

Identification of the First Synthetic Steroidogenic Factor 1 Inverse Agonists: Pharmacological Modulation of Steroidogenic Enzymes

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ABSTRACT

Steroidogenic factor SF-1, a constitutively active nuclear hormone receptor, is essential to the development of adrenal and gonadal glands and acts as a shaping factor of sexual determination and differentiation. Its effects are exerted primarily through the control of the synthesis of steroid hormones. The functional cell-based assay Receptor Selection and Amplification Technology (R-SAT) was used to identify potent and selective SF-1 inverse agonists through the screening of a chemical library of drug-like small-molecule entities. Among them,

4-(heptyloxy)phenol (AC-45594), a prototype inverse agonist lead, was used to show that SF-1 constitutive activity can be pharmacologically modulated by a synthetic ligand. In a physiological system of endocrine function, the expression of several reported SF-1 target genes, including SF-1 itself, was inhibited by treatment with AC-45594 and analogs. Thus, pharmacological modulation of SF-1 is critical to its function as an endocrine master regulator and has potentially important consequences to diseases in which SF-1 activity is critical.

The orphan nuclear hormone receptor steroidogenic factor-1 (SF-1, AD4BP, NR5A1) has emerged in recent years as a key regulator of endocrine function. First identified as a transcription factor with limited tissue distribution, it is found primarily in steroidogenic tissues (Morohashi et al., 1992). As such, SF-1 is highly expressed in the testes and ovaries but has also been reported to be present in other tissues, including the skin, spleen, and brain (Ikeda et al., 1994). SF-1 null mice lack gonads and adrenal glands and consequently display severe adrenocortical insufficiency (Luo et al., 1994). SF-1 modulates the transcription of many genes involved in steroidogenesis and reproduction, including cytochrome P450 steroid hydroxylases, steroidogenic acute regulatory protein (StAR), and others (reviewed in Parker and Schimmer, 1997). Naturally occurring SF-1 mutations in the human population have been reported and are associated with XY sex reversal, primary adrenal deficiency, and dysgenetic testes (Correa et al., 2004). These mutations result in significant loss of function.

More recently, SF-1 has been linked to metabolic disorders. SF-1-null mice display altered development of the ventromedial hypothalamic nucleus (Ikeda et al., 1995), a region involved in satiety, homeostasis, and metabolism. SF-1 knockout mice rescued by adrenal transplantation develop late-onset obesity (Majdic et al., 2002). Leptin, a master switch of satiety and energy expenditure, increases the firing of SF-1-positive neurons in the ventromedial hypothalamic nucleus (Dhillon et al., 2006). Moreover, persons carrying loss-of-function SF-1 mutations are mildly to severely obese (Correa et al., 2004). Finally, such mutations have also been associated with insulin resistance and type 2 diabetes in Chinese patients (Liu et al., 2006).

Whether SF-1 transcriptional activity is (or can be) regulated by physiological ligands still remains largely unanswered. In various cell systems, heterologous expression of SF-1 leads to a constitutively active receptor (Ito et al., 1998), modulating the transcription of target genes in absence of a ligand. Nonligand modes of regulation have been described, including tissue-specific repressors (Ito et al., 1998) and phosphorylation of the hinge region (Desclozeaux et al., 2002). A report suggesting that SF-1 could be activated by

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ABBREVIATIONS: SF-1, steroidogenic factor-1; LBD, ligand binding domain; R-SAT, receptor selection and amplification technology; DMEM, Dulbecco's modified Eagle's medium; SFRE, SF-1 response element; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction; AC-45594, 4-(heptyloxy)phenol; LRH-1, liver receptor homolog-1; StAR, steroidogenic acute regulatory protein; HTS, high-throughput screening; ER, estrogen receptor; SFRE, SF-1 response element.

various oxysterols (Lala et al., 1997) could not be confirmed by other investigators (Mellon and Bair, 1998; Desclozeaux et al., 2002). The crystal structure of the ligand binding domain (LBD) of SF-1 was solved by several groups independently (Madauss et al., 2004; Krylova et al., 2005; Li et al., 2005; Wang et al., 2005). The SF-1 LBD binds to a coactivator-derived peptide in a manner consistent with the adoption of a transcriptionally active conformation. A large binding pocket was uncovered that was filled with phospholipids. Similar findings were reported with LRH-1, the receptor most closely related to SF-1 (Krylova et al., 2005; Ortlund et al., 2005; Wang et al., 2005). Further characterization indicated that SF-1 preferentially binds eukaryotic phosphatidyl inositol bis- and triphosphates, as well as various C12-C16 fatty acids, with high affinity. These phospholipids can be readily exchanged and modulate the interaction of SF-1 with coactivators. In addition, SF-1 mutations that disrupt the predicted interactions of the phospholipids within the binding pocket or alter the pocket size reveal a strong correlation between phospholipid binding and transcriptional activity. Attempts to extend these results to biological systems have failed so far to demonstrate a phospholipid-dependent regulation of SF-1 activity in vitro (Wang et al., 2005), with the exception of one recent study reporting that sphingosine acts a negative regulator of SF-1 activity (Urs et al., 2006).

In an attempt to identify small molecule modulators of SF-1, our internal chemical library was screened against constitutively active human SF-1 receptor. Here we report on the identification and biochemical characterization of selective SF-1 inverse agonists in steroidogenic cells.

Materials and Methods

Chemicals. The purities of the six compounds 4-(butoxy)phenol, 4-(pentyloxy)phenol, 4-(hexyloxy)phenol, 4-(heptyloxy)phenol, 4-(octyloxy)phenol, and 4-(decyloxy)phenol were analyzed by liquid chromatography/mass spectroscopy and were determined to be >99% by ultraviolet/mass spectroscopy. The analyses were performed on a liquid chromatography/mass spectroscopy instrument (Micromass ZQ2000; Waters, Milford, MA) consisting of a ZQ single quadrupole mass spectrometer equipped with an electrospray ionization interface, and a Waters Alliance HT with a 2795 Separation Module and 996 photodiode array detector. High-performance liquid chromatography method: *Mobile phase*: A, 10 mM NH₄OAc H₂O; B, 10 mM NH₄OAc CH₃CN-H₂O (95:5). *Column*: Waters Xterra MS C₁₈ 3.5 μ m, 30 \times 4.6-mm inner diameter with a guard column cartridge system. *Program*: 5-min gradient starting at 30% B (initial hold for 0.5 min.) to 100% B, hold for 1.5 min, over 0.5 min to 30% B, hold for 2.5 min. The flow rate was 1 ml/min. *Photodiode array detector range*: 190 to 450 nm.

