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A novel steroidal inhibitor of estrogen-related receptor α (ERRα)

Sarah J. Duellman [a,*], Joy M. Calaoagan [a], Barbara G. Sato [a], Richard Fine [b], Boris Klebansky [b], Wan-Ru Chao [a], Peter Hobbs [a], Nathan Collins [a], Lidia Sambucetti [a], Keith R. Laderoute [a]

[a] Biosciences Division, SRI International, 333 Ravenswood Ave., Menlo Park, CA 94025, United States
[b] BioComputing Group, Inc., Oradell, NJ 07649, United States

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ABSTRACT

The orphan nuclear receptor estrogen-related receptor α (ERRα) has been implicated in the development of various human malignancies, including breast, prostate, ovary, and colon cancer. ERRα, bound to a co-activator protein (e.g., peroxisome proliferator receptor γ co-activator-1α, PGC-1α), regulates cellular energy metabolism by activating transcription of genes involved in various metabolic processes, such as mitochondrial genesis, oxidative phosphorylation, and fatty acid oxidation. Accumulating evidence suggests that ERRα is a novel target for solid tumor therapy, conceivably through effects on the regulation of tumor cell energy metabolism associated with energy stress within solid tumor microenvironments. This report describes a novel steroidal antiestrogen (SR16388) that binds selectively to ERRα, but not to ERRβ or ERRγ, as determined using a time-resolved fluorescence resonance energy transfer assay. SR16388 potently inhibits ERRα's transcriptional activity in reporter gene assays, and prevents endogenous PGC-1α and ERRα from being recruited to the promoters or enhancers of target genes. Representative *in vivo* results show that SR16388 inhibited the growth of human prostate tumor xenografts in nude mice as a single agent at 30 mg/kg given once daily and 100 mg/kg given once weekly. In a combination study, SR16388 (10 mg/kg, once daily) and paclitaxel (7.5 mg/kg, twice weekly) inhibited the growth of prostate tumor xenografts in nude mice by 61% compared to untreated xenograft tumors. SR16388 also inhibited the proliferation of diverse human tumor cell lines after a 24-h exposure to the compound. SR16388 thus has utility both as an experimental antitumor agent and as a chemical probe of ERRα biology.

1. Introduction

Estrogen-related receptor α (ERRα) belongs to the nuclear receptor (NR) superfamily, which is a group of 48 structurally related, ligand-activated transcription factors [1–3]. The ERR family (the NR3B subgroup) consists of ERRα, -β, and -γ [3]. The ERRs are classified as orphan receptors because they do not bind any known natural or endogenous small-molecule ligands [2,3]. For example, although the ERRs are highly similar at both the primary sequence and structural levels to the classical estrogen receptors (ERα and -β), the ERRs do not bind estrogen (e.g., 17β-estradiol; E2). Substantial evidence supports a physiological model of ERR function in which the receptors regulate energy metabolism by directly interacting with certain transcriptional co-regulators, including peroxisome-proliferator activated receptor γ coactivator-1α (PGC-1α) and PGC-1β, steroid receptor co-activators, and the co-repressor nuclear receptor interacting protein 140 (RIP140) [2,3]. Co-activators of ERRs (e.g., PGC-1α) positively regulate fundamental metabolic processes, including mitochondrial genesis, oxidative phosphorylation, fatty acid oxidation, and generation of reactive oxygen species. Co-repressors, such as RIP140, that bind to ERRs compete with ERR co-activators to negatively regulate ERR-dependent gene expression. Organism-wide expression profiling of the ERR isoforms determined that ERRα is widely distributed, with significant protein expression in most adult tissues [4]. In general, ERRβ and -γ show restricted expression patterns and are found at lower levels compared to ERRα. Knockout studies of the ERR family members have revealed that each receptor has tissue- and function-specific metabolic phenotypes that are important for adaptation to energy stress at the whole body level. The knockout studies also indicate limited *in vivo* compensation among the ERR family members [1–3,5,6].

The pleiotropic effect of ERR activity on energy metabolism has generated interest in the possibility that specific ERRs could be

Abbreviations: 4-OHT, 4-hydroxytamoxifen; ChIP, chromatin immunoprecipitation; ERR, estrogen-related receptor; ER, estrogen receptor; ERRE, estrogen-related receptor response element; ERE, estrogen response element; EC_{50}, one-half maximal effective concentration; E2, 17β-estradiol; FBS, fetal bovine serum; LBD, ligand binding domain; NR, nuclear receptor; PGC, peroxisome-proliferator activated receptor coactivator; PDB, protein data bank; RLU, relative light units; TR-FRET, time-resolved fluorescence resonance energy transfer.

* Corresponding author. Tel.: +1 650 859 3505; fax: +1 650 859 5816.
E-mail address: sarah.duellman@sri.com (S.J. Duellman).

