

# Identification of a New RXR $\alpha$ Antagonist Targeting the Coregulator-Binding Site

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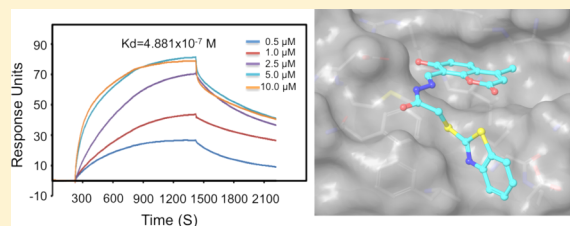
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## Supporting Information

**ABSTRACT:** Retinoid X receptor- $\alpha$  (RXR $\alpha$ ) is implicated in the regulation of many biological processes and also represents a unique intracellular target for pharmacologic interventions. Efforts on discovery of small molecules targeting RXR $\alpha$  have been primarily focused on the molecules that bind to its classical ligand-binding pocket (LBP). Here, we report the identification and characterization of a new RXR $\alpha$  transcriptional antagonist by using structure-based virtual screening. The new antagonist binds with submicromolar affinity to RXR $\alpha$  ( $K_d = 4.88 \times 10^{-7}$  M) and selectively inhibits RXR $\alpha$  transactivation. The compound does not bind to the LBP but to a hydrophobic groove on the surface of RXR $\alpha$ . The new compound also effectively suppresses AKT activation and promotes apoptosis of cancer cells in a RXR $\alpha$ -dependent manner by inhibiting tRXR $\alpha$  interaction with the p85 $\alpha$  subunit of PI3K. Thus, the compound represents a new RXR $\alpha$  modulator that regulates the nongenomic actions of RXR $\alpha$  by surface binding.

**KEYWORDS:** RXR $\alpha$ , coregulator-binding site, RXR $\alpha$  antagonist, nongenomic actions, virtual screening



Retinoid X receptor- $\alpha$  (RXR $\alpha$ ), a unique member of the nuclear receptor (NR) superfamily, plays an important role in many biological processes ranging from apoptosis, cell differentiation, and growth to lipid metabolism.<sup>1–4</sup> Altered expression and function of RXR $\alpha$  is implicated in the development of a number of diseases and cancer.<sup>1–4</sup> Thus, RXR $\alpha$  has been an attractive and important target for pharmacologic interventions and therapeutic applications.<sup>1–4</sup> The first identified natural RXR $\alpha$  ligand was the vitamin A derivative retinoid 9-*cis* retinoic acid (9-*cis*-RA).<sup>1–4</sup> Some fatty acids such as docosahexaenoic acid, oleic acid, and phytanic acid also serve as ligands for RXR $\alpha$ . 9-*cis*-RA and synthetic ligands (rexinoids) are effective in preventing tumorigenesis and treating inflammatory diseases. Targretin (bexarotene), a rexinoid, was approved for treating human cutaneous T-cell lymphoma.<sup>1,5</sup> RXR $\alpha$  acts primarily as a ligand-dependent transcription factor through forming homodimer with itself or heterodimer with other members of the NR family.

Structurally, RXR $\alpha$  is composed of three main functional domains: an N-terminal transcriptional activation function (AF-1) region, a DNA-binding domain and a ligand-binding domain (LBD).<sup>3,4</sup> The LBD possesses a canonical ligand-binding pocket (LBP), a transactivation function domain 2 (AF-2) composed of Helix 12 of the LBD, a coregulator binding surface, and a dimerization surface.<sup>3,4</sup> The ligand-dependent transcription regulation is predominately mediated through H12 that is highly mobile. The coregulator binding surface is a region where the binding sites of corepressor and the coactivator overlap. Canonical ligands bind to the LBP to mediate directly the transcriptional activity and so identifying

and optimizing molecules that bind to its classical LBP has been the focus of drug discovery efforts targeting RXR $\alpha$ . A large pool of RXR $\alpha$  ligands that bind to the LBP have been designed and reported.<sup>1,6</sup> However, there are key limitations of treatment with rexinoids including unwanted side effects such as rising of plasma triglyceride levels, suppression of the thyroid hormone axis, and induction of hepatomegaly.<sup>1,4–6</sup> The current challenge is to discover selective RXR $\alpha$  modulators with the desired pharmacological activities but lacking undesired side effects.<sup>1,4,6</sup> Therefore, targeting potential binding sites other than LBP could become a new paradigm for RXR $\alpha$ -based drug discovery. One of these potential binding sites is the coregulator-binding site.

