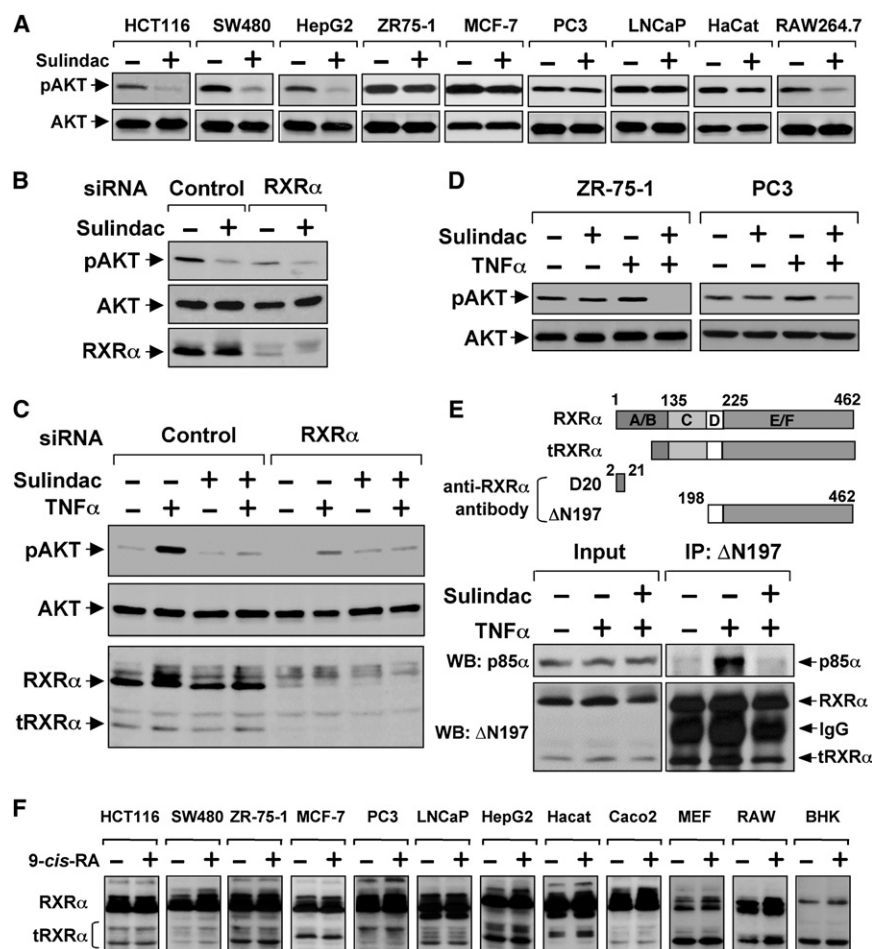


Figure 3. Sulindac Inhibits TNF α -Induced AKT Activation and tRXR α -p85 α Interaction

(A) Inhibition of AKT activation by Sulindac. The indicated cells starved overnight and treated with Sulindac (100 μ M) for 1 hr were analyzed for AKT activation by immunoblotting.

(B) Inhibition of basal AKT activation by RXR α siRNA. HepG2 cells transfected with RXR α siRNA for 48 hr were treated with Sulindac (100 μ M) for 1 hr. AKT activation and RXR α expression were analyzed by immunoblotting.

(C) Inhibition of TNF α -induced AKT activation by Sulindac and RXR α siRNA. A549 lung cancer cells transfected with RXR α or control siRNA for 48 hr were pretreated with Sulindac (100 μ M) for 1 hr before exposed to TNF α (10 ng/ml) for 30 min.

(D) Synergistic inhibition of AKT activation by TNF α and Sulindac. ZR-75-1 and PC3 cells were pre-treated with Sulindac for 1 hr before exposed to TNE α (10 ng/ml) for 30 min.

(E) Induction of RXR α -p85 α interaction by TNF α . A549 cells treated with TNF α (10 ng/ml) and/or Sulindac (100 μ M) for 30 min were analyzed for RXR α -p85 α interaction by coimmunoprecipitation using Δ N197 antibody. The top portion shows a schematic representation of anti-RXR α antibodies used in coimmunoprecipitation and immunoblotting assays. D20 antibody recognizes amino acids 2-21 in the N-terminal A/B domain, while Δ N197 antibody recognizes the C-terminal E/F domain. A RXR α truncated protein with about 44 kDa is also shown.

(F) Expression of RXR α in various cancer cell lines. The indicated cell lines treated with or without 9-*cis*-RA (10^{-7} M) for 30 min were analyzed by immunoblotting using the Δ N197 RXR α antibody. One of three to six similar experiments is shown. See also [Figure S3](#).

Regulation of tRXR α Production and its Activation of AKT

We reported previously that cell density plays a critical role in determining the cytoplasmic localization of RAR γ (Han et al., 2009). We similarly observed that the level of the 44 kDa tRXR α reduced as the density of cells increased, which was accompanied with production of a smaller RXR α fragment. Interestingly, the levels of the 44 kDa tRXR α protein correlated with AKT activation (Figure 4A), suggesting that cell density-dependent proteolytic cleavage of RXR α might be an important mechanism regulating AKT activation. Consistent with cytoplasmic localization of tRXR α (Figure S4A), immunostaining of MEFs with the Δ N197 antibody revealed RXR α staining predominantly in the cytoplasm and occasionally on the plasma membrane (Figure 4B), likely due to the high levels of tRXR α in MEFs. Thus, deletion of the N-terminal sequences of RXR α could alter its subcellular localization, conferring its ability to interact with p85 α .

In an effort to study the regulation of tRXR α production, we found that expression of the N-terminal region of RXR α , RXR α /1-134, enhanced the tRXR α level (Figure S4B). To study the biological function of the endogenous tRXR α , we stably expressed RXR α /1-134 in HeLa cells, which resulted in production of a

significant amount of 44 kDa tRXR α protein. Compared with parental HeLa cells, HeLa/RXR α /1-134 stable clone had much higher AKT activation (Figure 4C) and was able to rapidly grow in soft agar (Figure 4D). Sulindac strongly reduced colonies formed by the stable clone in the colony formation assay (Figure 4E). Together, these results demonstrate that tRXR α may contribute to the growth and survival of cancer cells by activating AKT and that tRXR α -mediated activities can be negatively regulated by Sulindac.