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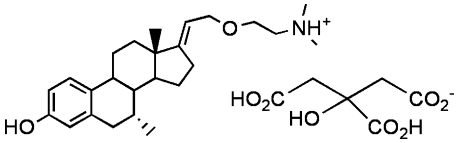

Fig. 1. Structure of SR16388. SR16388 (21-[2-(N,N-dimethylamino)ethyl]oxy-7a-methyl-19-norpregna-1,3,5(10),17(20)-tetraen-3-ol citrate salt) is an orally active compound that belongs to the antiestrogen class of therapeutic agents. SR16388 is a potent and selective inhibitor of human ERRα, which does not bind estrogen (E2).

targets for the discovery of new therapies for diseases such as type 2 diabetes, progressive heart failure, osteoporosis, and cancer [2,3]. Synthetic small-molecule ligands have been identified for the ERR family, such as diethylstilbestrol, and for ERRβ and -γ, such as 4-hydroxytamoxifen (4-OHT); the 4-OHT derivative GSK5182; and the phenolic acyl hydrazones DY131 and GSK5182 [2]. Selective ligands (inverse agonists) of ERRα have also been reported, including the thiadiazoleacrylamide XCT790 [7], N-[(2Z)-3-(4,5-dihydro-1,3-thiazol-2-yl)-1,3-thiazolidin-2-yl idene]-5H dibenzo[a,-d][7]annulen-5-amine [8], and cyclohexylmethyl-(1-*p*-tolyl-1*H*-indol-3-ylmethyl)-amine [9].

Here we report a novel, purely steroidal antiestrogen, designated SR16388 (21-[2-(N,N-dimethylamino)ethyl]oxy-7a-methyl-19-norpregna-1,3,5(10),17(20)-tetraen-3-ol; Fig. 1) with strong selectivity for binding to ERRα over both ERRβ and ERRγ. Since SR16388 is essentially an estrogen molecule with a basic side chain attached at position C17, the affinity of SR16388 for ERRα is striking considering the lack of significant E2 binding to this receptor [5].

Representative *in vitro* and *in vivo* results provided below show that SR16388 inhibits the proliferation of diverse human tumor cell lines and substantially delays the growth of human tumor xenografts—independently of estrogen receptor status. Mechanistically, we show that SR16388 inhibits the binding of ERRα to a critical peptide from its co-activator PGC-1α in a cell-free assay, and strongly inhibits ERRα's ability to occupy promoter or enhancer elements and activate transcription from selected target genes in human cancer cells. We also provide a computational model of the ligand-binding domain (LBD) of human ERRα containing SR16388, which suggests how this antiestrogen could act as an inhibitor of cellular ERRα activity.

2. Materials and methods

2.1. Cell culture and reagents

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. PC3 human prostate cancer cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). MCF7 breast cancer cells were grown in Dulbecco modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% FBS and 20% F12 nutrient mixture (Invitrogen). All cells were grown at 37 °C in a humidified 5% CO_2 atmosphere. Both cell lines were obtained from American Type Culture Collection (Manassas, VA).

2.2. Cell viability assay

The alamarBlue reagent (Invitrogen) was used to determine the effect of SR16388 on the proliferation and viability of cancer cell lines. Cells were plated in 96-well plates at 1000 cells per well, and 6-point serial dilution dose–response curves were obtained. Briefly, cells were incubated with SR16388 at 37 °C for 24 h, and then the medium was replaced with fresh medium. On day 4 after treatment, alamarBlue was added to the cells, and the incubation was continued for 3 h. Fluorescence from the reduced reagent was measured using a BioTek Synergy 2 fluorescence plate reader (Winooski, VT) with excitation at 530 nm and emission at 590 nm (30 nm bandwidth).

2.3. TR-FRET assay

The LanthaScreen time-resolved fluorescence resonance energy transfer (TR-FRET) co-activator assay series (Invitrogen) was used to analyze the interaction of the LBD of ERRα, -β, or -γ, and ERα, or -β with a PGC-1α peptide according to the manufacturer's instructions. A 12-point 1:3 serial dilution dose–response curve was obtained by incubating the binding reaction in the presence of compounds for 1 h at room temperature. TR-FRET was measured with an Analyst HT fluorescence plate reader (Molecular Devices, Sunnyvale, CA) with excitation at 360 nm and emission at 495 nm (10 nm bandwidth) and 520 nm (25 nm bandwidth), 100 μs lag time, and 200 μs integration time. To determine the one-half maximal effective concentrations (EC_{50}), nonlinear regression, sigmoidal dose–response (variable slope) curves were calculated using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA).

2.4. Luciferase reporter assay

The dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine ERRα's transcriptional activity in the presence of various compounds. Cells were plated in 6-well plates and transiently transfected with 1 μg pERRE(5x)TAffLuc [10] (which contains an estrogen-related receptor response element, ERRE), 20 ng pTA-srLuc [10], 450 ng pcDNA3.1-hERRα1 [11], and 6 μg pcDNA3/HA-hPGC-1α [12] using the TurboFectin 8.0 transfection reagent (OriGene, Rockville, MD) according to the manufacturer's instructions. After 24 h, transfected cells were treated with DMSO, 5 μM ICI 182,780 (Fulvestrant), or 5 μM SR16388. At 48 h posttransfection, cells were washed with PBS and lysed according to the dual-luciferase reporter assay instructions. A total of 20 μl of each lysate was analyzed for luciferase activity; relative light units (RLU) were normalized for transfection efficiency by comparison to the *Renilla* luciferase signal. Statistical analysis was done using the unpaired Student's *t*-test.