Compounds that bind to the coregulator-binding site have been successfully demonstrated for other NRs, including estrogen receptor (ER),<sup>7</sup> androgen receptor,<sup>8,9</sup> vitamin D receptor, and thyroid receptor.<sup>10–12</sup> However, compounds that bind to the coregulator-binding site of RXR $\alpha$  have not been reported. Inspired by the successes reported for other NRs, we employed docking-based virtual screening (VS) to identify RXR $\alpha$  modulators targeting the coregulator-binding site. Here we report the identification and characterization of a small molecule that binds to the coregulator-binding site of RXR $\alpha$  to regulate its nongenomic actions.

Docking-based VS is a popular approach used in drug discovery where the structure of the target or target homologue

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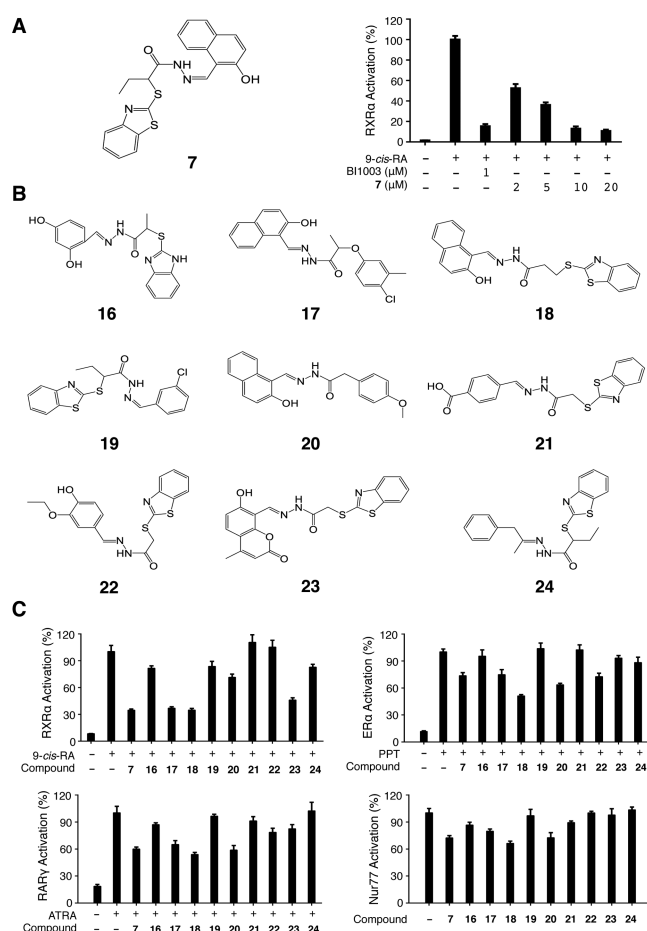
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is available.<sup>13</sup> Many crystal structures of RXR $\alpha$  LBD have been determined either in apo form or in complex with ligands or with both ligand and coregulator peptide,<sup>4,6</sup> offering an excellent opportunity to identify new RXR $\alpha$  binding compounds using docking-based VS. A chemical library of 200,000 compounds, commercially available from Specs (www.specs.net), was subjected to a Pipeline Pilot protocol<sup>14</sup> to filter out compounds that failed the Lipinski rules<sup>15</sup> and that are potentially reactive and contain undesired groups.<sup>16</sup> About 102,000 compounds left were then docked using Glide<sup>17</sup> to the coactivator binding site on RXR $\alpha$  using the structure of RXR $\alpha$  LBD in complex with CD3254 and a coactivator peptide (PDB code 3FUG).<sup>18</sup> Fourteen compounds (Figure S1A, Supporting Information) were selected for purchase and biological testing after visual evaluation of the first 300 compounds with the best docking score. Compound 7 (Figure 1A) showed the strongest antagonist activity (Figure S1B, Supporting Information) among these candidate compounds. Interestingly, part of 7 is similar to a recently reported androgen receptor inhibitor that is a diarylhydrazide and functions also via binding to the coactivator-binding site.<sup>8</sup> Similar to the classical RXR $\alpha$  antagonist BI1003,<sup>19</sup> 7 inhibited 9-*cis*-RA-induced RXR $\alpha$  transactivation in a dose-dependent manner (Figure 1A). So analogues of 7 were searched and selected for preliminary SAR studies. Nine analogues (Figure 1B) were available commercially and ordered and tested for their RXR $\alpha$  antagonist effect and their selectivity toward other nuclear receptors including ER, retinoic acid receptor- $\gamma$  (RAR $\gamma$ ), and Nur77 (Figure 1C), as well as glucocorticoid receptor (GR), PPAR $\gamma$ , and LXR $\alpha$  (Figure S2, Supporting Information). Among these compounds, 23 (ordered from www.specs.net under catalog number AE-848/34436002) showed an antagonist activity similar to 7, but demonstrated the best selectivity for RXR $\alpha$ . It significantly inhibited the activity of RXR $\alpha$ , but not ER, GR, and Nur77, and showed slight inhibition of transactivation of RAR $\alpha$ , RAR $\gamma$ , PPAR $\gamma$ , and LXR $\alpha$  (Figures 1C and S2, Supporting Information), which are known to heterodimerize with RXR $\alpha$ .<sup>20</sup> Further, compound 23 showed very little inhibitory effect on transactivation of RXR $\alpha$ /RAR $\alpha$  and RXR $\alpha$ /LXR $\alpha$  heterodimers (Figure S3, Supporting Information). We also tested the antagonist effect of 23 toward the RXR subtypes RXR $\gamma$  and RXR $\beta$ . The results showed that overall 23 is more selective toward RXR $\alpha$  though it demonstrated some inhibition of transactivation of RXR $\gamma$  and RXR $\beta$  (Figure S4, Supporting Information). Thus, 23 is a new RXR $\alpha$ -selective antagonist.

