Title: 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.
INTRODUCTION

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 primarily found 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 as a consequence 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, the 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 (VMH) (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 VMH (Dhillon et al., 2006). Moreover, individuals 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. Non-ligand 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 various oxysterols (Lala et al., 1997) could not be confirmed by other investigators (Desclozeaux et al., 2002; Mellon and Bair, 1998). Recently, the crystal structure of the ligand binding domain (LBD) of SF-1 was solved by several groups independently (Krylova et al., 2005; Li et al., 2005;
Madauss et al., 2004; 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, filled with phospholipids. Similar findings were reported with LRH-1, the most closely related receptor to SF-1 (Krylova et al., 2005; Ortlund et al., 2005; Wang et al., 2005). Further characterization indicates that SF-1 preferentially binds eukaryotic PIP2 and PIP3 phosphatidylinositols, 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-butoxyphenol, 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 UV/MS. The analyses were performed on a Waters/Micromass ZQ2000 LC/MS instrument consisting of a ZQ single quadropole mass spectrometer equipped with an electrospray ionization interface, and a Waters Alliance HT with a 2795 Separation Module and 996 Photodiode Array Detector (PDA). HPLC Method: Mobile Phase: A: 10mM NH4OAc H2O; B: 10mM NH4OAc CH3CN-H2O (95:5). Column: Waters Xterra® MS C18 3.5µm, 30x4.6mm ID 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. PDA range: 190-450nm.

R-SAT® assays

R-SAT® (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 GPCRs, RTKs, cytokine receptors and nuclear receptors (Burstein et al., 2006; Piu et al., 2006; Piu et al., 2002). 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 transformation via the loss of contact inhibition and of growth factor dependency. Monitoring is achieved by transfecting the cells with a β-galactosidase reporter gene vector whose expression is under a constitutively active promoter. Briefly, NIH/3T3 fibroblasts were plated overnight in 96-wells plates in DMEM 10 % calf serum (Hyclone) and grown to 60-70 % confluency prior to transfection. Transient transfections were performed using Polyfect (Qiagen) according to manufacturer's instructions. Typically a transfection mix would consist of the receptor and the β-galactosidase expression vectors. Sixteen hours post-transfection, cells were incubated with different doses of ligand in DMEM containing 30% Ultraculture (Hyclone) 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 ONPG (in phosphate-buffered saline with 5% Nonidet P-40 detergent) as described (Piu et al., 2002). Plates were read using a microplate reader at 420nm. 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, \( c = \) EC50, \( r = \) response, and \( x = \) concentration of ligand. Curves were generated using the curve fitting softwares Excel Fit and GraphPad Prism (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': CTAGCTCAAGGTCACTACAGTGCAAGTCAAGTCAAGGCATAA; 3': GAGTTCCAGTGTCCAGTCTAG (SFRE underlined). The StAR promoter (-1300, +39) was kindly provided by Dr. Strauss III. HEK-293T cells were maintained in DMEM containing 10% FBS. Cells were plated at 20,000 cells/well in a 96-well plate 18-24 hours prior to transfection. Transient transfections were performed in 10% Charcoal stripped FBS-DMEM using Polyfect (Qiagen, Valencia, CA) according to manufacturer's instructions. Cells were transfected with 60 ng/well SF-1 encoding plasmid (or same amount of LRH-1) and the 20 ng/well SFERE luciferase vector (or StAR promoter), in addition to 0.4 ng/well vectors encoding GRIP1, SRC-1 and DRIP205. Sixteen hours post-transfection, compounds were added at various doses in media without serum. After 36 hours of incubation, media was removed, cell extracts isolated and luciferase activity was measured using a commercially available kit (SteadyGlo, Promega, Madison, WI).

**cAMP-induced mRNA expression of SF-1 target genes**

Briefly, H295 adrenocortical carcinoma cells (ATCC, Manassas, VA) were plated at 1.7 million cells/35 mm dish in DMEM/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 (CS media) (Hyclone, Logan, UT). All subsequent additions were made in CS media. One hour
after media exchange, cells were treated with compound or DMSO. After 48 hours of treatment, cells were treated with compound and (Bu)$_2$cAMP (300 µM). After 24 hours of (Bu)$_2$cAMP 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 for that 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 MgSO$_4$. Oligonucleotide primers were synthesized by MWG Biotech (High Point, NC). Primer sequences were: 5’-CCAGATGTGGGCAAGGTG-3’ (sense) and 5’-CAGCGCACGCTCACAAAG-3’ (antisense) for StAR, 5’-CGAGCCACATCGCTCAGACAC-3’ (sense) and 5’GCTAAGCAGGGTGGTGCAGG-3’ (antisense) for GAPDH, 5’-GACAAGGTTGTCCG GCTACCAC-3’ (sense) and 5’-GTCTCCAGGTGAAGCCATTGG-3’ (antisense) for SF1, 5’-CAAGACCTGGAAGGACCATGTG-3’ (sense) and 5’-GATATCTCTGCAGGGTCACGGAG-3’ (antisense) for CYP11A1. The sizes of the amplified bands were 227 bp (StAR), 495 bp (GAPDH), 413bp (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 seconds), 58°C (30 seconds), and 68°C (50 seconds) for 30 cycles. GAPDH RT-PCR cycling conditions were 95°C (50 seconds), 58°C (30 seconds), and 68°C (50 seconds) for 20 cycles. SF-1 RT-PCR cycling conditions were 95°C (50 seconds), 56°C (30 seconds), and 68°C (50 seconds) for 40 cycles. CYP11A1 RT-PCR cycling conditions were 95°C (50 seconds), 58°C (30 seconds), and 68°C (50 seconds) for 25 cycles. Following RT-PCR, products were visualized on a 1.5% agarose gel, and the bands were quantitated using Scion Image.