2.5. Chromatin immunoprecipitation assay

MCF7 cells were treated with DMSO or 5 μM SR16388 for 6 or 24 h. The cells were fixed, and a chromatin immunoprecipitation (ChIP) assay was performed using a ChIP-IT Express kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. The following primary antibodies were used for the ChIP assays: anti-PGC-1α (H-300, Santa Cruz Biotechnology, Santa Cruz, CA); anti-ERRα (07-662, Millipore, Temecula, CA); and a negative control IgG antibody (Active Motif). Primers used for quantitative PCR (qPCR) were the following: VEGF, forward 5′ CACCAGCTCACCCTGGTATT and reverse 5′ ACTTCCCTCTCCCTGCTCTC; ERRα, forward 5′ CTTCCCCGTGACCTTCATT and reverse 5′ AGCCGACTTAAAACATGCAATA; Acadm, forward 5′ AACGCAGAAAACCAAACCAG and reverse 5′ CATGCTCCGTGACCCTTG. Three independent replicate measurements were acquired for each experiment, and each qPCR measurement was done in duplicate. Results were reported as fold enrichment, which represents the difference in signal relative to that for the IgG negative control antibody. Statistical analysis was performed with the Wilcoxon signed rank test.

2.6. PC3 prostate cancer xenografts

PC3 human prostate cancer cells (3×10^6) were suspended in 100 μl of a 1:1 mixture of cell culture medium and Matrigel and

implanted subcutaneously in the right flank region of nude mice (BALB/c [nu/nu], Taconic, Germantown, NY). Mice were monitored for tumor growth daily after cell implantation, and when tumor volumes reached 80–100 mm^3, mice were randomized into appropriate groups of 10 mice each. Treatment with SR16388 alone or in combination with paclitaxel was initiated on the day after randomization. SR16388 was orally administered once daily at 10 mg/kg or 30 mg/kg, and once weekly at 100 mg/kg. For the combination study, SR16388 was orally administered once daily at 10 mg/kg, and paclitaxel was administered twice weekly by intraperitoneal injection at 7.5 mg/kg. Treatments were continued for 4 weeks. During the study, tumor volumes were measured twice weekly and body weights once weekly. Tumor volumes were measured using the formula $V = L \times W \times H \times \pi/6$, where L and W represent the longer and shorter tumor diameters, respectively, and H represents tumor depth. Tumor growth rates were evaluated statistically between the control and each treatment group using the Student–Newman–Keuls method. No significant compound-related effects on body weight or any other signs of overt toxicity were observed in any of the groups. All procedures were approved by the SRI Institutional Animal Care and Use Committee.

2.7. Molecular modeling

The primary structure of human ERRα that was used for the current study was Protein Data Bank (PDB) entry 2PJL, which reveals an inverse agonist bound to the ERRα LBD [9]. In placing SR16388 in this structure, we were guided by two other crystal structures of related compounds bound to the related receptor ERα. The fused-ring portion of SR16388 is highly similar to that of E2, for which a crystal structure bound to ERα (PDB entry 1ERE) is known [13]. The tail portion of SR16388 resembles that of 4-OHT, for which a crystal structure with the compound bound to ERα (PDB entry 1ERT) is also known [14]. Structural superposition of 1ERE and 1ERT onto 2PJL yielded a compelling template for the construction of the model of bound SR16388, with the 4-OHT tail spatially occupying the region where the SR16388 tail would be placed if it were attached to the homologous fused-ring portion of E2. The model was built by superimposing the fused-ring portion of SR16388 onto that of E2, and subsequently orienting its tail portion to follow the tail of 4-OHT in the superimposed active sites of 1ERE, 1ERT, and 2PLJ. The resulting model was then subjected to energy minimization using the program Gromacs [15].

Table 1
Alamar Blue proliferation/viability toxicity assays were used to determine the IC_{50} of SR16388 in various cancer cell lines after a 24-h exposure to the compound.

Cell line	IC_{50} (μM)
PC3 androgen-independent prostate cancer	3.0 ± 0.1
LNCaP C4-2B androgen receptor-positive, androgen-independent prostate cancer	0.9 ± 0.1
MDA-MB-231 ER-negative breast cancer	6.2 ± 0.1
MCF7 ER-positive breast cancer	2.2 ± 0.1
Panc-1 pancreatic cancer	2.8 ± 0.1
MiaPaCa2 pancreatic cancer	1.3 ± 0.1
RCC4 renal cell carcinoma	2.9 ± 0.1
RCC4/VHL renal cell carcinoma	0.5 ± 0.1
786-0 renal cell carcinoma	1.1 ± 0.1
786-0/VHL renal cell carcinoma	0.7 ± 0.1
A498 renal call carcinoma	1.0 ± 0.1
A498/VHL renal cell carcinoma	0.6 ± 0.1

3. Results

3.1. SR16388 inhibits human tumor cell proliferation

We performed cell proliferation/viability assays using alamar-Blue to determine the effect of SR16388 (Fig. 1) on the proliferation of a panel of human tumor cell lines (Table 1). SR16388 showed strong antiproliferative effect toward these cell lines, which included both ER-positive and ER-negative cells. Although