A dose-dependent study showed that 23 could inhibit 9-*cis*-RA-induced RXR $\alpha$  transactivation with an IC<sub>50</sub> of 2.45  $\mu$ M (Figure 2A). By using Biacore's Surface plasmon resonance (SPR) technology, we found that 23 could bind to RXR $\alpha$  with a K<sub>d</sub> of 4.88  $\times 10^{-7}$  M (Figure 2B). The binding of 23 to RXR $\alpha$  is unlikely due to its binding to the RXR $\alpha$  LBP, as it failed to compete with the binding of [<sup>3</sup>H]9-*cis*-RA to the RXR $\alpha$  LBP. By contrast, 9-*cis*-RA competed well with [<sup>3</sup>H]9-*cis*-RA for binding to RXR $\alpha$  (Figure 2C).

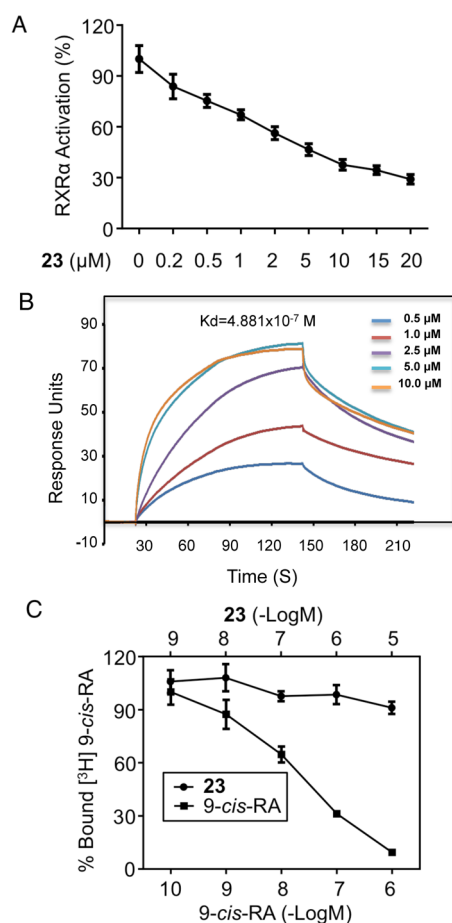
To confirm that 23 binds to the coregulator-binding site on the surface of RXR $\alpha$ , mutagenesis in the site was carried out to validate its importance in the antagonist effect of 23. Mutations in the coactivator-binding site might impact not only the binding of 23 but also the binding of coactivator, which would preclude our evaluation by the reporter assay that depends on the binding of the coactivator. However, comparison of the interactions of 23 and coactivator to RXR $\alpha$  identified that



**Figure 1.** Identification of RXR $\alpha$ -selective antagonist 23. (A) Compound 7 and its antagonist effect on RXR $\alpha$  transactivation. MCF-7 cells cotransfected with the reporter plasmids pG5-Luc and pBind-RXR $\alpha$ -LBD were treated with 9-*cis*-RA ( $10^{-7}$  M) alone or together with 7 or BI1003 for 18 h. Luciferase reporter activities were measured by using the