To study the possible pathological function of tRXR α , we examined its expression in tumor tissues. Immunoblotting of tissue samples showed the presence of tRXR α in breast and liver cancer tissues but not in tumor surrounding tissues or distant normal tissues from the same patients (Figure 4F). Previous studies revealed an extensive cytoplasmic RXR α immunostaining in malignant human prostatic tumor (Zhong et al., 2003) and thyroid tumor (Takiyama et al., 2004) specimens. Immunohistochemical analysis using the Δ N197 antibody also revealed a strong cytoplasmic RXR α staining in liver tumor tissue but not in the surrounding tissue (Figure 4G), confirming that tRXR α produced in tumor tissues is cytoplasmic. Together, these results suggest that tRXR α may play a role in the development of cancer through its ability to activate AKT.

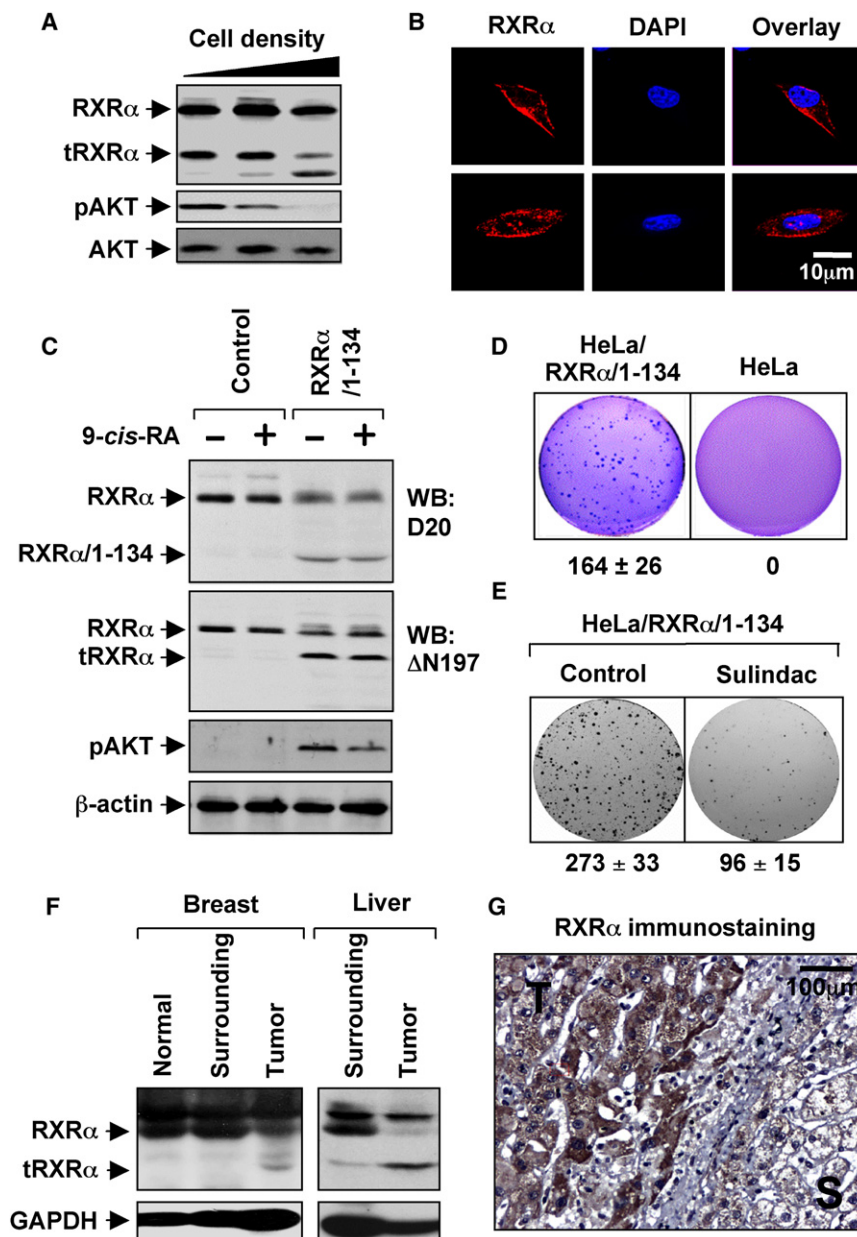


Figure 4. Role of tRXR α in AKT Activation and Anchorage-Independent Cell Growth

(A) Cell density dependent production of tRXR α and AKT activation. MEFs seeded at different cell density were analyzed for RXR α expression using Δ N197 antibody and for AKT activation by immunoblotting.

(B) Subcellular localization of endogenous RXR α in MEFs was visualized by confocal microscopy after immunostaining with anti-RXR α (Δ N197). Cells were also stained with DAPI to visualize the nucleus. More than 60% of cells showed the images presented.

(C) Stable expression of RXR α /1-134 induces RXR α cleavage and AKT activation. HeLa or HeLa cells stably expressing RXR α /1-134 were treated with 9-*cis*-RA for 30 min and analyzed for AKT activation and expression of RXR α .

(D) Growth of HeLa/RXR α /1-134 and HeLa cells in soft agar.

(E) Sulindac inhibits clonogenic survival of HeLa/RXR α /1-134 cells. Cells grown in 6-well plates for 5 days were treated with Sulindac (25 μ M) for 3 days.

(F) Production of tRXR α in human tumor tissues of breast (five out of six) or liver (four out of six) compared with tumor surrounding and normal tissues.

(G) Cytoplasmic localization of RXR α in liver tumor specimens immunostained by Δ N197 antibody. T, tumor tissue; S, tumor surrounding tissue. One of three to five similar experiments is shown. See also Figure S4.

N-Terminally Truncated RXR α Mediates TNF- α Activation of the PI3K/AKT Pathway and Promotes Cancer Cell Growth and Survival

To directly address the role of N-terminally truncated RXR α , we constructed a RXR α mutant lacking its N-terminal 80 amino acids (RXR α / Δ 80) with a molecular weight similar to the endogenous tRXR α . Also similar to tRXR α , RXR α / Δ 80 interacted with p85 α , which was strongly enhanced by TNF- α (Figure 5A). In contrast, the full-length RXR α did not interact with p85 α either in the absence or presence of TNF- α , suggesting that the N-terminal sequences of RXR α prevented its binding to p85 α . Interestingly, RXR α mutant lacking the N-terminal 100 amino acids (RXR α / Δ 100) was unable to interact with p85 α (Figure 5A). RXR α /1-134 but not RXR α /223-462 could interact with p85 α

(Figure 5B). RXR α / Δ 80 but not RXR α / Δ 100 strongly activated AKT in different cell types (Figure 5C), demonstrating the role of RXR α / Δ 80 in AKT activation. Consistent with cytoplasmic localization of tRXR α (Figure 4B and Figure S4A), RXR α / Δ 80 predominantly resided in the cytoplasm, with occasional punctate plasma membrane localization (Figure 5D). Thus, deletion of the N-terminal sequences of RXR α alters its subcellular localization and confers its ability to interact with p85 α .