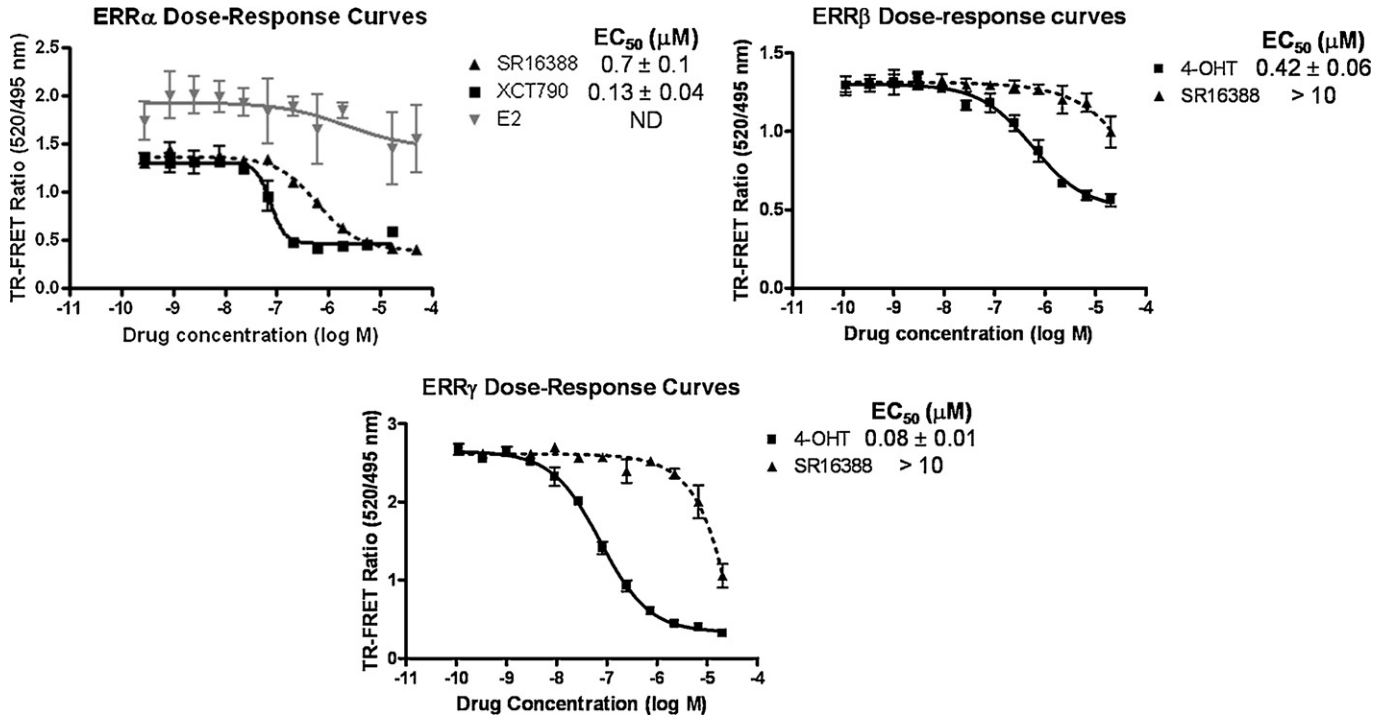

Fig. 2. SR16388 disrupts the binding of the ERRα-LBD, but not the ERRβ or -γ LBDs, to a PGC-1α peptide. A TR-FRET assay was used to determine whether various compounds could inhibit the interaction of an ERRα, -β, or -γ LBD-glutathione-S-transferase fusion protein with a co-activator peptide from PGC-1α (aa 135–153, EAEEPSLLKKLLLAPANTQ). Known inhibitors of the ERRs were used as positive controls: XCT790 for ERRα, and 4-hydroxytamoxifen (4-OHT) for ERRβ and -γ. E2 was included in the ERRα TR-FRET assay to confirm that E2 does not bind ERRα. ND: not determined.

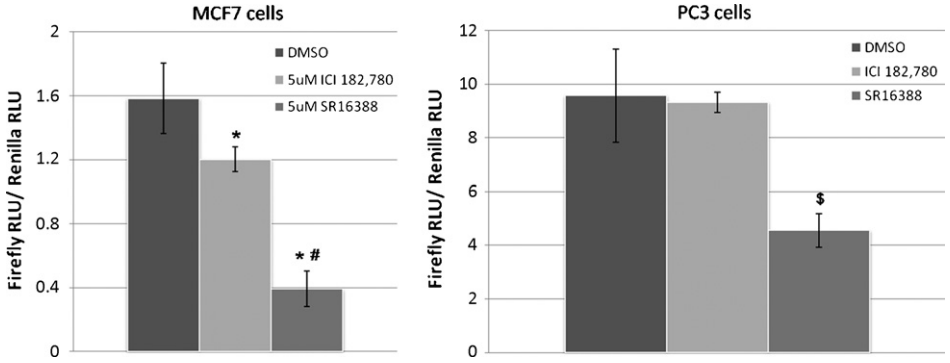

Fig. 3. The ability of ERRα to activate ERRE-directed transcription was analyzed in the presence of ICI 182,780 and SR16388. A dual-luciferase assay was performed using MCF7 and PC3 cells that were transiently co-transfected with ERRα and PGC-1α expression plasmids. $^{*}p$-Value ≤0.01 compared to DMSO. $^{\#}p$-Value ≤0.02 compared to ICI treatment. $^{\$}p$-Value ≤0.04 compared to DMSO. Statistical analysis was performed using the unpaired Student's *t*-test.