R-SAT Assays. Receptor Selection and Amplification Technology is a functional cell-based assay that allows one to monitor receptor-dependent proliferative responses and has been described elsewhere (Piu et al., 2002). The technology has been validated for a number of receptors including G protein-coupled receptors, receptor tyrosine kinases, cytokine receptors, and nuclear receptors (Piu et al., 2002, 2006; Burstein et al., 2006). Its principle resides in the genetic selection and amplification of the nuclear receptors in a ligand-dependent manner. This process is achieved by partial cellular transfection via the loss of contact inhibition and of growth factor dependence. Monitoring is achieved by transfecting the cells with a β -galactosidase reporter gene vector whose expression is under a constitutively active promoter. In brief, NIH/3T3 fibroblasts were plated overnight in 96-well plates in DMEM 10% calf serum (Hyclone) and grown to 60 to 70% confluence before transfection. Tran-

sient transfections were performed using Polyfect (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Typically, a transfection mix would consist of the receptor and the β -galactosidase expression vectors. Sixteen hours after transfection, cells were incubated with different doses of ligand in DMEM containing 30% Ultraculture (Hyclone, Logan, UT) and 0.5% calf serum (Hyclone) to generate a dose-response curve. After 5 days, plates were developed by adding onto the washed cells a solution containing the β -galactosidase substrate *o*-nitrophenyl-D-galactopyranoside (in phosphate-buffered saline with 5% Nonidet P-40 detergent) as described previously (Piu et al., 2002). Plates were read using a microplate reader at 420 nm. Data from R-SAT assays were fit to the equation: $r = A + B/(x/(x + c))$, where A = minimum response, B = maximum response minus minimum response, $c = EC_{50}$, r = response, and x = concentration of ligand. Curves were generated using the curve-fitting software Excel Fit and Prism (GraphPad Software, San Diego, CA).

Reporter Gene Assays. SFRE luciferase vector was generated by replacement of the three ERE sequences of the ER2 luciferase reporter vector (Panomics, Fremont, CA) with three SFRE sequences using the following primers: 5'-CTAGCTCAAGGTCACAGTCAAGGTCACAGTCAAGGTCAG; 3'-GAGTTCAGTGTGAGTTCAGTGTGAGTTCAGTTCAGTTCAG (SFRE underlined). The StAR promoter (−1300, +39) was kindly provided by Dr. J. F. Strauss III (University of Pennsylvania, Philadelphia, PA). Human embryonic kidney-293T cells were maintained in DMEM containing 10% fetal bovine serum. Cells were plated at 20,000 cells/well in a 96-well plate 18 to 24 h before transfection. Transient transfections were performed in 10% charcoal-stripped fetal bovine serum-DMEM using Polyfect (QIAGEN) according to the manufacturer's instructions. Cells were transfected with 60 ng/well SF-1 encoding plasmid (or same amount of LRH-1) and 20 ng/well SFRE luciferase vector (or StAR promoter), in addition to 0.4 ng/well vectors encoding GRIP1, SRC-1 and DRIP205. Sixteen hours after transfection, compounds were added at various doses in media without serum. After 36 h of incubation, media was removed, cell extracts were isolated, and luciferase activity was measured using a commercially available kit (SteadyGlo; Promega, Madison, WI).

cAMP-Induced mRNA Expression of SF-1 Target Genes. In brief, H295 adrenocortical carcinoma cells (American Type Culture Collection, Manassas, VA) were plated at 1.7×10^6 cells/35-mm dish in DMEM/Ham's F12 media supplemented with 10% fetal bovine serum and ITS mix (insulin, transferrin, selenium). The next day, media was exchanged for media with charcoal-stripped fetal bovine serum (Hyclone). All subsequent additions were made in charcoal-stripped fetal bovine serum. One hour after media exchange, cells were treated with compound or DMSO. After 48 h of treatment, cells were treated with compound and (Bu)₂cAMP (300 μ M). After 24 h of (Bu)₂cAMP treatment, total RNA was isolated and treated with DNase I using the RNAqueous-4PCR kit (Ambion, Austin, TX). Two micrograms of total RNA were converted to cDNA using the SuperScript III Reverse Transcriptase and oligo dT (Invitrogen, Carlsbad, CA). PCR reactions were performed using Platinum Taq (Invitrogen) using manufacturer's suggested conditions, except that the StAR reaction was done in the presence of 5% DMSO, the GAPDH, CYP11A1 reactions were done in the presence of 2.5% DMSO, and the SF-1 reaction was done in the presence of 3 mM MgSO₄. Oligonucleotide primers were synthesized by MWG Biotech (High Point, NC). Primer sequences were: for StAR: sense, 5'-CCAGATGTGGGCAAGGTG-3'; antisense, 5'-CAGCGC-ACGCTCACAAAG-3'; for GAPDH: sense, 5'-CGAGCCACATCGCTCAGACAC-3'; antisense, 5'-GCTAAGCAGTTGGTGGTGCCAGG-3'; for SF1: sense, 5'-GACAAGGTGTCCG GCTACCAC-3'; antisense, 5'-GTCTCCAGCTTGAAGCCATTGG-3'; for CYP11A1: sense, 5'-CAAGACCTGGAAGGACCATGTG-3'; antisense, 5'-GATATCTCTGCA-GGGTCACGGAG-3'. The sizes of the amplified bands were 227 bp (StAR), 495 bp (GAPDH), 413 bp (CYP11A1), and 314 bp (SF-1). RT-PCR conditions were optimized by cDNA amount and number of cycles so that band intensities were in the linear range. StAR RT-PCR cycling conditions were 95°C (50 s), 58°C (30 s), and 68°C (50 s) for 30 cycles. GAPDH RT-PCR cycling conditions were 95°C (50 s), 58°C (30 s), and

68°C (50 s) for 20 cycles. SF-1 RT-PCR cycling conditions were 95°C (50 s), 56°C (30 s), and 68°C (50 s) for 40 cycles. CYP11A1 RT-PCR cycling conditions were 95°C (50 s), 58°C (30 s), and 68°C (50 s) for 25 cycles. After RT-PCR, products were visualized on a 1.5% agarose gel, and the bands were quantitated using Scion Image (Scion Corporation, Frederick, MD).

cAMP-induced Protein Expression of SF-1 Target Genes. H295 adrenocortical carcinoma cells were treated as described in the cAMP-induced mRNA expression of SF-1 target genes section. Protein extracts were isolated by harvesting cells in Laemmli buffer, and total protein concentration was determined by the BCA protein assay (Pierce Biotechnology, Inc., Rockford, IL). Between 22 and 25 μ g of protein were loaded onto each gel, and Western blotting was performed using the antibody manufacturer's instructions. Antibodies used were StAR (ABR Affinity Bioreagents, Golden, CO), GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), SF-1 (Santa Cruz Biotechnology), and CYP11A1 (Santa Cruz Biotechnology). To quantify expression levels, blots were scanned and pixels in each band were measured using Scion Image.