To determine how tRXR α /p85 α interaction induced AKT activation, we examined whether RXR α / Δ 80 immunocomplex possessed PI3K activity in vitro. The PI3K activity exhibited by the Myc-RXR α / Δ 80 immunocomplex was dramatically enhanced by TNF- α treatment (Figure 5E and Figures S5A and S5B), which correlated well with its ability to interact with p85 α (Figure 5A) and activation of AKT (Figure 5C). Thus, TNF- α -induced tRXR α /p85 α interaction can activate the PI3K/AKT signaling.

To further study the role of tRXR α , we stably expressed RXR α / Δ 80 in SW480 and HCT116 colon cancer cells. The resulting stable cells, SW480/RXR α / Δ 80 and HCT116/RXR α / Δ 80, showed elevated AKT activation and induction of its downstream targets c-Myc and cyclin D1 (Figure 5F) and increased clonogenic survival than do the control cells (Figure 5G). We then

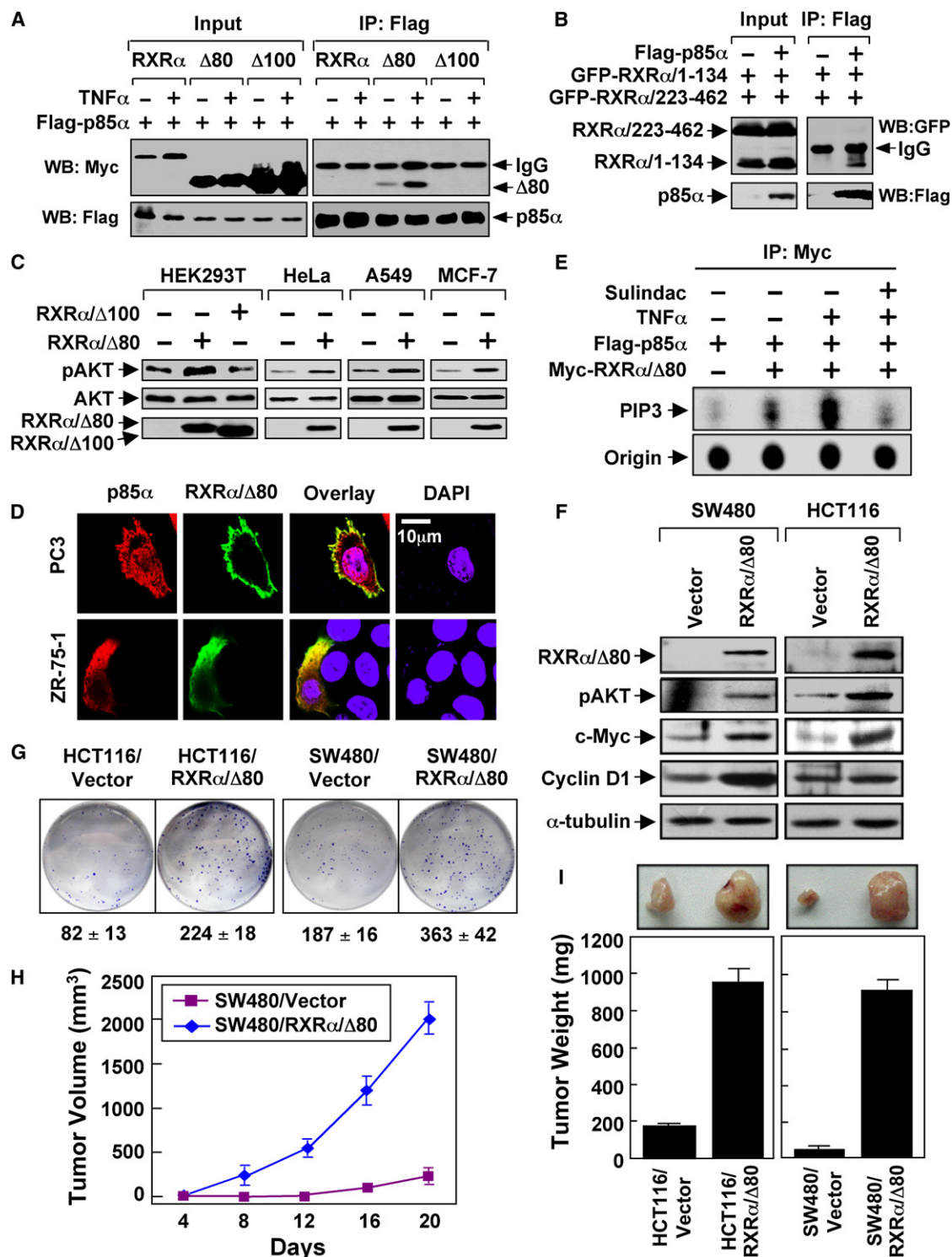


Figure 5. Role of N-Terminally Truncated RXR α in PI3K/AKT Activation by TNF α and Cancer Cell Growth

(A) HEK293T cells were transfected with Flag-p85 α and RXR α , RXR α /80, or RXR α /100 tagged with the Myc epitope, treated with TNF α (10 ng/ml) for 30 min, and analyzed by coimmunoprecipitation with anti-Flag antibody.

(B) N-terminal A/B domain of RXR α interacts with p85 α . Flag-p85 α was cotransfected with GFP-RXR α /1-134 and GFP-RXR α /223-462 into HEK293T cells, and analyzed for their interaction by coimmunoprecipitation with anti-Flag antibody.

(C) RXR α /80 is a potent AKT activator. AKT activation of the indicated cells transfected with RXR α /80 or RXR α /100 was determined by immunoblotting.

examined the effect of RXR α /Δ80 on the growth of cancer cells in animals by injecting the same number of RXR α /Δ80 expressing cells and the control cells into different flanks of same nude mice. Our results showed that tumors formed by SW480/RXR α /Δ80 and HCT116/RXR α /Δ80 cells grew much faster than those formed by the control cells (Figures 5H and 5I). Together, these results demonstrate that the N-terminally truncated RXR α is a potent promoter of cancer cell growth.

Sulindac Activates TNF- α -Induced Extrinsic Apoptotic Pathway

We next determined whether and how synergistic inhibition of AKT activation by Sulindac and TNF- α induced apoptosis. Treatment of various cancer cell lines with Sulindac and TNF- α effectively induced PARP cleavage and caspase-8 activation (indicated by cleaved caspase-8 products, p43/p41 and p18), whereas treatment of these cells with either Sulindac or TNF- α alone had little effect (Figure 6A and Figure S6). The apoptotic effect of Sulindac/TNF- α combination was partially suppressed by RXR α -selective ligand SR11237 (Figure 6A) or transfection of RXR α siRNA (Figure 6B).