SR16388 is an antiestrogen [manuscript submitted], these results demonstrate that SR16388 has cytotoxicity toward diverse human cancer cells that does not depend on the presence of the ERs. Indeed, it is unlikely that all the tumor cell types in this diverse panel require the ERs for survival (e.g., ER-negative MDA-MB-231 cells [16] are sensitive to SR16388). Furthermore, an NCI-60 Developmental Therapeutics Program Human Tumor Cell Line screen showed that SR16388 was cytotoxic to most cell lines in the screen (data not shown). In this screen, all sensitive tumor cell lines showed a percentage of growth between −50 and −100 in response to treatment with SR16388 (e.g., 10 μM for 48 h) relative to untreated cells, which is consistent with a cytotoxic endpoint in this screen [17]. In part because ERs were not required for the cytotoxicity of SR16388 in the alamarBlue assay, we investigated whether the compound could target the closely related ERR orphan nuclear receptors. As mentioned above, the ERRs (ERRα, -β, and -γ) do not bind any known endogenous ligand, including estrogen.

3.2. SR16388 selectively inhibits the interaction of ERRα with PGC-1α

To investigate the effect of SR16388 on the ability of ERRα, -β, and -γ to regulate transcription, we first used a TR-FRET assay that measures the direct interaction between the LBD of each ERR and a target peptide from PGC-1α, which is an established co-activator of both the classic ERs and the ERRs (reviewed in [18]). SR16388 inhibited the interaction of ERRα with the PGC-1α peptide (EC_{50} = 0.7 ± 0.1 μM), but did not comparably inhibit the interaction of ERRβ (EC_{50} > 10 μM) or ERRγ (EC_{50} > 10 μM) with the peptide (Fig. 2). The ERRα inverse agonist XCT790 had a lower EC_{50} than SR16388 in the ERRα TR-FRET cell-free assay; however, as discussed below, XCT790 was not as effective as SR16388 in cell-based assays of ERRα activity. Because SR16388 belongs to a group of related molecules that includes an established antiestrogen (TAS-108 [19]), we tested the ability of SR16388 to inhibit ERα and ERβ in this TR-FRET assay as well. SR16388 inhibited both ERα (EC_{50} = 0.2 ± 0.2 μM) and ERβ (EC_{50} = 0.5 ± 0.2 μM). These findings indicate that SR16388 could have antiestrogenic activity in tumor cells containing significant expression of ERα or -β. As mentioned above, however, the general antiproliferative effect of SR16388 toward diverse human tumor cell lines (Table 1) suggests that this molecule has targets in addition to the classic ERs, such as ERRα.

3.3. SR16388 inhibits ERRα's transcriptional activation function

ERRα constitutively activates transcription through site-specific DNA binding to ERREs in the promoter or enhancer regions of its target genes [2,3]. Therefore, we next determined whether SR16388 could inhibit the ability of ERRα to stimulate expression of an ERRE-dependent luciferase reporter gene in MCF7 human breast cancer cells and PC3 human prostate cancer cells. The cells were transfected with separate expression vectors for ERRα and PGC-1α, along with an ERRE-firefly luciferase reporter construct and a TATA-*Renilla* luciferase transfection control construct. In

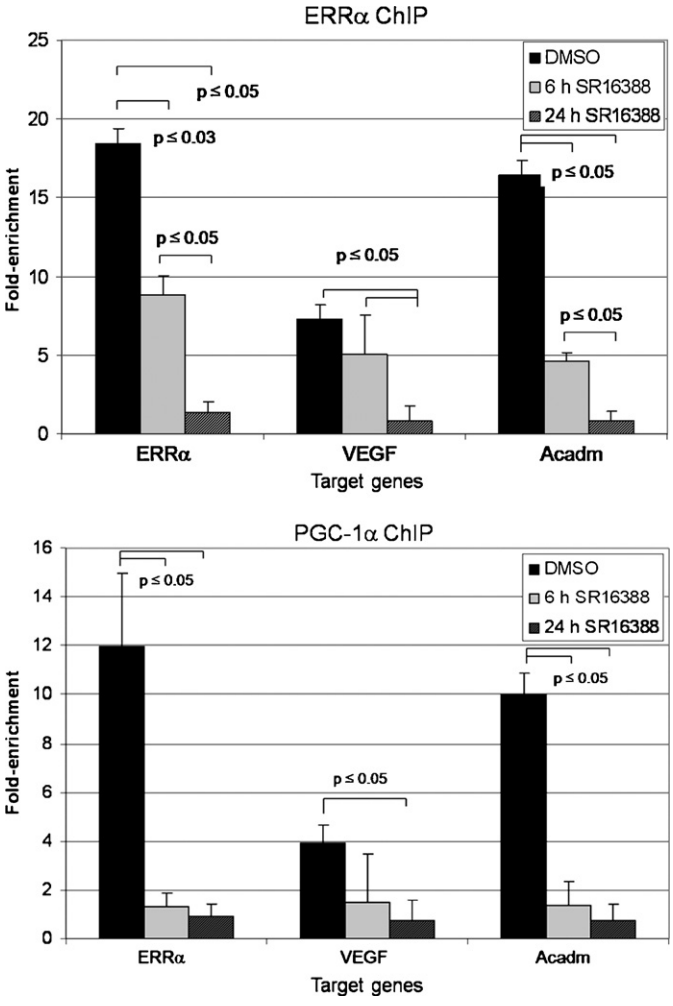

Fig. 4. Treatment with SR16388 decreases the association of ERRα and PGC-1α with target gene promoters. MCF-7 cells were treated with DMSO or 5 μM SR16388 for 6 or 24 h followed by ChIP using anti-ERRα, anti-PGC-1α, or control IgG antibodies. qPCR was performed using primer sets designed around established ERREs in the promoter or enhancer regions of the target genes [ERRα (*ESRRA*), *VEGFA*, *Acadm*]. Three independent replicate measurements were acquired for each experiment. The fold enrichment represents the difference in signal relative to the IgG negative antibody control. Statistical analysis was performed using the Wilcoxon signed rank test.