Results

We characterized the signaling and intrinsic activity properties of the human SF-1 nuclear receptor using a functional cell-based assay. R-SAT defines a broadly applicable cell proliferation assay that allows for the pharmacological study of various receptor targets and their ligands, including G protein-coupled receptors, cytokine receptors, and nuclear hormone receptors (Piu et al., 2002; Burstein et al., 2006; Piu et al., 2006). As expected, SF-1 was highly constitutively active in this assay (Fig. 1A): up to 25-fold activation over baseline was evident when 40 ng/well of the SF-1 expression vector was transiently transfected compared with reporter alone. Higher levels (80 μ g/ml) reached the maximum of DNA that could effectively be transfected and produced slightly decreased activation. Significant activation (8–10-fold) was seen with amounts as little as 4 ng/well. We then confirmed that the intrinsic activity of SF-1 could be positively modulated by classic coactivators. In conditions where SF-1 displays little constitutive activity (low amounts of DNA transfected), we showed that the coactivators SRC-1, and to a lesser extent DRIP205, significantly increased SF-1 activity, whereas GRIP1 had a marginal but reproducible effect in our system (Fig. 1B). When all three coactivators were expressed together, an additive effect was evident. This effect was dependent upon the presence of SF-1, because expression of the coactivators alone had no effect. The strong stimulatory effect of SRC-1 association with SF-1, as well as the much weaker interaction between SF-1 and GRIP1, are consistent with published literature (Ito et al., 1998; Borud et al., 2003; Li et al., 2005). We also discovered that DRIP205, a critical member of the mediator DRIP/TRAP complex, which serves as a physical bridge between transcription factors and the transcription machinery (Belakavadi and Fondell, 2006), strongly augments SF-1 constitutive activity, a finding not previously reported.

Subsequently, a high-throughput screen of SF-1 to identify inverse agonist chemistries was initiated. An internal library of 280,000 small-molecule drug-like compounds was screened (Piu et al., 2005). The compound library is diverse, sharing a significant overlap (67%) in chemical space with the reference MDDR (MDL Drug Data Report) library but also exhibits unique chemical space properties. The library is also heavily biased in favor of compounds displaying drug like-

ness characteristics, as defined by Lipinski rules. The HTS campaign relied on a robust assay (-fold: 11.1 ± 2.4 ; Z' factor: 0.54 ± 0.06), which resulted in the identification of several hits defining a unique pharmacophore (Hit Rate: 0.03%). The 4-(heptyloxy)phenol AC-45594, a prototype lead, displayed full inverse agonist activity at SF-1, with an IC_{50} of 50 to 100 nM in the R-SAT assay (Fig. 2A). Analysis of analogs of AC-45594 with different alkyloxy chain lengths revealed that

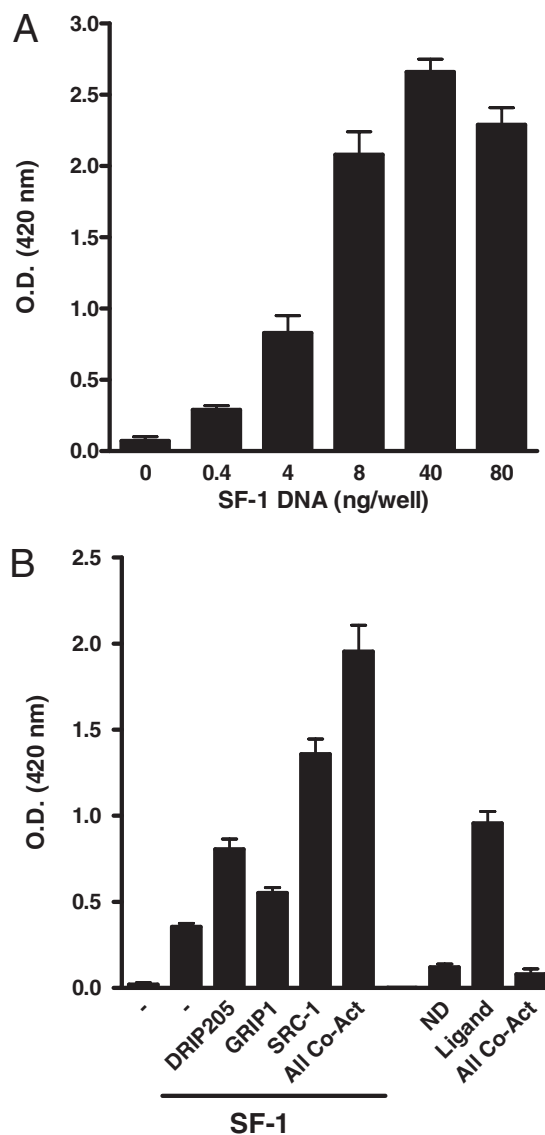


Fig. 1. Pharmacological properties of the human SF-1 nuclear receptor. A, the levels of constitutive activity displayed by SF-1 were evaluated using R-SAT. Various DNA amounts of an expression vector encoding human SF-1 were transiently transfected in NIH/3T3 cells, and the resulting activity was assessed using R-SAT. Activity was reported as colorimetric absorbance values (optical densities measured at 420 nm). B, the influence of coactivators (GRIP1, SRC-1, and DRIP205) on the constitutive activity of SF-1 was assessed using R-SAT. Low DNA amounts of SF-1 (0.4 ng/well) were transiently transfected alone or in presence of the different coactivators (0.4 ng/well). Activity was reported as colorimetric absorbance values [optical densities (O.D.) measured at 420 nm]. The RAR β 2-liganded receptor was used as a control to define the dynamic range of the assay. ND, no drug; ligand, unique dose of AM-580 (10 μ M), a reported agonist ligand of RAR β 2 (Piu et al., 2006); All CoAct, GRIP1, SRC-1, DRIP 205 transfected together in absence of SF-1. All experiments (A and B) represent the average of three experiments performed in duplicates.

the inverse agonist properties of this class of compounds are severely restricted by the length of the alkyloxy chain (Table 1). Only compounds with a 6–10 carbon chain were active, with potencies ranging from 100 nM to 3 μ M. In addition, the conversion of the hydroxy group on the phenol moiety to a methyl led to a complete loss of activity. Furthermore, the analogous hexyl, heptyl, octyl, and nonyl-phenols reported to be micromolar estrogen receptor ligands were evaluated (Tabira et al., 1999). As expected, they display low-affinity estrogen receptor agonism but, more importantly, lacked any crossover activity at the SF-1 receptor (data not shown).