Our observation that Sulindac/TNF- α activated caspase-8 suggested that apoptosis induction might be due to the activation of TNF- α -mediated extrinsic apoptotic pathway. To address this, we treated cells with the caspase-8 inhibitor Z-IETD-fmk or with caspase-8 siRNA and observed suppression of Sulindac/TNF- α -induced PARP cleavage (Figures 6C and 6D and Figures S6B–S6D). Thus, Sulindac/TNF- α -induced apoptosis is mediated by the extrinsic apoptotic pathway.

We also examined whether Sulindac/TNF- α activation of the extrinsic apoptotic pathway resulted in Bax activation by immunostaining cells using conformation-sensitive Bax/6A7 antibody. Significant activated-Bax staining was observed only when cells were treated with both TNF- α and Sulindac (Figure 6E). Crosstalk between extrinsic and intrinsic apoptotic pathways can be linked through Bid cleavage and activation (Li et al., 1998). Indeed, we observed that Bid was significantly degraded in cells treated with TNF- α and Sulindac (Figure 6A), suggesting that Sulindac/TNF- α -induced Bax activation might be mediated through Bid activation.

Our observation that Sulindac/TNF- α combination synergistically induced apoptosis and inhibited AKT activation suggested that AKT activity might be critical for their induction of apoptosis. Indeed, Sulindac/TNF- α -induced PARP cleavage was inhibited by the expression of a constitutive-active AKT (CA-AKT) and enhanced by the expression of a dominant-negative AKT (DN-AKT) (Figure 6F). Consistently, induction of apoptosis and activation of caspase-8 and Bax by Sulindac/TNF- α combination were inhibited by CA-AKT (Figures 6G and 6H, and Figure S6E). To study how Sulindac promoted

apoptosis through its inhibition of AKT, we examined the expression of c-FLIP, a downstream target gene of AKT signaling (Panka et al., 2001), which acts as a potent inhibitor of the extrinsic apoptotic pathway by inhibiting caspase-8 activation (Irmiler et al., 1997). Treatment of cells with TNF- α resulted in strong induction of both the short form (c-FLIP_s) and long form (c-FLIP_l) of c-FLIP, which was inhibited by Sulindac (Figure 6I). Thus, Sulindac might induce apoptosis by suppressing the induction of c-FLIP expression by TNF- α .

Design and Synthesis of RXR α -Selective Sulindac Analogs

Our finding that RXR α served as an intracellular target of Sulindac action provided an opportunity to design RXR α -selective Sulindac derivatives for cancer therapy. Thus, we conducted docking of Sulindac to three-dimensional structures of the RXR α LBD to identify strategies for structural modifications of Sulindac in order to dissociate its COX inhibition from RXR α -binding activity. Docking of Sulindac to RXR α (Figure 7A) showed that Sulindac bound in a mode where its carboxylate group was aligned with the carboxylate group found in all RXR α ligands examined (Figure 7B), interacting with Arg³¹⁶ in the RXR α LBP. The benzyl methyl sulfide portion of Sulindac bound to the hydrophobic region of the RXR α LBP, overlapping with the α -ion-one ring of 9-*cis*-RA. In this binding mode, van der Waals interaction of the –SCH₃ group at position 4 (Figure 7C) with the RXR α protein was not optimal and there was room around it for modification to improve the binding to RXR α . The idea of making use of position 4 to design RXR α -selective analogs was fully supported by the fact that Sulindac prodrug sulindac sulfoxide and the metabolite sulindac sulfone show no COX-inhibiting activity, whereas the metabolite sulindac sulfide (used in this study) is a potent COX inhibitor (Haanen, 2001). As shown in Figure 7A, the carboxylate group of Sulindac was positioned away from Arg³¹⁶ compared with the equivalent ones in RXR α ligands DHA, BMS649, and 9-*cis*-RA. Replacing –CH₂COOH at position D with a bulkier group such as –CH₂CH₂COOH would help place the carboxylate group closer to Arg³¹⁶ to achieve good charge-charge interaction with RXR α as observed in 9-*cis*-RA. Our candidate compounds were also examined by docking to the crystal structure of COX-2 (Kurumbail et al., 1996) (Figure S7) to identify non-COX binders. Based on these considerations, five analogs were designed and synthesized (Figure 7C and Figure S7). Their evaluation showed that all analogs retained RXR α -binding activity, with K-80003 being the most potent, likely due to its isopropyl group at position 4, which has improved interaction with the hydrophobic residues on Helix 7 of RXR α . Significantly, K-80003 and K-80005 had no detectable inhibition of COX activities and failed to inhibit constitutive and TNF- α - or interleukin-1 β -induced prostaglandin E₂

(D) Cytoplasmic colocalization of RXR α /Δ80 and p85 α . Myc-RXR α /Δ80 and p85 α were cotransfected into the indicated cell lines, immunostained with anti-Myc and anti-p85 α antibody, and their subcellular localization revealed by confocal microscopy. RXR α /Δ80 predominantly resided in the cytoplasm of about 80% of cells. Approximately 15% of cells showed the images presented.

(E) Activation of PI3K by RXR α /Δ80 immunoprecipitates. A549 cells transfected with Flag-p85 α and Myc-RXR α /Δ80 were treated with TNF α and/or Sulindac, immunoprecipitated with anti-Myc antibody, and subjected to in vitro PI3K assay.

(F) AKT activation by stable expression of RXR α /Δ80. Cells stably transfected with GFP-RXR α /Δ80 or control GFP vector were analyzed by immunoblotting.

(G) RXR α /Δ80 promotes clonogenic survival of cancer cells grown in 6-well plates for 8 days.

(H and I) RXR α /Δ80 promotes cancer cell growth in nude mice (n = 6) for 3 weeks. One of three to five similar experiments is shown. Error bars represent SEM. See also Figure S5.