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 22-25 ug of protein were loaded onto each gel and Western blotting was performed using
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 quantitate 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. Receptor Selection and Amplification Technology (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 (Burstein et al., 2006; Piu et al., 2006; Piu et al., 2002). 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 to reporter alone. Higher levels (80 mg/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 classical 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 as 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 (Borud et al., 2003; Ito et al., 1998; Li et al., 2005). We also uncovered 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 likeness 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 IC50 of 50-100 nM in the R-SAT® assay (Fig. 2A). Analysis of analogs of AC-45594 varying in their alkyloxy chain length revealed that 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 µM. 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). Interestingly, 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 (Mangelsdorf et al., 1995). Mapping studies have revealed that additional bases 5’ to the half-site determine the binding selectivity for each nuclear receptor, with 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 while 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, 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.
As SF-1 primarily functions 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 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 where 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 IC50s for all compounds tested ranged from 500-5000 nM. Additionally, we investigated 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 IC50 values consistent with the ones observed using the synthetic SFRE construct. Further, we confirmed that AC-45594 did not affect the activity of LRH-1 in that same system. In conditions where 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 µM (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. Similarly, steroidogenesis-stimulating hormones and their cognate receptors are primarily modulated through SF-1. While 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, Steroidogenic Acute Regulatory protein (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.
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 µM, and thus was only evaluated at 10 µM. Detectable levels of StAR mRNAs were evident in non treated H295 cells using quantitative RT-PCR (Fig. 4A). Treatment with cAMP increased StAR transcripts by about 3-fold within 24h 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, following cAMP treatment, StAR increased by 30-fold after 24 hours (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 about 3-fold (Fig 5A). That activation was completely abolished in the presence of AC-45594 at 30 µM, and partially with the octyloxy at 10 µM. At the protein level (Fig. 5B), cAMP induction increased CYP11A1 levels by about 3.5 –fold, an effect that was reversed in 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, as constitutive expression of the mutant impaired expression of endogeneous 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. Following cAMP addition, SF-1 mRNAs were increased reproducibly by about 2.5-fold (Fig. 5A). The addition of 10 µM AC-45594 did not inhibit SF-1 induction by cAMP, while 30 µM AC-45594 partially inhibited this response. The octyloxy analog partially reversed the SF-1 mRNAs induction at 10uM. Similarly, SF-1 protein levels were increased by a factor of about 3.5-fold by cAMP (Fig. 5b). Pretreatment with 30 µM AC-45594 and 10 µM 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.

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, non selective 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 pharmcophore. The extremely low hit rate observed in this screening effort and the fact that not all hits conform to the Lipinski’s rule speaks 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 the Lipinski’s rules. Thus, a scaffold such as one determined by AC-45594 and analogs represent a viable avenue for chemical optimization, as it prove 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) and 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 (Fayard et al., 2004; Parker and Schimmer, 1997). 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 PIP2 and PIP3 phosphatidyl inositol 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 non-coordinated regulation of SF-1 and LRH-1 biological functions. Synthetic ligands such as AC-45594 would permit to investigate such behaviors.

The relative chemical similarities between the alkyloxyphenol 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 alkyloxyphenols merely by the absence of an oxygen atom. While alkylphenols show estrogen agonism but no SF-1 activity, alkyloxyphenols 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 to either the hydrogen bond accepting properties of the oxygen or of a conformational change forced by the oxygen on the alkyl part compared to the carbon analog. While 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 only showed marginal activity at up to 10uM (data not shown). Similarly to the AC-45594 pharmacophore, sphingosine shares the alkyl chain fragment. However, AC-45594 contains a phenol moiety which has a crucial role in maintaining activity while 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 contain an alkyl chain. While not structurally interested to either AC-45594 or sphingosine, it is intriguing that chemistries modulating SF-1 activity identify 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). Altogether, these findings suggests 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, indirect activation of SF-1, have contributed to portray 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 ocyloxy 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. Interestingly, inhibition of SF-1 by AC-45594 and the ocyloxy 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 result 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 non amenable to
pharmacological modulation. Small molecule 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., 2006).