Conversely, the alkyloxy analogs including AC-45594 itself showed neither agonist nor antagonist activity at the estrogen receptors as well as no inverse agonist activity at the related LRH-1 receptor (Table 2).

Unlike many nuclear receptors, SF-1 modulates the transcription of target genes by interacting as a monomer with AGGTCA recognition motifs (Parker and Schimmer, 1997). It is noteworthy that this motif also constitutes the half-site of the estrogen response element ERE. In addition, a subset of nuclear receptors is known to primarily bind to this motif, including the Nurr, ROR, and ERR family members (Man-

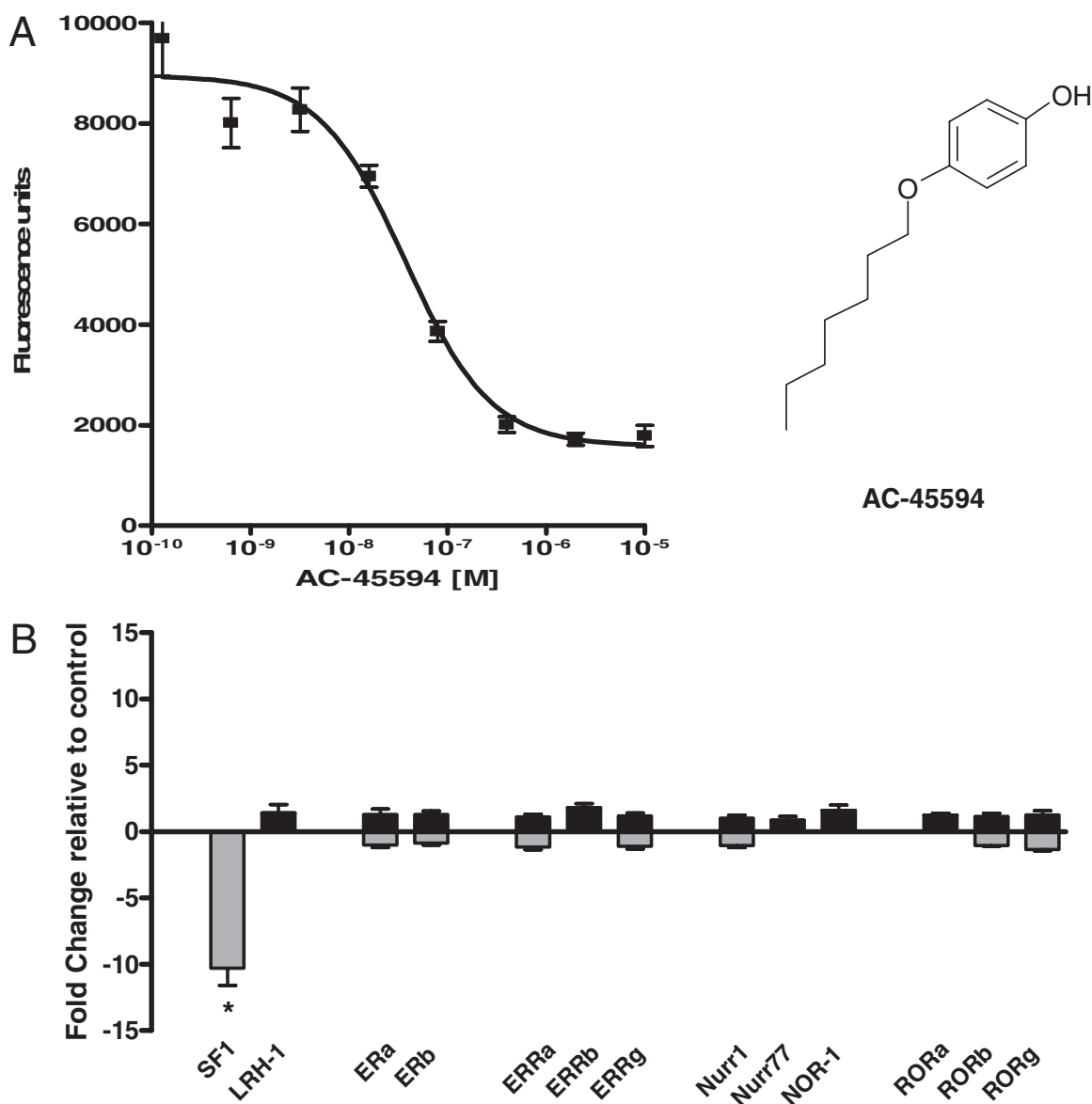


Fig. 2. AC-45594 is a selective and potent inverse agonist at human SF-1. A, high DNA amounts of human SF-1 were transiently cotransfected along with the coactivators DRIP205, GRIP1, and SRC-1, then treated with various doses of AC-45594, and the activity was measured using R-SAT. Data are reported as FLU (arbitrary fluorescent light units) because the assay was performed using a red fluorescent protein variant as a reporter gene. The chemical structure of AC-45594 is indicated. B, AC-45594 was extensively profiled using R-SAT. All receptors were transiently transfected in presence of a coactivator mix that included GRIP1, SRC-1, and DRIP205 treated with AC-45594 at various doses, and the relevant pharmacological response (agonism, inverse agonism) was evaluated through R-SAT. The respective amounts of transfected receptors vary in each assay to fine-tune it for sensitivity toward agonism (low receptor amount) and inverse agonism (high receptor amount). Data are reported as the maximum -fold change relative to nontreated conditions for each receptor (vehicle treated), and determined by the largest dynamic range seen in response to the various doses of AC-45594. A positive value refers to agonist activity (black bars), whereas inverse agonist activity is reported as a negative value (gray bars). Internal controls indicated that all receptors behaved in a manner consistent with previous experiments, including response to reference ligands and presence of constitutive activity. Marginal activities for ER α , ER β , LRH-1, ERR β , and NOR1 translate to an approximately 2-fold increase with weak potency (pEC₅₀, 5.5–6.0) (data not shown). *, $P < 0.05$.