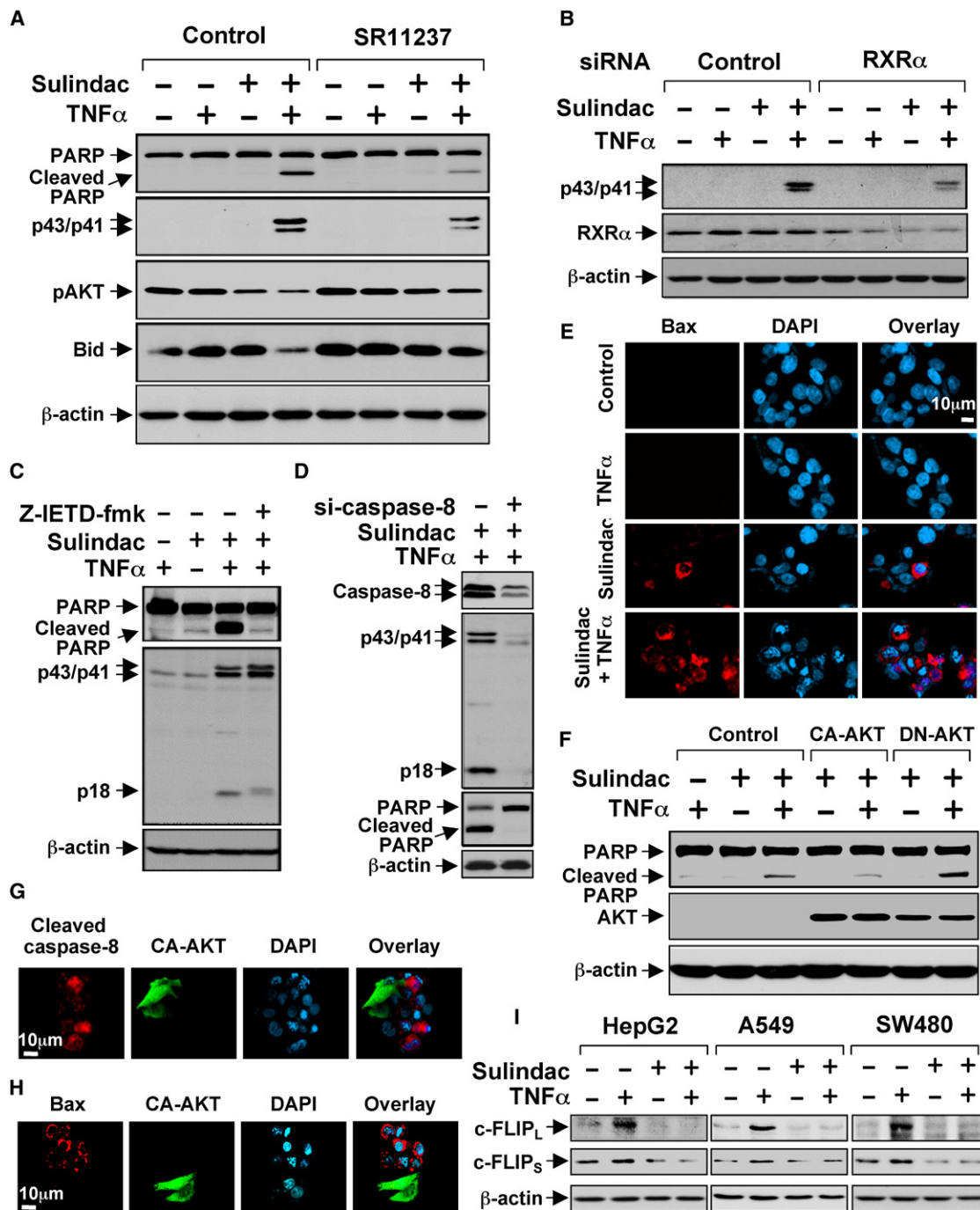


Figure 6. Activation of TNF α -Induced Extrinsic Apoptotic Pathway by Sulindac

(A) Synergistic induction of apoptosis by Sulindac/TNF α combination and its inhibition by RXR α ligand. HepG2 cells cultured in medium with 1% FBS were treated with SR11237 (1 μ M) for 1 hr, then TNF α (10 ng/ml) and/or Sulindac (75 μ M) for 4 hr, and analyzed by immunoblotting.

(B) Inhibition of Sulindac/TNF α -induced caspases-8 cleavage by RXR α siRNA. HepG2 cells transfected with control or RXR α siRNA were treated with TNF α and/or Sulindac and analyzed by immunoblotting.

(C and D) Inhibition of Sulindac/TNF α -induced PARP cleavage by caspase-8 inhibitor and siRNA. HepG2 cells transfected with control or caspase-8 siRNA or pretreated with Z-IETD-fmk (40 μ M) for 1 hr were treated with TNF α and Sulindac and analyzed by immunoblotting.

(E) Activation of Bax by Sulindac and TNF α . HepG2 cells treated with TNF α and/or Sulindac were immunostained with Bax/6A7 antibody. About 15% Sulindac-treated cells while about 60% Sulindac/TNF α -treated cells showed Bax staining.

(F) Regulation of Sulindac/TNF α -induced PARP cleavage by AKT. PC3 cells transfected with CA-AKT or DN-AKT were treated with TNF α and/or Sulindac, and analyzed by immunoblotting.

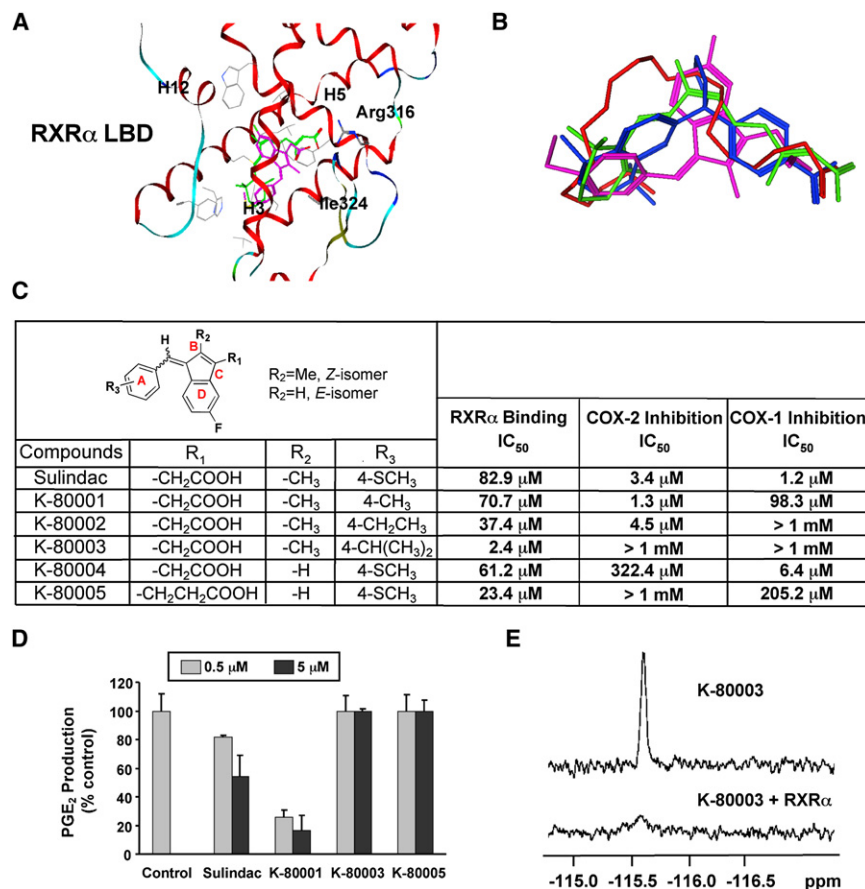


Figure 7. Design, Synthesis, and Evaluation of RXR α -Selective Sulindac Analogs

(A) Docking of sulindac sulfide (magenta) to the LBP of RXR α in reference to 9-*cis*-RA (green). Side chains within 4 Å of the ligands are displayed in gray.

