Identification of Small Molecule Agonists of the Orphan Nuclear Receptors Liver Receptor Homolog-1 and Steroidogenic Factor-1

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Abstract: We report the identification of substituted *cis*-bicyclo[3.3.0]-oct-2-enes as small molecule agonists of subfamily V orphan nuclear receptors (NR5A), liver receptor homolog-1 (LRH-1) and steroidogenic factor-1 (SF-1). Using fluorescence resonance energy transfer (FRET)-based biochemical assays, compound **5a** (GSK8470) was identified as a high-affinity ligand for LRH-1 and SF-1. In liver cells, **5a** increased the expression of the LRH-1 target gene small heterodimer partner (SHP). Synthesis of analogues modified at three positions led to the development of compounds with functional selectivity between LRH-1 and SF-1.

Nuclear receptors (NRs)[a] are a large family of mammalian transcription factors that function as the molecular targets for widely prescribed medicines and therapeutic agents in clinical development.[1] The activity of many NRs is controlled by small lipophilic ligands such as steroid hormones, retinoids, vitamin D, and thyroid hormone. On the other hand, a multitude of NRs for which the natural ligands are unknown have been termed "orphan" receptors.[2] We have a particular interest in the liver receptor homolog-1 (LRH-1)[3] and steroidogenic factor-1 (SF-1),[4] members of the subfamily V nuclear receptors (NR5A) for which no small molecule ligands have been reported.

LRH-1 plays a critical role in embryonic development of the endoderm.[5] In adults it is expressed in the intestine, liver, exocrine pancreas, and ovary. LRH-1 regulates the expression of genes involved in hepatic bile acid biosynthesis and cholesterol homeostasis.[3] Thus, the receptor may be a target for the treatment of cardiovascular disease. Its regulation of aromatase expression also suggests a possible utility in cancer therapy.[6,7] SF-1 plays an important role in sex determination during development. SF-1 knockout mice were phenotypically female independent of genetic sex.[8] The receptor is expressed in the testes, ovaries, and adrenal cortex where it regulates many genes involved in steroid hormone production.[4,9] It is also expressed in the hypothalamus and has been implicated in the regulation of feeding behavior.[10,11]

Unlike most NRs, LRH-1 and SF-1 bind to DNA as monomers and show constitutive activation of transcription when expressed in cells.[3,4] Receptor activity can be regulated by phosphorylation, sumoylation, or through interaction with the atypical orphan receptors SHP (NR0B1)[12] and DAX-1 (NR0B2)[13] that lack their own DNA-binding domains (DAX-1 = dosage sensitive sex reversal - adrenal hypoplasia congenita gene on the X chromosome, gene 1). Recently, X-ray crystallography coupled with mass spectroscopy has revealed the presence of phospholipids in the ligand binding pockets of both human LRH-1 and SF-1.[14−17] The functional role of phospholipids in the regulation of receptor activity remains to be established and is further confounded by the absence of a lipid in the pocket of the mouse LRH-1.[18] However, the knowledge that human LRH-1 and SF-1 can bind to phospholipids suggests that the receptors are chemically tractable and has further stimulated interest in their potential role as therapeutic targets for drug discovery. The identification of a synthetic activating ligand would be a valuable chemical tool to elucidate their function in mammalian physiology.[2]

A high throughput screen using a fluorescence resonance energy transfer (FRET)-based assay to detect the interaction of the ligand binding domain of human LRH-1 with a peptide derived from the coactivator TIF2 (amino acids 737−757) led to the identification of the unusual bicyclic compound **5a**[19] as a potent ligand with EC_{50} = 430 nM [TIF2 = transcriptional intermediary factor 2 (NCOA2)]. It also proved to be an effective ligand for human SF-1 with EC_{50} = 54 nM in a FRET-based assay for recruitment of a peptide derived from DAX-1 (amino acids 1−23). In both assays, the apo-receptors were initially present with 1 equiv of phospholipid bound, and it is remarkable that the small nonpolar molecule **5a** was able to displace these large endogenous lipids from their pockets. The potent binding affinity of **5a** and rigid *cis*-bicyclo[3.3.0]oct-2-ene core structure made the series an attractive template for structure−activity studies. As well as establishing the requirements for high-affinity binding, we were particularly keen to discover compounds with selectivity for LRH-1 over SF-1 and vice versa. Compounds were designed to define the ligand−receptor pharmacophore at three positions (R^1−R^3 in Table 1).