gelsdorf et al., 1995). Mapping studies have revealed that additional bases 5' to the half-site determine the binding selectivity for each nuclear receptor, PyCAAGGTCA defining the consensus sequence for the SF-1 response element (SFRE) (Wilson et al., 1993). In addition, cross-talk at the SFRE site involving ERs and ERRs has been reported (Vanacker et al., 1999). Because of the functional cross-talk between SF-1 and other nuclear receptors, we investigated whether AC-45594 had any activity at additional nuclear receptors. Molecular profiling of AC-45594 and analogs indicated that although all were active at SF-1, none displayed activity (neither agonism nor antagonism) at the estrogen ER subtypes (Table 2, Fig. 2B). In addition, no activity was evident at LRH-1 or the ERR, Nurr, and ROR family members (Fig. 2B). Thus, the chemical class defined by AC-45594 and analogs provides for a selective SF-1 inverse agonist pharmacophore.

Because SF-1 functions primarily as a transcription factor, we investigated whether AC-45594 and related analogs could modulate the transcriptional properties of SF-1 in a reporter gene assay. In the absence of any ligands, transient transfection of increasing amounts of SF-1 translated to higher transcriptional activity through a synthetic SFRE (data not shown). In conditions in which the constitutive transcriptional activity of SF-1 was maximal, addition of AC-45594 led to a dose-dependent inhibition of SFRE-mediated transcription (Fig. 3A). Analogs of AC-45594 were similarly active, indicating that this class of compounds inhibits SF-1 activity by interfering with its ability to modulate the transcription of target genes. The observed IC_{50} values for all compounds tested ranged from 500 to 5000 nM. In addition, we investi-

gated whether the transcriptional effects of AC-45594 could be extended to natural promoters. To that end, we evaluated the StAR promoter (−1300, +39), which contains several SF1 binding sites (Sugawara et al., 1996) (Fig. 3B). AC-45594 dose dependently inhibited SF-1 induced transcription through the StAR promoter. The hexyloxy and octyloxy analogs were similarly active at the StAR promoter, with IC_{50} values consistent with the ones observed using the synthetic SFRE construct. Furthermore, we confirmed that AC-45594 did not affect the activity of LRH-1 in that same system. Under conditions in which high amounts of transfected LRH-1 increased transcriptional activity through the synthetic SFRE (which is also a response element for LRH1), the addition of AC-45594 and analogs had no effects at doses up to 10 μ M (data not shown), confirming the selectivity of this class of ligands.

SF-1 is a master regulator of the expression of almost every gene involved in steroidogenesis (Parker and Schimmer, 1997; Val et al., 2003). For instance, the expression of most steroidogenic enzymes, as well as many cholesterol transporters, is under the transcriptional control of SF-1. Likewise, steroidogenesis-stimulating hormones and their cognate receptors are primarily modulated through SF-1. Although it is evident that the constitutive activity displayed by SF-1 allows for gene regulation in absence of ligand, we sought to investigate whether ligand-dependent modulation of SF-1 activity using inverse agonists would lead to regulatory effects on target genes.

Among the SF-1 target genes, StAR constitutes the rate-limiting factor in the steroid hormone synthesis pathway. Two SFRE *cis*-acting elements in the StAR promoter have been identified and, through SF-1, are responsible for both basal and cAMP-dependent gene regulation (Sugawara et al., 1997). In human adrenocortical H295 cells, expression of StAR can be induced by cAMP and is dependent upon the presence of SF-1 (Brand et al., 2000). We investigated how AC-45594 and the octyloxy analog would affect cAMP induction of StAR at the mRNA and protein levels. The octyloxy analog showed significant toxicity at 30 μ M, and thus was only evaluated at 10 μ M. Detectable levels of StAR mRNAs were evident in nontreated H295 cells using quantitative RT-PCR (Fig. 4A). Treatment with cAMP increased StAR transcripts by approximately 3-fold within 24 h of induction. Incubation with AC-45594 reduced StAR mRNA to basal levels in a dose-dependent manner. A similar effect was seen with the octyloxy analog. At the protein level, after cAMP

TABLE 1

Influence of the length of the alkoxy chain on SF-1 activity

Compounds with an alkoxy chain ranging from $n = 4$ to $n = 10$ were tested using R-SAT. Full dose response experiments were performed multiple times in triplicate. Results represent the average of all experiments. AC-45594 was defined as the reference compound, and its activity was defined as 100% inhibition.

IDs	Alkoxy Chain		SF-1	
	Length	Name	Inhibition %	pIC_{50}
AC-45594	4	Butyloxy	10 ± 8	N.A.
	5	Pentyloxy	10 ± 5	N.A.
	6	Hexyloxy	88 ± 12	6.7 ± 0.2
	7	Heptyloxy	100 ± 5	7.2 ± 0.3
	8	Octyloxy	109 ± 7	7.1 ± 0.1
	10	Decyloxy	103 ± 14	5.5 ± 0.3

N.A., not active at 10 μ M.

TABLE 2

Selectivity profiling of AC-45594 and analogs

Compounds were tested for activity at several nuclear receptors using R-SAT. Data from agonist experiments are reported as efficacy (% Eff) and potency ($pEC_{50} = -\log[EC_{50}]$), relative to a standard reference. Antagonist and inverse agonist data are reported as pK_i ($-\log[K_i]$) and pIC_{50} ($-\log[IC_{50}]$), respectively. Efficacy and inhibition values were normalized to that of the reference drug defined as 100%: for SF-1 inverse, AC-45594; for LRH1, inhibition was normalized to the dynamic range of the constitutive activity displayed by LRH1 because no reference ligand is available; for ER α and ER α agonism, estrone (pEC_{50} : ER α , 9.9 ± 0.4 ; ER β , 9.8 ± 0.3); for ER α and ER β antagonism, 4OH-tamoxifen (pK_i : ER α , 8.6 ± 0.2 ; ER β , 9.1 ± 0.3). Potencies for the reference compounds were consistent with published literature values. Data are the average of several experiments performed in triplicates.