(B) Comparison of orientation and position of docked sulindac sulfide (magenta) to the crystal structures of 9-*cis*-RA (green), DHA (red) and BMS649 (blue).

(C) RXR α binding and inhibition of COX-1 and COX-2 activities by Sulindac analogs. RXR α binding was measured by competition ligand-binding assays. COX inhibition assays used Cayman's COX Fluorescent Activity Assay Kits.

(D) Inhibition of PGE₂ production by Sulindac and analogs. A549 cells stimulated with TNF α (10 ng/ml) for 24 hr were treated with Sulindac or analogs for 30 min. PGE₂ production was measured and expressed as the ratio of PGE₂ produced in the presence of compound to that with vehicle. Error bars represent SEM.

(E) Comparison of ¹⁹F NMR spectra of K-80003 (100 μ M) in the absence and presence of 10 μ M RXR α LBD. One of three to five similar experiments is shown. See also Figure S7.

(PGE₂) production (Figures 7C and 7D, and data not shown). The binding of K-80003 to RXR α was also confirmed by ¹⁹F NMR binding assays (Figure 7E). Thus, Sulindac's RXR α -binding can be dissociated from its COX-binding.

RXR α -Selective Analog K-80003 Is a Potent Inhibitor of AKT Activation and Cancer Cell Growth

Because of its much-improved affinity to RXR α and lack of COX inhibitory effect, K-80003 was chosen for further evaluation. Immunoblotting showed that K-80003 was much more effective than Sulindac in inhibiting RA- and TNF α -induced AKT activation (Figure 8A and Figure S8). The inhibitory effect of K-80003 on AKT activation in PC3 cells is largely impaired by reducing RXR α , but not RAR γ , expression by siRNA (Figure 8B). Thus, inhibition of AKT activation by K-80003 was also dependent on RXR α expression. The interaction of RXR α /Δ80 with p85 α either in the absence or presence of TNF α was more potently inhibited by K-80003 than by Sulindac (Figure 8C). K-80003 was also more effective than Sulindac in inducing PARP cleavage when

used together with TNF α in ZR-75-1 cells (Figure 8D). Similar to Sulindac, K-80003 combination with TNF α synergistically induced PARP cleavage and caspase-8 activation (Figure 8E). In clonogenic survival assays, colony formation of HeLa/RXR α /1-134 and RXR α /Δ80 cells was almost completely suppressed by K-80003 (Figure 8F). Significantly, K-80003 exhibited much more potent inhibitory effect than Sulindac on the growth of RXR α /Δ80 tumor in animals (Figure 8G). Together, the RXR α -selective Sulindac analog K-80003 is a potent inhibitor of RXR α -mediated PI3K/AKT signaling and cancer cell growth.

DISCUSSION

RXR α is an attractive molecular target for drug development. Here, we report that Sulindac could bind to RXR α in the range of concentrations commonly used to study the anticancer effects of Sulindac. Conventional administration of Sulindac could result in about 10–15 μ M Sulindac in the serum of patients and up to ~50 μ M Sulindac could be detected in the plasma of humans (Yamamoto et al., 1999). Sulindac could be also concentrated in epithelial cells at concentrations that are at least 20-fold higher than those in the serum (Duggan et al., 1980). Thus, the binding affinity of Sulindac to RXR α is relevant to in vivo cancer prevention by this drug. The findings that Sulindac

(G and H) CA-AKT inhibits activation of caspase-8 (G) and Bax (H) by Sulindac and TNF α . HepG2 cells transfected with CA-AKT were treated with TNF α and Sulindac, and immunostained with anti-cleaved caspase-8 or Bax/6A7 antibody. About 80% nontransfected and 15% CA-AKT-transfected cells showed caspase-8 staining. Approximately 60% nontransfected and about 13% CA-AKT-transfected cells exhibited Bax staining.

(I) Regulation of c-FLIP expression by TNF α and Sulindac. Cells treated with TNF α and/or Sulindac for 6 hr were analyzed by immunoblotting. One of three to five similar experiments is shown. See also Figure S6.