Dual-Luciferase Reporter Assay System. Transfection efficiency was normalized to Renilla luciferase activity. (B) Compounds related to 7 identified by computational approach. (C) Antagonist effect of compound 7 and analogues. MCF-7 cells cotransfected with pG5-Luc and pBind-RXR $\alpha$ -LBD, pBind-ER-LBD, pBind-RAR $\gamma$ -LBD, or pBind-Nur77-LBD were treated, respectively, with 9-*cis*-RA ( $10^{-7}$  M), propyl pyrazole triol (PPT) (10  $\mu$ M), and all-*trans*-RA ( $10^{-7}$  M), in the presence or absence of compound (10  $\mu$ M) for 18 h. Reporter activities were measured as described above. Data shown are mean  $\pm$  SD.

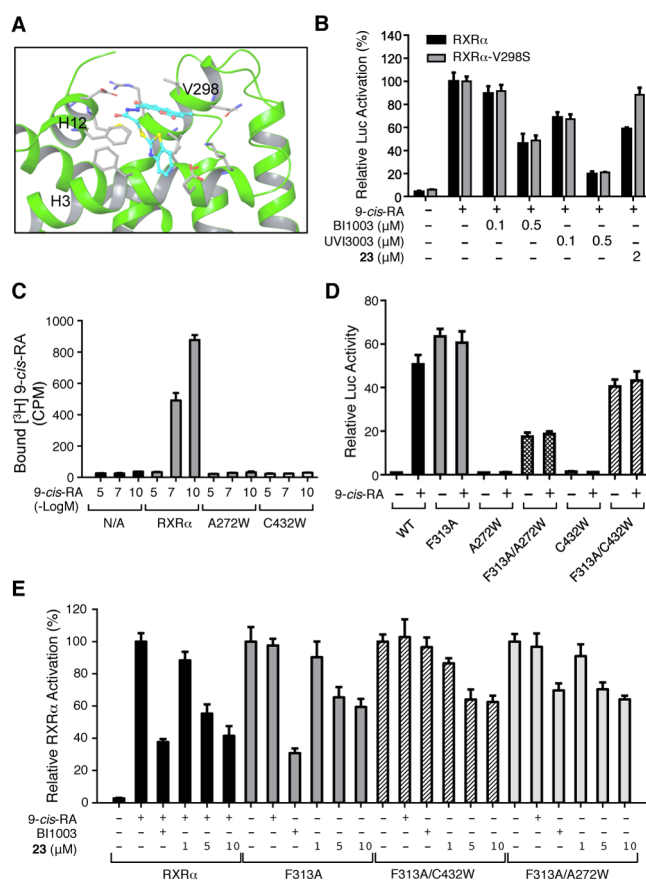
Val298 in the Helix 4 of RXR $\alpha$  is critical for the binding of 23 but not the coactivator (Figure 3A). Thus, Val298 was mutated to Ser, and the resulting mutant, RXR $\alpha$ -V298S, was subjected to evaluation by the reporter assay. 9-*cis*-RA was able to activate RXR $\alpha$ -V298S, similar to its effect on RXR $\alpha$  (Figures 3B and S5, Supporting Information), suggesting that mutation of Val298 did not impair the ability of RXR $\alpha$  to bind to 9-*cis*-RA and to recruit the coactivator. Classical antagonists, such as BI1003<sup>19</sup> and UVI3003,<sup>21</sup> also potently inhibited the 9-*cis*-RA-induced RXR $\alpha$ -V298S activity. By contrast, the antagonist effect of 23 was largely reduced as compared to its inhibitory effect on RXR $\alpha$ . Thus, Val298 is crucial for the binding of 23 but not classical RXR $\alpha$  ligands and coregulators.