IDs	SF-1		LRH-1		ER α			ER β		
	Inhibition	pIC_{50}	Inhibition	pIC_{50}	Efficacy	pEC_{50}	pK_i	Efficacy	pEC_{50}	pK_i
	%		%		%			%		
Hexyloxy	88 ± 12	6.7 ± 0.2	9 ± 2	N.A.	4 ± 5	N.A.	N.A.	15 ± 11	N.A.	N.A.
AC-45594	100 ± 5	7.2 ± 0.3	10 ± 5	N.A.	21 ± 8	N.A.	N.A.	9 ± 8	N.A.	N.A.
Octyloxy	109 ± 7	7.1 ± 0.1	8 ± 3	N.A.	1 ± 3	N.A.	N.A.	6 ± 9	N.A.	N.A.
Decyloxy	103 ± 14	5.5 ± 0.3	4 ± 1	N.A.	8 ± 9	N.A.	N.A.	12 ± 7	N.A.	N.A.

N.A., not active at 10 μ M.

treatment, StAR increased by 30-fold after 24 h (Fig. 4B). Treatment with the SF-1 inverse agonist AC-45594 completely reversed in a dose-dependent manner the cAMP-dependent activation of StAR. Related analogs of AC-45594 produced a similar response, with degrees of inhibition ranging from 50 to 75%, including the octyloxy molecule.

The effects of AC-45594 on another SF1 target gene were evaluated. CYP11A1 (CYP450scc) catalyzes the side chain cleavage of cholesterol, the first and rate-limiting step in the steroid biosynthetic pathway. SF-1 plays a major role in the tissue specificity and hormonally regulated expression of this enzyme. Treatment of H295 cells with cAMP led to a weak but reproducible increase in CYP11A1 mRNA levels of ap-

proximately 3-fold (Fig. 5A). That activation was completely abolished in the presence of AC-45594 at 30 μ M, and partially with the octyloxy at 10 μ M. At the protein level (Fig. 5B), cAMP induction increased CYP11A1 levels by approximately 3.5-fold, an effect that was reversed in the presence of AC-45594 and the octyloxy analog.

Studies using a dominant negative SF-1 mutant have suggested the involvement of SF-1 in an auto-regulatory feedback loop, because constitutive expression of the mutant impaired expression of endogenous SF-1 in adrenocortical cells (Li et al., 2004). We thus investigated whether SF-1 inverse agonists would also modulate SF-1 expression at the mRNA and protein levels. After cAMP addition, SF-1 mRNAs were increased reproducibly by approximately 2.5-fold (Fig. 5A). The addition of 10 μ M AC-45594 did not inhibit SF-1 induction by cAMP, whereas 30 μ M AC-45594 partially inhibited this response. The octyloxy analog partially reversed SF-1 mRNA induction at 10 μ M. Likewise, SF-1 protein levels were increased by a factor of approximately 3.5-fold by

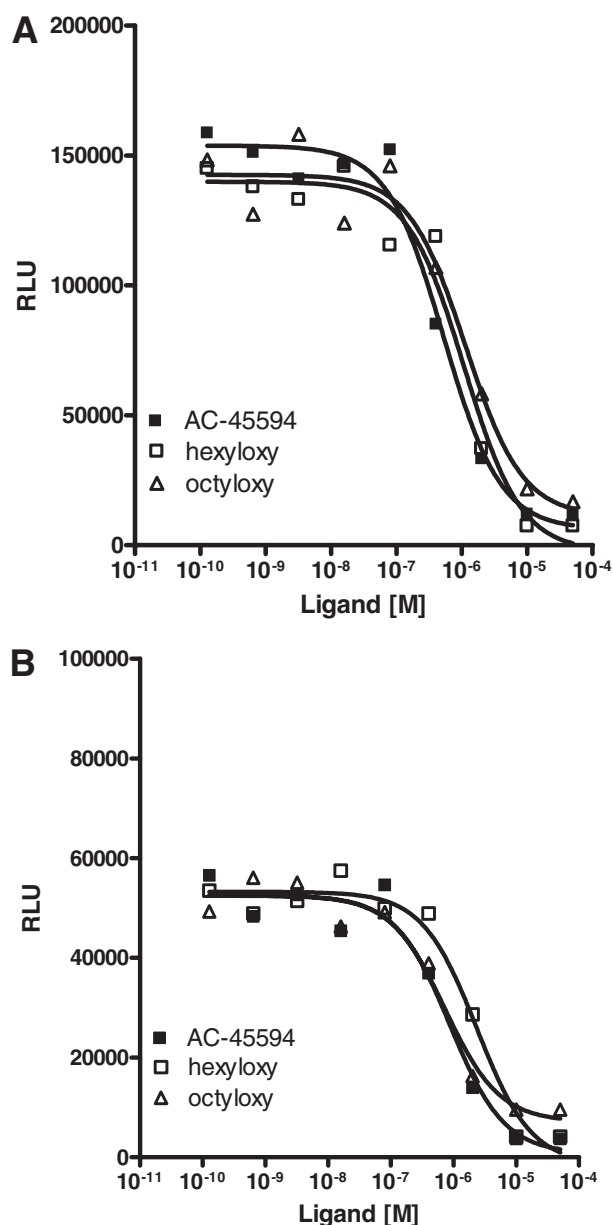


Fig. 3. AC-45594 and analogs inhibits SF-1 transcriptional activation. High DNA amounts of human SF-1 were transiently cotransfected along with the coactivators DRIP205, GRIP1, and SRC-1, along with a synthetic Luciferase reporter gene containing three copies of the consensus SF-1 response element (A) or with the natural StAR promoter (-1300, +39) fused to Luciferase (B). Cells were subsequently exposed to various doses of AC-45594 and related analogs for 48 h. Data are reported as luciferase (RLU, relative luminescent units).