Compound **5a** was produced in a one-pot tandem reaction sequence in which initial co-cyclization of a 1,6-enyne **1** using the Negishi reagent (Cp_2ZrBu_2)[20] to afford a zirconacyclopentene **2** was followed by insertion of phenyl isonitrile to generate the potent carbometalating reagent, zirconocene η^2-imine **3** (Scheme 1). Subsequent insertion of 4-octyne into the carbon−zirconium bond followed by protonolysis gave the bicyclic amine **4a**. Unexpectedly, **4a** underwent a facile rearrangement to afford the 1-amino-*cis*-bicyclo[3.3.0]oct-2-ene compound **5a** as revealed by a small molecule X-ray structure determination.[19] Variation of the starting enyne **1** allowed the synthesis of a series of analogues **5b**−**5g** with modification in the R^1 group (Table 1). Two methods were used to accomplish the rearrangement of the initial products **4** to the required bridgehead amines **5**. Initially, for compounds **5a**−**5c**, **5h**, and **5j**, we relied on the spontaneous rearrangement that occurred partly during the initial workup with methanol/water and completed during chromatography on silica (method A). A drawback was that the product **5** decomposes on silica, making the yields and initial purity of the products capricious. Subsequently, for compounds **5d**−**5g**, **5i**, and **5k** we isolated the initial amine product **4** by protonation

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[a] Abbreviations: DAX-1, dosage sensitive sex reversal - adrenal hypoplasia congenita gene on the X chromosome, gene 1; FRET, fluorescence resonance energy transfer; LRH-1, liver receptor homolog-1; NR, nuclear receptor; SHP, small heterodimer partner; SF-1, steroidogenic factor-1; TIF2, transcriptional intermediary factor 2 (NCOA2).

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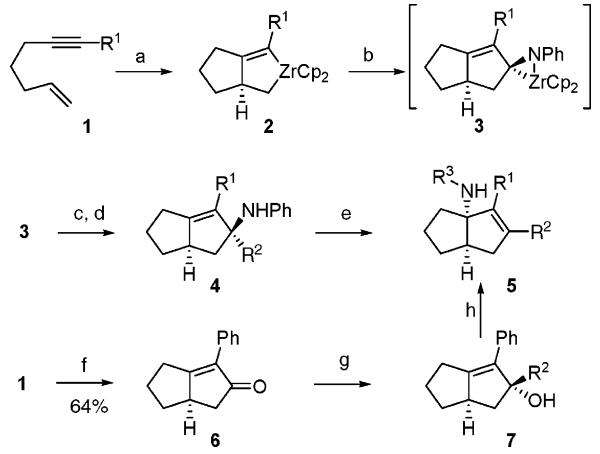
Table 1. Synthesis and Biological Activity of 1-Arylamino-*cis*-bicyclo[3.3.0]oct-2-enes

						LRH-1[c]			SF-1[c]		
cmpd	method[a]	yield (%)[b]	R^1	R^2	R^3	EC_{50} (μM)	±SD	RE[d] (%)	EC_{50} (μM)	±SD	RE[d] (%)
5a	A	53	Ph	(*E*)-4-oct-4-enyl	Ph	0.43	0.07	100	0.054	0.008	50
5b	A	61	Me	(*E*)-4-oct-4-enyl	Ph	0.09	0.04	15	>10		
5c	A	68	*n*-C_4H_9	(*E*)-4-oct-4-enyl	Ph	0.022	0.007	50	0.030	0.001	95
5d	B	53	*c*-C_6H_{11}	(*E*)-4-oct-4-enyl	Ph	0.012	0.002	50	0.010	0.002	90
5e	B	19	4-Br-C_6H_4	(*E*)-4-oct-4-enyl	Ph	0.16	0.01	70	0.17	0.07	35
5f	B	27	3-MeO-C_6H_4	(*E*)-4-oct-4-enyl	Ph	0.10	0.01	115	0.14	0.01	45
5g	B	35	2-naphthyl	(*E*)-4-oct-4-enyl	Ph	0.06	0.01	55	0.13	0.05	30
5h	A	34	Ph	(*E*)-3-hex-3-enyl	Ph	1.4	0.2	50	0.8	0.1	80
5i	B	49	Ph	(*E*)-6-dodec-6-enyl	Ph	>10			0.014	0.003	30
5j	A	7	Ph	CH(Me)C_4H_9	Ph	0.07	0.01	45	0.13	0.05	90
5k	B	42	Ph	CH(Me)C_8H_{17}	Ph	0.32	0.05	25	0.13	0.04	95
5l	C	58	Ph	Me	Ph	2.1	0.7	20	>10		
5m	D	29	Ph	*n*-C_6H_{13}	Ph	0.034	0.003	45	0.043	0.008	100
5n	D	27	Ph	*c*-C_6H_{11}	Ph	0.23	0.02	25	0.18	0.07	65
5o	C	39	Ph	*n*-$C_{12}H_{25}$	Ph	>10			>10		
5p	D	34	Ph	Ph	Ph	0.15	0.02	35	0.12	0.02	55
5q	D	29	Ph	*n*-C_6H_{13}	3-F-C_6H_4	0.33	0.004	45	0.56	0.02	70
5r	C	20	Ph	*n*-C_6H_{13}	4-Cl-C_6H_4	1.0	0.7	15	0.7	0.3	25
5s	D	13	Ph	*n*-C_6H_{13}	2,3-diMe-C_6H_4	0.03	0.01	30	0.011	0.008	20
5t	D	30	Ph	*n*-C_6H_{13}	4-OCF_3-C_6H_4	>10			>10		
5u	D	16	Ph	*n*-C_6H_{13}	4-*tert*-Bu-C_6H_4	>10			>10		
5v	D	28	Ph	*n*-C_6H_{13}	4-I-C_6H_4	>10			>10		
5w	C	14	Ph	*n*-C_6H_{13}	1-naphthyl	1.2	0.3	45	>10		