To further exclude the possibility that 23 binds to the LBP of RXR $\alpha$ , we designed and constructed RXR $\alpha$  mutants with its LBP blocked. Computational modeling suggested that sub-



**Figure 2.** RXR $\alpha$  antagonist 23 binds RXR $\alpha$  at a site other than LBP. (A) Dose-dependent effect of 23 on inhibiting RXR $\alpha$  transactivation. HEK293T cells cotransfected with pG5-Luc and pBind-RXR $\alpha$ -LBD were treated with 9-*cis*-RA (10<sup>-7</sup> M) alone or together with the indicated concentration of 23 for 18 h. (B) SPR assay. Gradient concentrations of 23 were injected through flow cells immobilized with RXR $\alpha$ -LBD. The kinetic profiles are shown. The dissociation constant ( $K_d$ ) of the 23/RXR $\alpha$ -LBD complex was calculated to be 4.881 × 10<sup>-7</sup> M. (C) Compound 23 fails to compete with 9-*cis*-RA for binding to RXR $\alpha$  LBP. The bacterially expressed His-tagged RXR $\alpha$ -LBD was incubated with 7.5 nM [<sup>3</sup>H]-9-*cis*-RA in the presence or absence of the indicated concentrations of 9-*cis*-RA or 23. The RXR $\alpha$  LBD was captured by nickel-coated beads. Bound [<sup>3</sup>H]-9-*cis*-RA was quantitated by liquid scintillation counting. Data shown are mean ± SD.