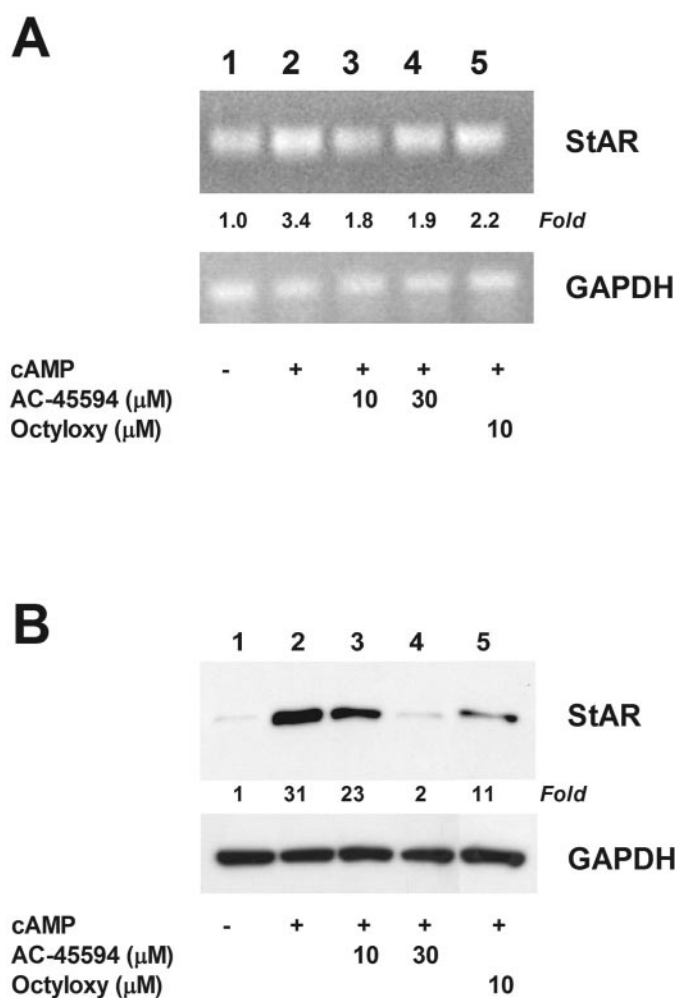


Fig. 4. SF-1 inverse agonists inhibit StAR expression in human adrenocortical cells. Human adrenocortical H295 cells were treated for 24 h with cAMP (300 μ M) to induce endogenous mRNA (A) and protein (B) expression of StAR protein, a known SF-1 target. Cells were pretreated or not for 48 h with AC-45594 and the octyloxy analog at different doses (10 and 30 μ M). Cell extracts were made, and mRNA and protein levels were detected and quantified by RT-PCR and Western blots, respectively. Fold activation was relative to nontreated cells and normalized relative to the GAPDH control.

cAMP (Fig. 5b). Pretreatment with 30 μ M AC-45594 and 10 μ M concentrations of the octyloxy analog prevented almost completely this induction, thus maintaining SF-1 proteins at basal levels.

Taken together, our findings strongly demonstrate that pharmacological inhibition of SF-1 activity through selective inverse agonists in a physiological system has a direct effect on the expression of several well described SF-1 target genes, including SF-1 itself.

Discussion

In summary, using a functional cell-based platform technology, we have discovered synthetic bona fide selective and potent SF-1 inverse agonist chemistries. In an *in vitro* assay system of steroidogenesis, we demonstrated that the pharmacological inhibition of SF-1 constitutive activity can be achieved using selective inverse agonists, such as AC-45594, and leads to the down-regulation of key SF-1 target genes, including enzymes critical to steroid biosynthesis and SF-1 itself.

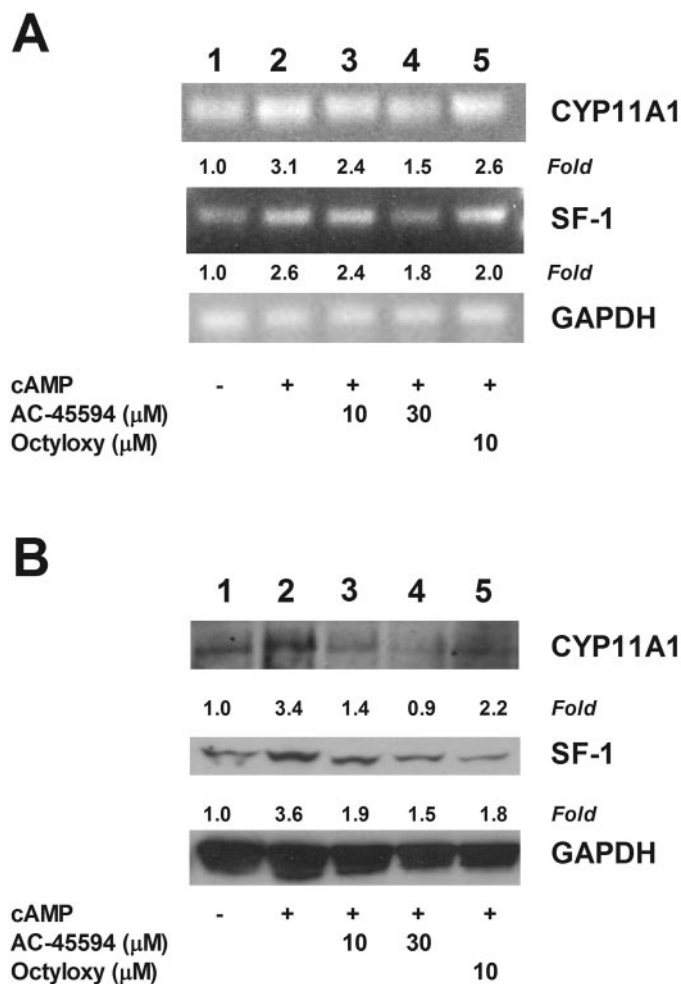


Fig. 5. cAMP-induced expression of CYP11A1 and SF-1 is negatively modulated by a SF-1 inverse agonist. To induce expression of CYP11A1 (CYP450scc) and SF-1, human H295 cells were treated with cAMP (300 μ M) for 24 h. Before that, a subset of cells was pretreated for 48 h with AC-45594 and the octyloxy analog at different doses (10 and 30 μ M). mRNA (A) and protein (B) cell extracts were analyzed by RT-PCR and Western blot, respectively. Expression levels were quantified and normalized relative to the GAPDH control.

We have developed a novel way of assessing the functional activity of the orphan nuclear receptor SF-1. R-SAT offers a number of unique features that distinguish it from other commonly used HTS technologies. For instance, the length of the assay and the large biological separation between stimulus and response offer several distinct advantages, including simple, low-cost detection, improved assay enablement, signal amplification, and ultra-high throughput. Having the measured response located well downstream of the ligand-receptor interaction allows for the capture and integration of multiple signaling responses into a single homogeneous output. Therefore, when dealing with assay enablement, it becomes possible to employ a large number of signaling intermediates to enable and augment the functional response of SF-1. Moreover, the high sensitivity to constitutive responses (i.e., in absence of ligand) paves the way for rapidly developing nuclear orphan receptor assays. Limitations also exist primarily relating to the nature of the cell-based assay (cell toxicity, nonselective effects at high drug concentration). Using such strategies, it is possible to build a nearly homogeneous functional screening platform within and across genetic families to support a chemical genomics approach to drug discovery. Indeed, we have successfully developed R-SAT assays for all of the reported (known and orphan) human nuclear hormone receptors (data not shown). Overall, R-SAT applied to the nuclear receptors constitutes a powerful tool to identify and follow-up with novel chemistries.