cells treated with the vehicle control (DMSO), ERRα and PGC-1α were able to co-activate transcription of the luciferase construct. In cells treated with SR16388, however, transcriptional activation of this co-activator complex was significantly inhibited (Fig. 3). ICI 182,780 was also able to inhibit ERRα-mediated transcription to an intermediate level, but only in MCF7 cells (Fig. 3). Because ICI 182,780 is an ER inhibitor [18], we hypothesize that the negative effect on ERRα-dependent gene expression detected in ICI 182,780-treated MCF7 cells was a result of their relatively high level of ERα expression, which is not observed in PC3 cells. Equimolar XCT790 did not inhibit luciferase expression in this assay after a 24-h treatment (data not shown). In general, to inhibit ERRα activity XCT790 seems to require longer exposure times at higher concentrations (e.g., 10 μM for 48 h, [19–21]) than that used here (5 μM for 24 h), which explains why SR16388 was much more effective than XCT790 as an inhibitor of ERRα-dependent gene expression in the present assay. It is important to note that the signal for the renilla luciferase transfection control construct was not significantly different for the DMSO and SR16388 treated samples (data not shown), indicating that the inhibitory effect of SR16388 on ERRE-dependent firefly luciferase activity was due to disruption of ERRα-dependent transcription of the ERRE-firefly luciferase construct rather than a cytotoxic effect.

The binding of ERRα to ERREs in the promoter or enhancer regions of selected endogenous target genes, and thus the recruitment of PGC-1α to these specific DNA-binding elements, was also disrupted by SR16388. Here, we used ChIP assays and qPCR to monitor the effect of SR16388 on endogenous ERRα and PGC-1α occupancy at established ERREs that regulate the genes for ERRα (*ESRRA*) itself [22–25], vascular endothelial growth factor (*VEGFA*) [26], and acyl-coenzyme A dehydrogenase (*Acadm*) [27,28]. MCF7 cells were treated with either DMSO or 5 μM SR16388 for 6 or 24 h. Chromatin was immunoprecipitated using anti-PGC-1α, anti-ERRα, or negative control IgG antibodies, and qPCR amplification was performed for each of the indicated ERRα-regulated genes. Fold enrichment values were calculated relative to that of the IgG control to evaluate the relative occupancy of each endogenous gene by ERRα or PGC-1α. After 24 h of SR16388 treatment, the relative occupancy of all ERREs for both proteins was significantly decreased ($p \leq 0.05$, Fig. 4). After 6 h of treatment, both ERRα and PGC-1α relative occupancies were decreased compared to those in DMSO treated cells for the ERRα