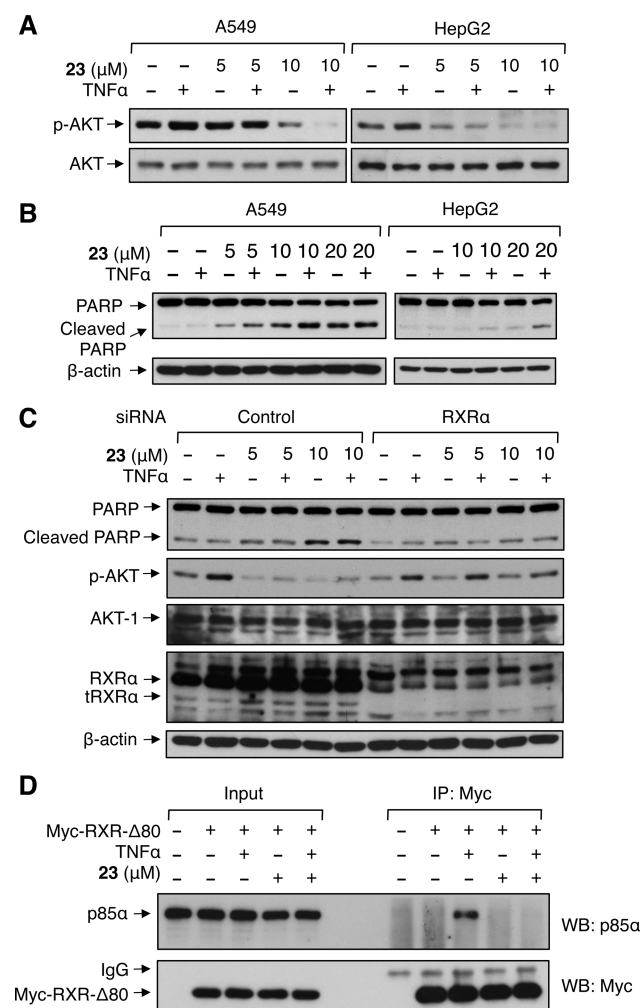
stitution of Cys432 and Ala272 in the LBP with a bulky amino acid such as Trp could block the passage of a ligand to the LBP. Indeed, RXR $\alpha$ /C432W and RXR $\alpha$ /A272W failed to show any binding to [<sup>3</sup>H]-9-*cis*-RA (Figure 3C), even though the mutant proteins were well expressed (Figure S6, Supporting Information). In contrast, RXR $\alpha$  bound well to [<sup>3</sup>H]-9-*cis*-RA, which was competed away by unlabeled 9-*cis*-RA. F313A mutation is known to shift RXR $\alpha$  from an apo-receptor to a constitutively active form.<sup>22</sup> In order to utilize LBP-blocked mutants to evaluate the antagonist effect of 23, Phe313 in RXR $\alpha$ /C432W and RXR $\alpha$ /A272W was substituted with Ala. As reported,<sup>22</sup> RXR $\alpha$ -F313A is constitutively active in the absence of 9-*cis*-RA. Similar to RXR $\alpha$ -F313A, both RXR $\alpha$ -C432W/F313A and RXR $\alpha$ -A272W/F313A mutants showed strong constitutive transcriptional activity (Figure 3D), making them ideal mutants to evaluate the LBP-independent antagonist



**Figure 3.** Coregulator-binding site of RXR $\alpha$  but not its LBP is critical for the antagonist effect of 23. (A) Val298 is critical for 23 binding revealed by modeling. (B) Mutation of Val298 impairs the antagonist effect of 23. HEK-293T cells cotransfected with pG5-Luc and pBind-RXR $\alpha$ /V298S were treated with the indicated compounds for 18 h. (C) Substitution of Cys432 and Ala272 in RXR $\alpha$  with Trp impairs 9-*cis*-RA binding. Lysates of HEK-293T cells transfected with RXR $\alpha$ , RXR $\alpha$ -C432W, or RXR $\alpha$ /A272W were incubated with 7.5 nM [<sup>3</sup>H]-9-*cis*-RA in the presence or absence of unlabeled 9-*cis*-RA. The Myc-RXR $\alpha$  was captured by hydroxylapatite. Bound [<sup>3</sup>H]-9-*cis*-RA was quantitated. (D,E) Transactivation of RXR $\alpha$  LBP mutants. HEK-293T cells cotransfected with pG5-Luc and the indicated RXR $\alpha$  or mutant expression vector were treated with 9-*cis*-RA (10<sup>-7</sup> M), BI1003 (1 μM), or the indicated concentration of 23 for 18 h. Data shown are mean ± SD.

effect of 23. Treatment of cells with 23 could effectively inhibit their constitutive activity in a dose-dependent manner, similar to its effect on RXR $\alpha$  (Figure 3E). By contrast, BI1003, which showed potent inhibitory effect on the transactivation of RXR $\alpha$  and RXR $\alpha$ -F313A by binding to their LBP, had much reduced inhibitory effect on the constitutive transactivation of both mutants. Thus, the blockage of the LBP of RXR $\alpha$ , which affects the activity of 9-*cis*-RA and the classical RXR $\alpha$  antagonist BI1003, has no effect on the antagonist activity of 23, further confirming unique RXR $\alpha$  binding activity of 23.