[a] See text and Scheme 1 for description of methods A−D. [b] Based on enyne **1** for methods A and B, and on cyclopentenone **6** for method C and D. [c] All biological data $n = 3$. [d] RE, relative efficacy normalized to **5a** for LRH-1 and **5m** for SF-1, ±5%.

Scheme 1[a]



[a] Reagents and conditions: (a) Cp_2ZrBu_2, −78 °C to 20 °C, then 20 °C, 2 h; (b) PhNC (0.95 equiv) −35 °C to 20 °C; (c) RC≡CR Or RCH=CH_2, 20 to 60 °C, 2 h; (d) MeOH/H_2O or aq. $NaHCO_3$; (e) Method a: MeOH, then SiO_2 or Method B: aq. $NaHCO_3$ then $PhNH_2$ (10 equiv), camphorsulfonic acid (0.1 equiv) 20 or 50 °C, 2 h; (f) $Co_2(CO)_8$, DMSO (6 equiv), THF, 50 °C, 4.5 h; (g) R^2MgBr, $CeCl_3$; (h) R^3NH_2 (10 equiv), camphor sulfonic acid (0.1 equiv), 20 °C, 16 h or 65 °C, 2−6 h.

of the zirconacycle under basic conditions (aqueous $NaHCO_3$) and treated the crude product with 10 equiv of aniline and 0.1 equiv of camphor sulfonic acid to effect the rearrangement to **5**. Purification of the final product was accomplished by chromatography on basic alumina (Method B). Generally, the yields of the latter method were lower than the former but less variable.

Variation of the substituent R^2 was initially accomplished by using different alkyne and alkene traps for the intermediate η^2-imine complex **3** (Scheme 1) to provide compounds **5h**−**5k** (Table 1). An alternative route to the same *cis*-bicyclo[3.3.0]-oct-2-ene structures was developed based on Pauson−Khand cyclization[21,22] of enyne **1** to afford the bicyclic cyclopentenone **6**. The cerium chloride promoted[23] addition of various Grignard reagents (R^2MgX) gave the tertiary alcohols **7** in good yield. Subsequent acid-catalyzed displacement of the hydroxyl moiety with aniline gave the desired amines **5l**−**5p** (Table 1) in variable yields (methods C and D). Although the tertiary alcohols **7** could be obtained pure, their instability toward acid meant that higher overall yields often resulted if they were used crude (method D). The exceptionally facile acid-catalyzed displacement of the hydroxyl with an amine is probably driven by release of strain energy from the starting bicyclo[3.3.0]oct-1-ene. It was inconvenient to use different isonitriles to allow variation in the group R^3 in the zirconium route, so we chose to employ a range of anilines to displace the alcohol in compound **7** (R^2 = *n*-hexyl) to afford the amines **5q**−**5w** (Table 1).