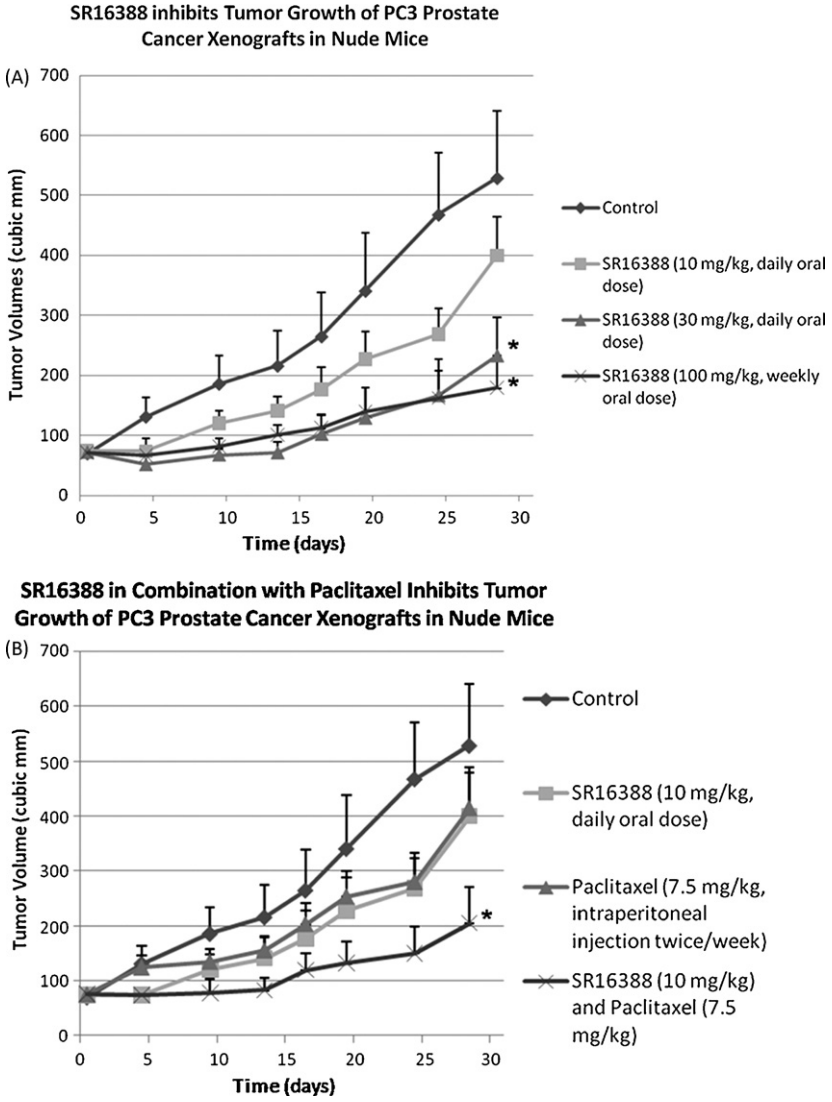

Fig. 5. SR16388 inhibits tumor growth of PC3 human prostate cancer xenografts in nude mice. PC3 cells were implanted subcutaneously in the right flank region of nude mice. When tumor volumes reached 80–100 mm^3, mice were treated with SR16388 with or without paclitaxel. Treatments were continued for 4 weeks, and differences in tumor growth rates between the control group and each treatment group were evaluated statistically. No significant compound-related effects on body weight or any other signs of overt toxicity were observed in any of the groups. *Tumor volumes were significantly reduced when compared with control, $p \leq 0.02$.

and Acadm ERREs ($p \leq 0.05$). The fold enrichment of ERRα occupancy at these ERREs, however, was higher at 6 h than at 24 h ($p \leq 0.05$) for all the ERREs tested. On the other hand, the fold enrichment of PGC-1α occupancy was equally diminished at both time points, indicating that PGC-1α was released from the ERREs shortly after the addition of SR16388 to the cells. It is possible that the observed decreases in ERRα occupancy were not the result of direct inhibition of the binding of ERRα to target genes, but rather of an overall decrease in the total cellular ERRα protein level due to the disruption of transcription of the ERRα gene, which has a positive autoregulatory feedback loop [2,24,25]. In summary, the luciferase reporter gene and ChIP assay experiments described here demonstrate that SR16388 significantly inhibited both transfected and endogenous ERRα activity in cultured human cancer cells.

3.4. SR16388 inhibits tumor growth in PC3 human prostate cancer xenografts

We performed PC3 human prostate cancer xenograft studies in nude mice to investigate the antitumor efficacy of SR16388. Fig. 5A shows that SR16388 alone had a strong inhibitory effect on the growth of PC3 tumor xenografts. At the final measurement, the volumes of tumors from the mice treated with SR16388 doses of 100 mg/kg weekly and with 30 or 10 mg/kg daily were 34%, 44%, and 75% of the size of the tumors in control mice, respectively. SR16388 also inhibited tumor growth when used in combination with paclitaxel, a chemotherapeutic used for hormone-refractory prostate cancer (reviewed in [29]; Fig. 5B). In the combination study, mice were treated with 10 mg/kg of SR16388 (oral daily dose) and 7.5 mg/kg paclitaxel (intraperitoneal injection, twice/week). At the final measurement, the tumor growth was inhibited by 22% after treatment with paclitaxel alone, 24% with SR16388 alone, and 61% with SR16388 and paclitaxel in combination. No significant compound-related effects on body weight or any other signs of overt toxicity were observed in any of the groups.

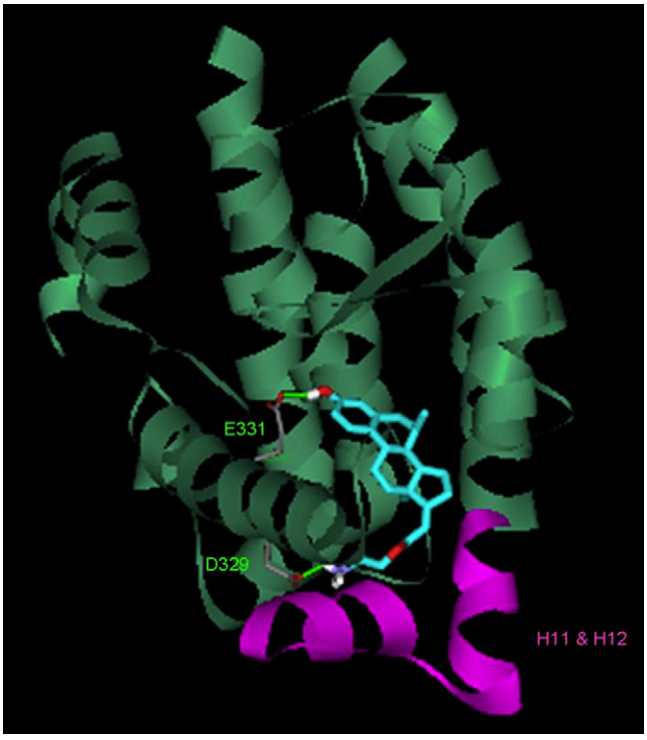

Fig. 6. Computational model of SR16388 bound to the LBD of ERRα. We hypothesize that the steroidal scaffold of SR16388 fits within the LBD of ERRα (amino acids 290–519) while the C-17 side chain extends toward helices 11 and 12 (H11 and H12), resulting in conformational changes that prevent or disrupt co-activator binding. Residues E331 and D329 are thought to be important for SR16388 binding and activity.