In conclusion, AC-45594 defines a class of alkyloxyphenol compounds which can be useful chemical tools to further probe into the biological function of SF-1 both in in vitro and in vivo systems. Preliminary pharmacokinetic data revealed that in rats AC-45594 has a half life of about 30 min and plasma exposure levels of about 1uM, suitable for in vivo testing. Studies to identify analogs with an improved profile are underway. Acute and chronic 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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REFERENCES


Vanacker JM, Pettersson K, Gustafsson JA and Laudet V (1999) Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER) alpha, but not by ERbeta. *Embo J* 18(15):4270-4279.


FIGURE LEGENDS

Figure 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 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 OD measured at 420 nm). The RAR beta2 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 uM), a reported agonist ligand of RAR beta2 (Piu et al., 2006). All Co-Act = 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.

Figure 2: AC-45594 is a selective and potent inverse agonist at human SF-1. (A) High DNA amounts of human SF-1 were transiently co-transfected along with the coactivators DRIP205, GRIP1 and SRC-1, then treated with various doses of AC-45594, and the activity measured using R-SAT®. Data is reported as FLU (arbitrary fluorescent light units) as the assay was performed using a RFP 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 includes GRIP1, SRC-1 and DRIP205, treated with AC-45594 at various doses, and the relevant pharmacological response (agonism, inverse agonism) evaluated through R-SAT®. The respective amounts of transfected receptors vary in each assay as to fine tune the assay for sensitivity towards agonism (low receptor amount) and inverse agonism (high receptor amount). Data are reported as the maximum fold change relative to non-treated conditions for each receptor (that is vehicle), 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 alpha, ER beta, LRH-1, ERR beta and NOR1 translate in an about 2-fold increase with weak potency (pEC50 5.5-6.0) (data not shown). * : p value <0.05.

**Figure 3: AC-45594 and analogs inhibits SF-1 transcriptional activation.** High DNA amounts of human SF-1 were transiently co-transfected along with the coactivators DRIP205, GRIP1 and SRC-1, along with a synthetic Luciferase reporter gene containing 3 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 48hrs. Data are reported as luciferase (RLU, relative luminescent units).

**Figure 4: SF-1 inverse agonists inhibit StAR expression in human adrenocortical cells.** Human adrenocortical H295 cells were treated for 24 hours with cAMP (300 uM) in order to induce endogeneous mRNA (A) and protein (B) expression of Steroidogenic Acute Regulatory (StAR) protein, a known SF-1 target gene. Cells were pretreated or not for 48 hours with AC-45594 and the octyloxy analog at different doses (10 and 30 uM). Cell extracts were made, mRNA and protein levels detected and quantified by RT-PCR and Western blots, respectively. Fold activation was relative to non-treated cells and normalized relative to the GAPDH control.

**Figure 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 uM) for 24 hours. Prior to that, a subset of cells were pretreated for 48 hours with AC-45594 and the octyloxy analog at different doses (10 and 30 uM). 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.
Table 1: Influence of the length of the alkyloxy chain on SF-1 activity.

Compounds with an alkyloxy chain ranging from \( n=4 \) to \( n=10 \) were tested using R-SAT\(^\circ\). 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 defined as 100% inhibition (\% Inh). NA = not active at 10 \( \mu \)M.

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<td>103 ± 14</td>
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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 (pEC50 = - Log [EC50]), relative to a standard reference. Antagonist and inverse agonist data are reported as pKi (- Log [Ki]) and pIC50 (- Log [IC50]), 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 as no reference ligand is available; for ER alpha and ER beta agonism, estrone (ERa pEC50 9.9 ± 0.4, ERb 9.8 ± 0.3); for ER alpha and ER beta antagonism, 4OH-tamoxifen (ERa pKi 8.6 ± 0.2, ERb 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. NA = not active at 10 μM.

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Figure 1

A. O.D. (420 nm) vs. SF-1 DNA (ng/well)

B. O.D. (420 nm) for different conditions
Figure 2

A. 

![Graph showing fluorescence units vs. AC-45594 concentration](image)

AC-45594 [M]

B. 

![Graph showing fold change relative to control](image)

Fold Change relative to control

SF1, LRH-1, ERα, ERβ, ERRα, ERRβ, ERRγ, Nur1, Nur77, NOR-1, RORα, RORβ, RORγ
Figure 3

A. [Graph showing RLU vs. Ligand [M] for different ligands: AC-45594, hexyloxy, octyloxy.]

B. [Graph showing RLU vs. Ligand [M] for different ligands: AC-45594, hexyloxy, octyloxy.]
**Figure 4**

**A.**

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**StAR**

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**GAPDH**

| cAMP | -  | +  | +  | +  | +  |
| AC-45594 (uM) | 10 | 30 |
| Octyloxy (uM) | 10 |

**B.**

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**StAR**

| 37 kD |  | 28 kD |  |

| 50 kD |  |      |  |

**GAPDH**

| cAMP | -  | +  | +  | +  | +  |
| AC-45594 (uM) | 10 | 30 |
| Octyloxy (uM) | 10 |
Figure 5

A.

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- 33 -