The HTS campaign took advantage of a very diverse library of small-molecule organic compounds and successfully identified the first synthetic SF-1 inverse agonist pharmacophore. The extremely low hit rate observed in this screening effort and the fact that not all hits conform to Lipinski's rule speak to the need for an extremely diverse chemical library. Indeed, recent discussions in the field support the fact that therapeutic drugs can be successfully developed even though they do not abide by Lipinski's rule. Thus, a scaffold such as the one determined by AC-45594 and analogs represents a viable avenue for chemical optimization, in that it proved to be a pharmacologically suitable tool *in vitro* and displayed favorable bioavailability *in vivo* (see below).

SF-1 is most closely related to the orphan nuclear receptor LRH-1 (FTF, CPF, NR5A2); as such, they both are classified within the same nuclear receptor subfamily NR5A. Both receptors are essential for normal embryonic development and adult steroid synthesis (Parker and Schimmer, 1997; Fayard et al., 2004). Unlike most nuclear receptors, they display high constitutive activity and act as monomers, rather than homo- or heterodimers. In addition, their ligand binding domains (LBDs) share significant structural homology, both in terms of sequence identity (Wang et al., 2005) and by the presence of constitutively bound phospholipids in the LBDs (Ingraham and Redinbo, 2005). Additional studies have demonstrated that both receptors preferentially bind phosphatidyl inositol bis- and trisphosphate species (Krylova et al., 2005). Uncovering a class of compounds that was selective for SF-1 and did not cross over to LRH-1 was both unexpected and encouraging. It follows that there might exist natural ligands (whether phospholipids or not) with distinguishable affinities for SF-1 versus LRH-1. Such ligands would promote a noncoordinated regulation of SF-1 and LRH-1 biological functions. Synthetic ligands such as AC-45594 would permit the investigation of such behaviors.

The relative chemical similarities between the alkoxyphenol class defined by AC-45594 and alkylphenols is intriguing. Alkylphenols are environmental substances that display weak estrogenic properties (Tabira et al., 1999) and differ from the alkoxyphenols merely by the absence of an oxygen atom. Although alkylphenols show estrogen agonism but no SF-1 activity, alkoxyphenols are potent SF-1 inverse agonists with no evident estrogenic activities. Thus, in addition to the contribution made to the activity by the length of the alkyl chain, the oxygen group linking the alkyl chain and the phenyl ring plays a determining role. This could be due either to the hydrogen bond accepting properties of the oxygen or to a conformational change forced by the oxygen on the alkyl part compared with the carbon analog. Although the methylene connected to the ether oxygen would be in the aromatic plane of the phenyl group, the corresponding carbon in the alkylphenols is more likely to be positioned out of the aromatic plane (above/below) in a low-energy conformation, placing the alkyl chain in a different position and direction.

Sphingosine was recently shown to behave as an inhibitor of SF-1 activity (Urs et al., 2006); in R-SAT, sphingosine showed only marginal activity at up to 10 μ M (data not shown). Similarly to the AC-45594 pharmacophore, sphingosine shares the alkyl chain fragment. However, AC-45594 contains a phenol moiety that has a crucial role in maintaining activity, whereas sphingosine contains an amine base function, indicating that these molecules are not related analogs.

Interestingly enough, the SF-1/LRH-1 dual agonist recently identified by Whitby et al. (2006) also contains an alkyl chain. Although not structurally interested to either AC-45594 or sphingosine, it is intriguing that chemistries modulating SF-1 activity identified so far share this alkyl moiety. This is consistent with recent structural studies describing phosphatidyl inositols (which contain an alkyl chain) as SF-1 ligands (Krylova et al., 2005; Ortlund et al., 2005; Wang et al., 2005). All together, these findings suggest that interaction with SF-1 can be limited in agreement with the relatively small size of the ligand binding pocket (Wang et al., 2005).

SF-1 has been proposed to be a key regulator of steroid biosynthesis. Studies involving gene knockout, heterologous expression of SF-1, and indirect activation of SF-1 have contributed to this picture. However, because of the lack of a natural or synthetic ligand for SF-1, no pharmacological studies were possible until very recently (Urs et al., 2006). In a physiological assay of steroidogenesis, we demonstrated through the administration of AC-45594 or the octyloxy analog that the pharmacological inhibition of SF-1 results in decreased expression of SF-1 target genes StAR and CYP11A1 at both the mRNA and protein levels. It is noteworthy that inhibition of SF-1 by AC-45594 and the octyloxy compound also results in reduced expression of SF-1 itself. In all cases, the effects on both the mRNAs and protein levels were consistent with each other, suggesting that the pharmacological inhibition of SF-1 by AC-45594 and analogs results in inhibition of SF-1-mediated transcription, leading to reduced expression of SF-1 and SF-1 target genes.

Our findings lend strong support to the evolving hypothesis of ligand regulation of constitutively active nuclear receptors, whose biological functions were previously thought to be nonamenable to pharmacological modulation. Small-mole-

cule dual agonists of LRH-1 and SF-1 were recently identified and shown to affect the expression of SHP, a target gene of LRH-1, in hepatocytes (Whitby et al., 2006). Other groups have reported findings that further strengthen this hypothesis, in particular with the identification of synthetic ligands for the Nurr family orphan nuclear receptors (Dubois et al., 2006; Hintermann et al., 2007).

In conclusion, AC-45594 defines a class of alkoxyphenol compounds that can be useful chemical tools to further probe into the biological function of SF-1 in both in vitro and in vivo systems. Preliminary pharmacokinetic data revealed that in rats, AC-45594 has a half-life of approximately 30 min and plasma exposure levels of approximately 1 μ M, suitable for in vivo testing. Studies to identify analogs with an improved profile are under way. Short- and long-term animal studies using AC-45594 or related SF-1 inverse agonists will help further define the therapeutic potential of this orphan nuclear receptor. For example, an SF-1 inverse agonist, by suppressing both adrenal androgen and gonadal testosterone synthesis, could have therapeutic utility in the treatment of prostate cancer.

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