3.5. Computational modeling of the binding pose of SR16388 in the ERRα LBD

The computational model of SR16388 bound in the ERRα LBD suggests a strong ionic interaction between the compound and charged amino acids E331 and D329 of ERRα (Fig. 6). The X-ray crystal structure used to generate this model [9] shows that the ERRα LBD contacts a leucine-rich nuclear receptor interacting motif (LxxLL) helix of PGC-1α through helices 11 and 12 of ERRα (Fig. 6). The crystal structure 2PJL [9] revealed displacement of helix 12 away from its active conformation (PDB structures 1XB7 [30] and 3D24 [31]) to accommodate introduction of an inverse agonist. In addition to this displacement, our model of SR16388 suggests additional displacement of the last turn of helix 11 to accommodate SR16388. Based on our model of SR16388 binding to the LBD, we hypothesize that the C17 side chain of SR16388 extends toward these helices and causes a conformational change that prevents or disrupts binding of PGC-1α. In future work it will be important to validate this model of SR16388 binding to ERRα experimentally through co-crystallization and mutational studies.

4. Discussion

SR16388 – originally developed as a selective estrogen receptor modulator (SERM) [18] – is a member of a compound series that includes TAS-108, which is under development as a hormonal therapy for breast cancer [32,33]. SR16388 is the first member of this series found to have significant inhibitory (inverse agonist) activity toward ERRα-dependent gene expression in human cancer cells. This conclusion is based on the following experimental findings: (1) SR16388 disrupted the interaction between the human ERRα LBD and a PGC-1α co-activator peptide in a cell-free TR-FRET assay (Fig. 2); (2) SR16388 inhibited constitutive human ERRα-dependent reporter gene expression in transient transfection assays of human cancer cells (Fig. 3); and (3) SR16388 disrupted the occupancy of ERREs by ERRα and PGC-1α in three endogenous genes in human cancer cells regulated by ERRα (*ESRRA*, *VEGFA*, and *Acadm*) (Fig. 4). Although the established ERRα inverse agonist XCT790 was more effective as an inhibitor of the interaction between ERRα and its PGC-1α target peptide in the TR-FRET assay (Fig. 2), XCT790 failed to show significant inhibition of ERRα activity in the ERRE-dependent reporter gene assay described above (data not shown).

The therapeutic focus of SR16388 and related molecules such as TAS-108 is the treatment of solid tumors such as breast and prostate carcinomas. SR16388, which inhibits the proliferation or survival of diverse human cancer cell lines *in vitro* (Table 1), also inhibited the growth of PC3 human prostate cancer xenografts in nude mice (Fig. 5). Taken with the findings presented here showing that SR16388 is an ERRα inhibitor, it is possible that the cytotoxicity of SR16388 could involve disrupting ERRα dependent gene expression, depending on the tumor cell line. ERR expression has been investigated in diverse human tumor cell lines and primary tumor types, including cancers derived from breast, prostate, ovary, endometrium, and colon [3,8,34–41]. Evidence supporting a role for deregulated ERR expression or activity in human cancer is still emerging, but recent research indicates that ERRα in particular is involved in the development or progression of certain tumor types. For example, ERRα expression was reported to be significantly higher in clinical specimens of human breast, ovarian, endometrial, and prostate cancer than it was in the

corresponding normal tissues [3,34–38,40]. ERRα can bind to the same DNA elements as ERs (estrogen-response elements, EREs) *in vitro* [35]. In breast cancer cells, ERα and ERRα can regulate the same genes through binding a hybrid DNA element containing an ERRE within an ERE [38,42]. The genes that are co-regulated by these two nuclear receptors are known to contribute to the development or progression of human breast cancer [42]. ERRα may thus compensate for the loss of ER activity in breast cancers that become resistant to hormonal (antiestrogen) therapy. ERRα is also able to occupy androgen receptor response elements in the regulatory regions of genes sensitive to androgens [43], which suggests that ERRα contributes to phenotypes associated with tumors such as prostate cancer. Finally, given the importance of ERRα as a positive regulator of cellular energy metabolism, it is possible that ERRα more generally contributes to tumor development as a key component of the adaptive response of tumor cells to energy stress within solid tumor microenvironments (e.g., reviewed in [44,45]). For example, using xenografts prepared from ER-negative MDA-MB-231 human breast cancer cells expressing siRNA targeting ERRα, it was found that growth of the ERRα knockdown tumors was significantly attenuated compared to that of tumors expressing control siRNA [38]. Moreover, the proliferation of the ERRα-knockdown cells was not inhibited *in vitro*, suggesting that ERRα is required for MDA-MB-231 cells to adapt to microenvironmental energy stress within the rapidly growing tumor xenografts. It will be important in future work to test the efficacy of SR16388 in ERRα-knockdown tumor xenograft models to investigate the specificity of the compound for ERRα.

In summary, SR16388 is a purely steroidal antiestrogen that selectively targets ERRα in the ERR subgroup of human nuclear hormone receptors. This selectivity is potentially important for breast cancer therapy because ERRα expression is correlated with an unfavorable clinical outcome in primary breast tumors, whereas ERRγ expression is correlated with a favorable outcome [46]. ERRα expression was also reported to be associated with an increased risk of recurrence and a poor prognosis in breast cancer [47]. While the mechanistic contribution of ERRα to tumor development or progression is not yet clear, we hypothesize that ERRα activity is required for the adaptation of tumor cell metabolism to energy stress within pathophysiological tumor microenvironments. Thus, SR16388 may be cytotoxic toward energetically stressed tumor cells in part by disrupting ERRα-dependent gene expression.

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