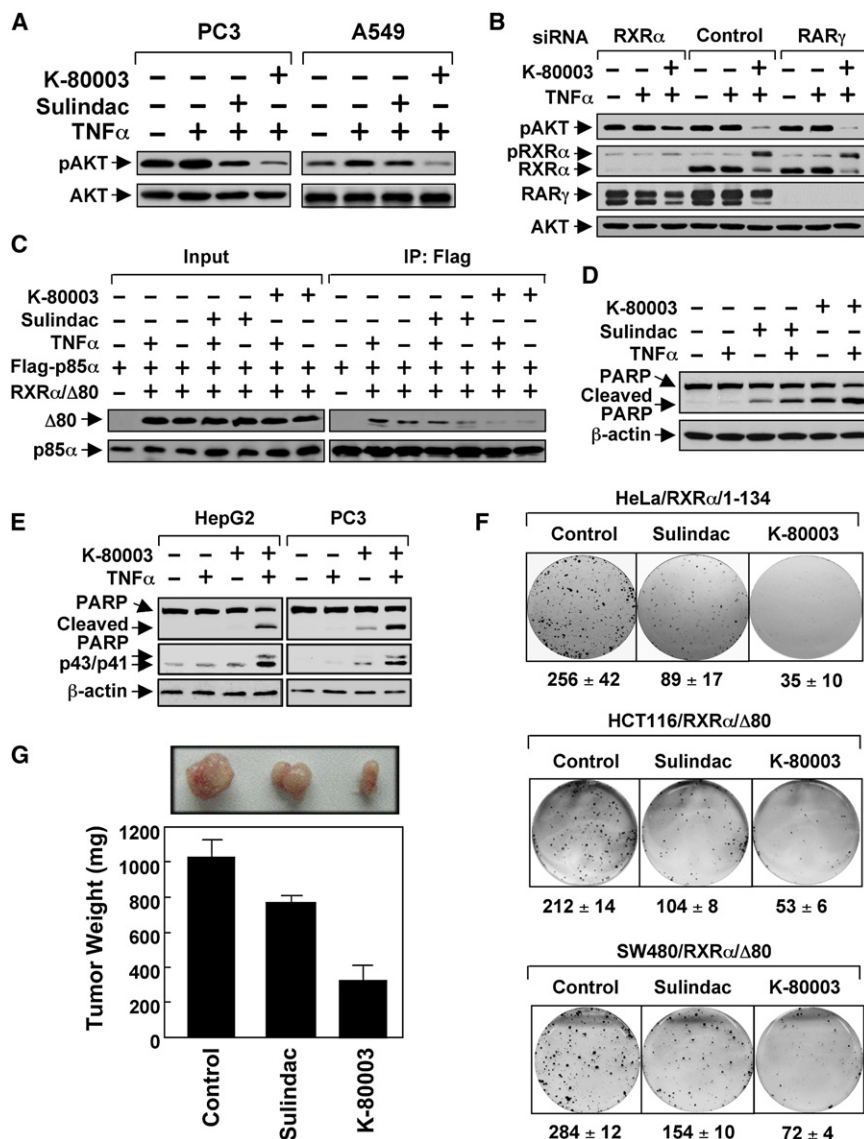


Figure 8. K-80003 Is a Potent Inhibitor of RXR α Dependent AKT Activation

(A) Inhibition of AKT activation by Sulindac (50 μ M) or K-80003 (50 μ M) in the presence of TNF α .

(B) RXR α -dependent inhibition of AKT activation by K-80003. PC3 cells transfected with RXR α or RAR γ siRNA were pretreated with K-80003 (50 μ M) for 1 hr before exposed to TNF α (10 ng/ml) for 30 min. pRXR α : phosphorylated RXR α .

(C) Inhibition of RXR α /Δ80 interaction with p85 α by Sulindac and K-80003. A549 cells were transfected with Flag-p85 α and Myc-RXR α /Δ80, treated with Sulindac (50 μ M) or K-80003 (50 μ M) for 1 hr before exposed to TNF α for 30 min, and analyzed by coimmunoprecipitation with anti-Flag antibody.

(D) Induction of PARP cleavage by Sulindac or K-80003 in the presence of TNF α . ZR-75-1 cells treated with TNF α and/or Sulindac (75 μ M) or K-80003 (50 μ M) for 6 hr were analyzed by immunoblotting.

(E) Activation of caspase-8 by K-80003 in the presence of TNF α . Cells treated with TNF α and/or K-80003 (50 μ M) were analyzed by immunoblotting.

(F) Inhibition of clonogenic survival of RXR α /1-134 cells and RXR α /Δ80 stable clones by Sulindac (25 μ M) and K-80003 (25 μ M).

(G) Inhibition of RXR α /Δ80 tumor growth in animals by Sulindac and K-80003. Mice (n = 6) were treated intraperitoneally with corn oil, Sulindac (60 mg/kg), or K-80003 (60 mg/kg) for 2 weeks. Tumors were removed and measured. Error bars represent SEM. One of three to five similar experiments is shown. See also Figure S8.

can bind to RXR α and that the apoptotic effect of Sulindac largely depends on RXR α expression and its intact LBP strongly suggest that RXR α is an intracellular target of Sulindac.

An important finding of this study is that the N-terminally truncated RXR α protein acts differently from the full-length RXR α protein. Cytoplasmic tRXR α interacted with p85 α to activate the PI3K/AKT survival pathway and induce anchorage-independent cell growth in vitro and tumor growth in animals, implying that tRXR α may serve as an important tumor promoter. Our mutational analysis suggested that amino acids from 80 to 100 in RXR α are critical for tRXR α binding to p85 α . The region is enriched with proline residues, which can presumably form several polyproline helices (PPII helix) known to bind to the SH3 domain (Kaneko et al., 2008) that is present in p85 α . The p85 α -binding motif(s) in RXR α are probably masked by the N-terminal end sequences and regulated by phosphorylation. This is consistent with the regulation of tRXR α production and AKT activation by cell density. Regulated proteolysis is a key

step in a number of different signaling pathways. Caspase-mediated cleavage of the BH3-only protein Bid into a truncated protein (tBid) and subsequent translocation of tBid to mitochondria are implicated in death receptor signaling (Li et al., 1998), whereas proteolytic processing of Notch and nuclear translocation of truncated product are crucial steps in transduction of the Notch signaling (Ye et al., 1999). STAT signaling is also regulated by proteolytic processing (Hendry and John, 2004). Thus, cleavage of RXR α may represent a mechanism that triggers non-genomic tRXR α signaling by removing the inhibitory N-terminal domain, allowing tRXR α to expose its p85 α -binding motif and activate the PI3K/AKT signaling.

Our finding that tRXR α is produced in tumor tissues but not in normal tissues is consistent with previous findings that RXR α is cleaved in tumor but not in premalignant or normal tissues from patients with prostate or thyroid cancer (Takiyama et al., 2004; Zhong et al., 2003). Thus, agents targeting tRXR α -mediated pathway can be effective and tumor specific. To this end, we showed that Sulindac could inhibit the tRXR α -mediated PI3K/AKT activation, suggesting that Sulindac represents a lead for a class of anticancer agents targeting this pathway.

Our observation that Sulindac and TNF α synergistically inhibit tRXR α -dependent AKT activation provides insight into the

crosstalk between retinoid receptor and TNF α signaling pathways. Retinoids in combination with cytokines, such as TNF α and TNF-related apoptosis inducing ligand (TRAIL), can synergistically induce differentiation or apoptosis of human transformed cells (Altucci et al., 2005), whereas combination of retinoids and TNF α can overcome RA resistance (Witcher et al., 2004). The fact that Sulindac and TNF α synergistically inhibit AKT activation in cancer cells implies that TNF α and probably other cytokines can prime cancer cells for their responsiveness to RXR α ligands such as Sulindac by converting AKT activation from a RXR α -independent to a RXR α -dependent manner.

TNF α plays important roles in diverse cellular events such as cell survival and death. However, it often fails to induce apoptosis in cancer cells because of its simultaneous activation of the NF- κ B and/or the PI3K/AKT pathway (Aggarwal, 2003; Balkwill, 2009). Our observation that tRXR α mediates AKT activation by TNF α suggests a possibility of using Sulindac or analogs to suppress TNF α -induced AKT-mediated survival function, thereby shifting its function from survival to death. Consistently, we have provided evidence that Sulindac in combination with TNF α potentially induce tRXR α -dependent caspase-8 activation and apoptosis, demonstrating that Sulindac is able to sensitize cancer cells to TNF α -induced death receptor-mediated extrinsic apoptotic pathway. The fact that TNF α -induced c-FLIP expression is completely prevented by Sulindac places c-FLIP in a central position for integrating TNF α -induced AKT activation and its inhibition by Sulindac and induction of apoptosis by Sulindac and TNF α combination.