We recently reported that modulation of RXR $\alpha$  activity by certain RXR $\alpha$  ligands, such as Sulindac and analogues, could inhibit AKT activation in cancer cells.<sup>23,24</sup> We therefore asked whether the LBP-independent binding of 23 could suppress AKT activation. A549 lung cancer and HepG2 liver cancer cells were treated with 23, and the activation of AKT was examined. As shown in Figure 4A, AKT activation in these cells was inhibited by 23 dose-dependently, with apparent inhibition



**Figure 4.** Biological evaluation of **23**. (A,B) Inhibition of AKT activation (A) and induction of apoptosis (B). Cells were pretreated with **23** for 24 h before being exposed to TNF $\alpha$  (20 ng/mL) for an additional 30 min. Lysates prepared were analyzed by Western blotting for AKT activation (A) or PARP cleavage (B). (C) RXR $\alpha$ -dependent effects of **23**. A549 cells transfected with RXR $\alpha$  siRNA or control siRNA for 48 h were treated with **23** for 24 h before being exposed to TNF $\alpha$  (20 ng/mL) for an additional 30 min. Lysates prepared were analyzed by Western blotting. (D) Inhibition of p85 $\alpha$  interaction with tRXR $\alpha$  by **23**. A549 cells transfected with myc-RXR $\alpha$ - $\Delta$ 80 expression vector were analyzed for their interaction with endogenous p85 $\alpha$  by coimmunoprecipitation assay using anti-Myc antibody. Immunoprecipitates were analyzed by Western blotting for the presence of p85 $\alpha$  and Myc-RXR $\alpha$ - $\Delta$ 80. One of three to five similar experiments is shown.

observed when 5–10  $\mu$ M of **23** was used. Since TNF $\alpha$  could induce RXR $\alpha$ -dependent AKT activation,<sup>24</sup> cells were also treated with TNF $\alpha$ , and its activation of AKT in the absence or presence of **23** was examined. Treatment of cells with TNF $\alpha$  enhanced AKT activation, which was also suppressed by **23** (Figure 4A). Similar results were obtained in PC-3 prostate cancer cells (Figure S7, Supporting Information) and other cancer cells including colon cancer and pancreatic cancer cells (data not shown). We also evaluated the apoptotic effect of **23** in cancer cells. A549 and HepG2 cells were treated with **23** in the absence or presence of TNF $\alpha$ , and the cleavage of PARP, an indication of apoptosis in cancer cells, was examined by immunoblotting (Figure 4B). Treatment of cancer cells with **23**

induced PARP cleavage, which was further enhanced by TNF $\alpha$  treatment. Such apoptosis induction by **23** correlated well with its inhibition of AKT activation, suggesting that AKT inhibition might play a role in its induction of apoptosis.

To determine whether the expression of RXR $\alpha$  plays a role in the inhibition of AKT activation and the induction of apoptosis by **23**, A549 cells were transfected with RXR $\alpha$  siRNA and evaluated for its effect on the role of **23** in AKT activation and apoptosis induction. Transfection of RXR $\alpha$  siRNA reduced the levels of RXR $\alpha$  and its truncated version, tRXR $\alpha$ ,<sup>24</sup> and diminished the effect of **23** on inducing PARP cleavage and inhibiting AKT activation (Figure 4C). Furthermore, the inhibitory effect of compound **23** on the growth of cancer cells can be significantly enhanced by RXR $\alpha$  (Figure S8, Supporting Information). These results demonstrate that RXR $\alpha$  plays a crucial role in mediating the biological effect of **23** on cell death.

