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

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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.

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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.

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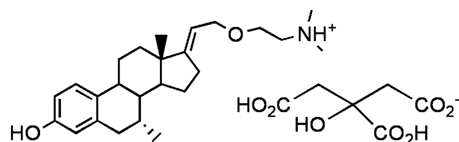

Fig. 1. Structure of SR16388. SR16388 (21-[2-(N,N-dimethylamino)ethyl]oxy-7a-methyl-19-norpregna-1,3,5(10),17(20)-tetraen-3-ol citrate salt) is an orally active compound that belongs to the antiestrogen class of therapeutic agents. SR16388 is a potent and selective inhibitor of human ERRα, which does 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

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