Our finding that RXR α serves as an intracellular target of Sulindac action provides a rationale to design RXR α -selective Sulindac derivatives for suppressing AKT activity. Our identification of a Sulindac analog, K-80003, with improved affinity to RXR α but lacking COX inhibitory effects offers an example to this approach. It is expected that K-80003 will lack or have much reduced COX-2-associated side effects. The fact that K-80003 could effectively inhibit the tRXR α pathway and the growth of cancer cells in vitro and in animals warrants its further development for cancer therapy.

EXPERIMENTAL PROCEDURES

Plasmids

Flag-p85 α , RXR α /F313S/R316E, RXR α /Δ80, and RXR α /Δ100 were constructed with standard methods.

Antibodies and Reagents

Antibodies for pAkt (Ser⁴⁷³, D9E), Bid, and cleaved caspase-8 (p43/p41) (Cell Signaling); Flag (M2), and Bax(6A7) (Sigma-Aldrich); p85 α (Millipore); AKT1(C-20), GFP(B-2), Hsp60(N-20), c-Myc(9E10), RAR γ (C-19), RXR α (D20), RXR α (ΔN197), Bax(Δ21), c-FLIP, PARP(H-250) (Santa Cruz Biotechnology) were used. Sulindac and analogs were dissolved in dimethyl sulfoxide.

Ligand Binding Assay

RXR LBD was incubated with [³H]-9-*cis*-RA in the presence or absence of unlabeled 9-*cis*-RA or Sulindac. Bound [³H]-9-*cis*-RA was determined.

HPLC Analysis of Sulindac Binding

HEK293 cells stably transfected with vector containing receptor fused to C-terminal TAP fusion were treated with or without 100 μ M Sulindac for 3 hr. Receptor proteins purified with InterPlay Mammalian TAP System (Stratagene) were analyzed by HPLC using microsorb-mv 100-3 C18 100 \times 4.6 column

(Varian). Sulindac was detected with a photoarray detector (Waters model 2996). A standard solution of Sulindac was used to obtain the calibration curve.

Proteolytic Protection Assay

In vitro translated [³⁵S]-labeled RXR α -LBD or GST-RXR α was incubated with solvent or Sulindac (100 μ M) for 30 min and then digested with chymotrypsin.

NMR Binding Assays

¹⁹F NMR Spectra on Bruker Avance 500 NMR Spectrometer with a F-H-D-O probe were recorded with 1024 transients using a sweep width of 18797 Hz. The repetition time was 2 s.

Apoptosis Assays

Apoptosis assays followed those described elsewhere (Kolluri et al., 2008; Li et al., 2000; Lin et al., 2004).

Bax Oligomerization Assay

Lysates were incubated with BMH (Pierce) to crosslink the oligomerized Bax protein. For studying Bax oligomerization on mitochondria, heavy membrane fractions were lysed for immunoblotting with anti-Bax antibody (Bax 2D2).

siRNA

RXR α (M-003443-02) and RAR γ (M-003439-01) siRNA siGENOME SMARTpool, Caspase-8 siRNA ON-TARGETplus siRNA (J-003466-14), and siRNA Nonspecific Control IX (D-001206-09-05) were from DHARMACON.

Coimmunoprecipitation and Immunoblotting Assays

The assays followed those described previously (Li et al., 2000; Lin et al., 2004).

Confocal Microscopy

Cells seeded on chamber slides were fixed, permeabilized, and stained with appropriate antibodies (Li et al., 2000).

PI3K Kinase Assay

Cells transfected with Flag-p85 α and Myc-RXR α /Δ80 were serum-starved for 4 hr, pretreated with Sulindac for 0.5 hr, and TNF- α for 15 min. Cell lysates immunoprecipitated with anti-Myc antibody were incubated with phosphatidylinositol and [γ -³²P] ATP and subjected to thin-layer chromatography.

Stable Transfection and Soft Agar Assay

RXR α /1-134 cloned into pNTAP vector was stably transfected into HeLa cells, while GFP-RXR α /Δ80 was stably transfected into SW480 and HCT116 cells. Cells seeded at 5 \times 10³ cells/well (6-well plate) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 0.35% agarose with 0.5% bed agar for 12 days were stained with 0.005% crystal violet.

Colony Formation Assay

Cells were seeded in 6-well plate for 5 days, treated with compound in 0.5% serum medium for 3 days, and fixed with 4% paraformaldehyde. Colonies were stained with 0.1% crystal violet.

Human Tissues and Evaluation

Breast and liver tumor tissues and their surrounding tissues were obtained by surgical resection from cancer patients. Histological normal specimens, which were about at least 3~5 cm distant from the tumor nodule, were obtained from the corresponding patients. The study was approved by Xiamen University Institute for Biomedical Research Ethics Committee, and all of the patients gave informed consent. Tissues with primary hepatocellular carcinoma (HCC, n = 6) or breast cancer (n = 6) were collected for detecting the expression of RXR α using ΔN197 antibody. For immunohistochemistry analysis, tissue sections were incubated with the ΔN197 antibody (1:500) overnight at 4°C and detected with goat anti-rabbit-specific immunoglobulins (1:100). The slides were counterstained with hematoxylin.

COX Assays

COX Fluorescent Activity Assay, COX Fluorescent Inhibitor Screening Assay, and Prostaglandin E₂ Enzyme Immunoassay Kits were from Cayman

Chemical. Assays were performed in accordance with the manufacturer's protocols.

Animal Studies

Nude mice (BALB/c, 4–5 weeks old) were injected subcutaneously with 100 μ l cells (2×10^6). For drug treatment, mice ($n = 6$) were treated intraperitoneally after 7 days of transplantation with Corn oil, Sulindac (60 mg/kg), or K-80003 (60 mg/kg) once every other day (six injections). Body weight and tumor sizes were measured every 4 days. All manipulations involving live mice were approved by the Animal Care and Use Committee of Xiamen University.

Chemical Synthesis

Compound synthesis followed schemes in [Supplemental Experimental Procedures](#). ^1H NMR and ^{13}C NMR spectra, IR spectra, and mass spectra were recorded on Bruker 400 AV spectrometer, Nicolet Avatar 360 FT-IR spectrophotometer, and Bruker Dalton Esquire 3000 plus or a Finnigan Mat-LCQ (ESI direct injection), respectively. ^1H NMR spectra were registered in CDCl_3 or $\text{DMSO}-d_6$, and chemical shifts were expressed in parts per million (δ) relative to internal Me_4Si . Elemental analyses were performed using a Vario RL analyzer. Melting points were determined on an X-4 Micro-melting point apparatus and were uncorrected.

Statistical Analyses

Data were analyzed using an analysis of variance or Student's t test and were presented as the mean and standard error of the mean (\pm SEM).

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.ccr.2010.04.023](https://doi.org/10.1016/j.ccr.2010.04.023).

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