We next determined whether **23** could affect tRXR $\alpha$  interaction with the p85 $\alpha$  regulatory subunit of PI3K, an event known to activate PI3K/AKT.<sup>24</sup> A549 cells were transfected with Myc-tagged RXR $\alpha$ - $\Delta$ 80, a mutant that mimics tRXR $\alpha$ ,<sup>24</sup> and treated with or without TNF $\alpha$  and/or **23**. Coimmunoprecipitation assays using anti-Myc antibody showed that p85 $\alpha$  was coimmunoprecipitated together with Myc-RXR $\alpha$ - $\Delta$ 80 in cells treated with TNF $\alpha$  (Figure 4D), demonstrating their interaction. However, when cells were cotreated with **23**, TNF $\alpha$ -induced interaction of Myc-RXR $\alpha$ - $\Delta$ 80 with p85 $\alpha$  was almost completely inhibited. Thus, **23** might induce apoptosis by suppressing AKT activation through its inhibition of tRXR $\alpha$  interaction with p85 $\alpha$ .

We report here through virtual screening our identification of a unique RXR $\alpha$  antagonist, **23**, which modulates RXR $\alpha$  activities through LBP-independent binding. Several lines of evidence showed that **23** acts through its binding to the surface of RXR $\alpha$ . First, despite its high affinity for binding to RXR $\alpha$  revealed by SPR study, **23** failed to compete with 9-*cis*-RA for binding to the LBP of RXR $\alpha$  revealed by the classical ligand competition assay (Figure 2C). Second, mutation of Val298, a critical amino acid residue in the hydrophobic groove on the surface of RXR $\alpha$ , impaired the antagonist effect of **23** (Figure 3A). Third, **23** could effectively inhibit the transcriptional activity of RXR $\alpha$  mutants with impaired LBP (Figure 3D). To our knowledge, compounds that bind to the surface site of RXR $\alpha$  have not been reported. Thus, **23** represents the first small molecule capable of functionally binding to the surface site of RXR $\alpha$  with submicromolar affinity.

Recent studies demonstrated that RXR $\alpha$  could crosstalk extensively with signal transduction pathways through its interaction with various signaling proteins,<sup>24</sup> which are likely mediated through its surface binding sites. When the effect of **23** on the PI3K/AKT signaling was examined, we found that it could suppress basal and TNF $\alpha$ -induced AKT activation (Figure 4A) and induce apoptosis (Figure 4B) in cancer cells in a RXR $\alpha$ -dependent manner (Figure 4C). Our coimmunoprecipitation assays demonstrated that **23** could block tRXR $\alpha$  interaction with p85 $\alpha$  (Figure 4D). Thus, the unique surface binding of **23** could interfere with the binding of some RXR $\alpha$ -interacting proteins, providing an opportunity to regulate RXR $\alpha$  activities by targeting the coregulator-binding sites. The report of the structure of estrogen receptor- $\beta$  with a second molecule of 4-hydroxytamoxifen bound in its coactivator-binding surface provides insight into the possible pharmacological effects of the drug through its binding to the surface site

on NR.<sup>25</sup> Thus, our demonstrations that **23** could bind to the coregulator-binding site of RXR $\alpha$  and regulate the PI3K/AKT signaling pathway and apoptosis in cancer cells provide a new approach to target a functionally important surface site on RXR $\alpha$  that could represent a new strategy to tackle the specificity issue.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Supplemental Figures 1 to 8 and detailed experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

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**Ying Su**, Ph.D., is a computational chemist with over 15 years of experience with computer-aided drug design and Cheminformatics. Ying Su received her Ph.D. from the University of California, San Diego. After a postdoctoral appointment at the Scripps Research Institute, she worked for several local biotech companies. She joined the Sanford-Burnham Medical Research Institute in 2005 to build and lead a HTS informatics and CADD group. Dr. Su is coauthor of over 40 peer-reviewed scientific publications.

## ■ ABBREVIATIONS

RXR $\alpha$ , retinoid X receptor alpha; tRXR $\alpha$ , N-terminally truncated RXR $\alpha$ ; PI3K, phosphatidylinositol-3-OH kinase; NR, nuclear receptor; LBD, ligand-binding domain; LBP, ligand-binding pocket; PPT, propyl pyrazole triol; TNF $\alpha$ , tumor necrosis factor- $\alpha$

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