The compounds **5a**−**5w** were screened for activity against both LRH-1 and SF-1 using FRET-based peptide recruitment assays, and the results are shown in Table 1. The data are presented as an EC_{50}, which serves as a measure of the binding affinity for the receptor, and the relative efficacy at peptide recruitment, which in the absence of a known standard was normalized to **5a** for LRH-1 and **5m** for SF-1. Several trends were discerned in the data. Changing R^1 from aryl to a similar size alkyl group increased the binding to LRH-1 but lowered the relative efficacy, as seen in the comparison of **5a** with **5c** and **5d**. For example, compound **5d** (EC_{50} = 12 nM) is around 40 times more potent than **5a**, although efficacy is reduced by half. On SF-1, a different structure−activity relationship was observed since **5c** and **5d** showed only a small increase potency compared to **5a**, but a large increase in relative efficacy. Larger aryl groups (**5e**−**5g**) gave mixed results on both receptors, suggesting a limit to the size of the lipophilic pocket. Compound **5f**, with a 3-methoxy substituent on the aryl ring, is interesting as it slightly increases both binding affinity and efficacy on LRH-1 relative to **5a**. A methyl group at either R^1 or R^2 (e.g., **5b** and **5l**) yielded compounds with low efficacy on LRH-1 and no measurable activity on SF-1. A larger branched alkenyl group at R^2 was tolerated in SF-1 but not in LRH-1, resulting in the identification of the functionally selective analogue **5i**. Other alkyl, cycloalkyl, and phenyl substituents at R^2 (e.g., **5m**−**5p**) showed parallel changes in activity. Increase in the size of R^3

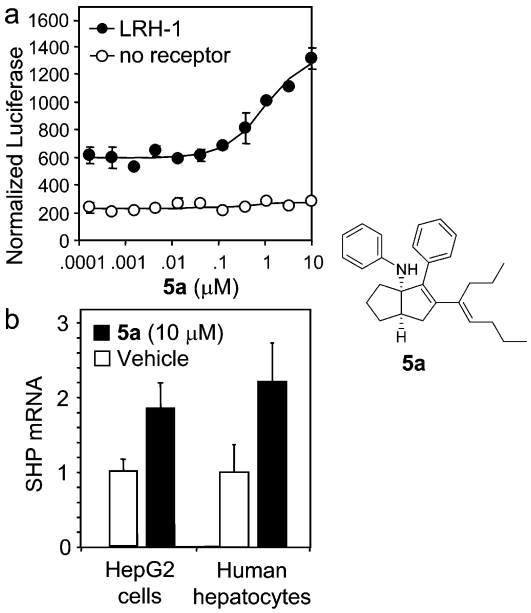


Figure 1. Agonist activity of **5a** in cells: (a) transactivation of a transiently expressed SHP reporter gene in CV-1 cells; (b) induction of SHP expression in hepatocytes.

or the presence of polar substituents (e.g., **5t**−**5v**) was poorly tolerated in both receptors. However, the 1-naphthylamine substituted analogue (**5w**) retained some activity on LRH-1 with functional selectivity over SF-1.

We had previously established that LRH-1 regulated the expression of SHP within an autoregulatory feedback loop to control cholesterol metabolism in the liver.[24] To demonstrate the cellular activity of the amino-*cis*-bicyclo[3.3.0]oct-2-enes, we used a heterologous reporter assay in which cells were transfected with an expression vector for human LRH-1 and a reporter construct engineered from the proximal promoter of the human SHP gene fused to luciferase. Coexpression of the receptor and reporter gene led to an increase in luciferase due to the constitutive activity of LRH-1 in the absence of an exogenous ligand (Figure 1a). However, micromolar concentrations of **5a** were able to double the reporter signal in a dose-responsive manner (EC_{50} = ∼1 μM). To further confirm the functional efficacy of **5a** on LRH-1, we treated intact human liver cells with 10 μM of the compound (Figure 1b). In both HepG2 cells and primary hepatocytes, a doubling of the expression of SHP mRNA was measured by quantitative PCR demonstrating that **5a** is a bone fide LRH-1 agonist.

In summary, we have characterized the first small molecule ligands for the orphan nuclear receptors LRH-1 and SF-1, including some that show functional selectivity. Compounds such as **5a** (GSK8470) can be used as chemical tools to investigate the biological function of LRH-1 and SF-1 in cells and to further define the therapeutic utility of these orphan receptors. The *cis*-bicyclo[3.3.0]oct-2-ene skeleton represents an interesting hydrophobic chemotype for development of new nuclear receptor ligands due to its rigidity, low molecular weight, and potential for functionalization at three sites. The primary limitation of the current 1-anilino series is its acid instability, with a typical half-life of around 12 h in the presence of 1 M acetic acid. Importantly, the compounds showed no significant decomposition upon storage for long periods under neutral conditions. Studies to identify analogues with improved acid stability and oral activity are underway.

Supporting Information Available: Synthetic methods and analytical data for compounds in Table 1, and biological methods for the Table 1 and Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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