Probe Report

Title: Discovery of Inverse Agonists for the Liver receptor homologue-1 (LRH1; NR5A2)

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Chemistry Center Name & PI: SRIMSC, Hugh Rosen

Assay Submitter & Institution: Patrick Griffin, The Scripps Research Institute (TSRI)

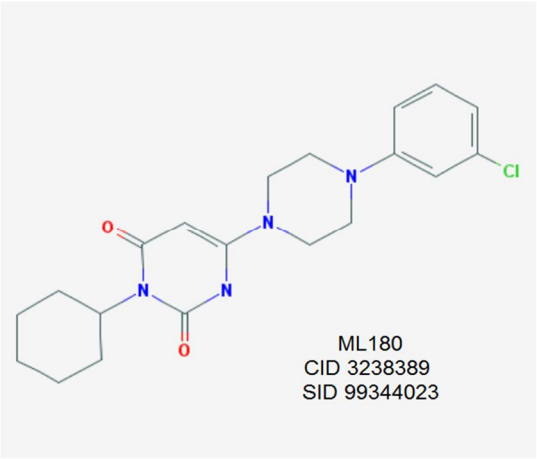
Summary Bioassay Identifier (AID): 488781

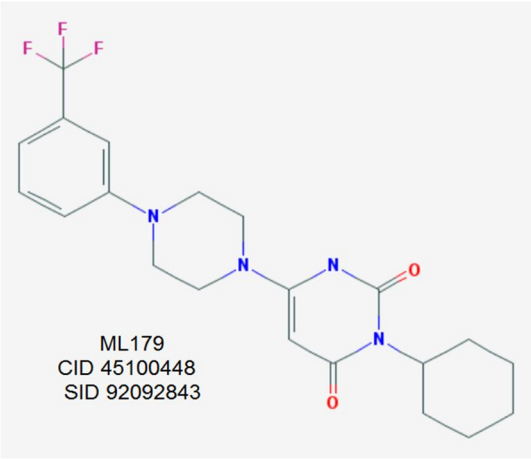
Abstract:

NR5A2 (Liver receptor homologue-1; LRH1) belongs to the four-member NR5A, or Ftz-F1, subfamily V of nuclear receptors. Murine LRH1 was originally identified due to its sequence homology to Drosophila Fushi tarazu factor-1. Orthologs were subsequently identified in other species including rat, chicken, horse, zebrafish and human. LRH1, and its closest family member steroidogenic factor-1 (SF-1, NR5A1), bind to identical DNA consensus sequences (response elements; REs) and both are able to bind phospholipids in their ligand binding domains (LBDs). LRH1 was shown to regulate expression of Cyp19 (aromatase), suggesting LRH1 as a target for inverse agonists for the treatment of ER-positive breast cancers. Interestingly, recently it was shown that LRH1 promotes motility and invasiveness in both ER-positive and ER-negative breast cancer cells (MDA-MB-231), with remodeling of the actin cytoskeleton and E-cadherin processing observed with LRH1 over-expression. These findings suggest that inhibition of LRH1 activity should be useful in attenuating both migration and invasion in ER-positive and ER-negative breast cancers. In addition to LRH1's role in cholesterol metabolism and bile acid homeostasis, the receptor can impact expression of markers of acute phase response (APR) to tissue injury. Thus, we endeavored to identify potent and selective LRH1 inverse agonists which may provide new approaches for the treatment of cancer and to blunt APR. We pursued a Center-based Initiative with transfection studies testing the ability of various chemical scaffolds to inhibit LRH-1-mediated activation of the Cyp19-Aromatase-luciferase and the StAR-luciferase reporter, followed by counterscreening against SF-1 and VP16, as well as studies examining the effect of compounds on LRH-1 modulation of the APR markers haptoglobin and serum amyloid A1 and A4 (SAA1 and SAA4). Combined, the studies led to identification of two novel LRH-1 inverse agonist probe compounds ML179 (PubChem CID 45100448) and ML180 (PubChem CID 3238389) with potent activity in breast cancer cells. In reporter assays ML179 and ML180 had potency IC_{50} values of 320nM and 3.7 µM and maximum efficacy of 40% and 64% repression, respectively. It is unclear at this stage if maximum repression is more important than potency; thus two probes were declared that differ from each other in terms of potency and efficacy. Further optimization is focused on improving both potency and efficacy. Also, it is likely that the selectivity of these probe compounds versus

SF1 is cell context- and promoter-dependent. The mechanism of action of these probes will require much more detailed studies which are currently underway.

Probe Structure & Characteristics: ML179, ML180





CID/ML#	Target Name	IC_{50} (nM) [SID, AID]	Anti-target	IC_{50} (μM) [SID, AID]	Fold Selective	Secondary Assay(s) Name: IC_{50} (nM) [SID, AID]
CID 3238389/ ML180	LRH1 (NR5A2)	3700 nM [SID 99344023, AID 488780] Active	SF1 (NR5A1)	> 10 μM [SID 99344023, AID 488780] Inactive	**>2.7**	**VP16 Fold Change Counterscreen Assay**: 1% [SID 99344023, AID488775] Inactive Mechanism of Action Assays (LRH1 Target Genes) **Haptoglobin QPCR Assay**: 0.12 fold change (at 10 μM) [SID 99344023, AID488769] Active **SAA1 QPCR Assay**: 0.09 fold change (at 10 μM) [SID 99344023, AID488769] Active **SAA4 QPCR Assay**: 0.45 fold change (at 10 μM) [SID 99344023, AID488769] Active **MTT Breast Cancer Cytotoxicity Assay:** [SID 99344023, AID 504928] Active **Star Promoter Counterscreen:** 2.05μM IC_{50} [SID 99344023, AID 504933] Active **Nuclear Receptor Profiling:** [SID 99344023, AID 504934], Active
CID 45100448/ ML179	LRH1 (NR5A2)	320nM [SID 92092843, AID 488780] Active	SF1 (NR5A1)	> 10μM [SID 92092843, AID 488780] Inactive	**>31.25**	**VP16 Fold Change Counterscreen Assay**: -6% [SID 92092843, AID488775] Inactive Mechanism of Action Assays (LRH1 Target Genes) **Haptoglobin QPCR Assay**: 0.14 fold change (at 10 μM) [SID 92092843, AID488769] Active **SAA1 QPCR Assay**: 0.07 fold change (at 10 μM) [SID 92092843, AID488769] Active **SAA4 QPCR Assay**: 0.45 fold change (at 10 μM) [SID 92092843, AID488769] Active **Star Promoter Counterscreen:** 2.12μM IC_{50} [SID 92092843, AID 504933] Active

Recommendations for scientific use of the probe:

- **What limitations in current state of the art is the probe addressing?** Currently there are no inverse agonists described for LRH1. Whitby and coworkers have reported the identification of cis-bicyclo[3.3.0]-oct-2-enes as synthetic agonists for both SF-1 and LRH1 with one compound in particular having the ability to induce the doubling of mRNA levels of SHP, a downstream target of both receptors, in HepG2 cells [14]. While these synthetic ligands are intriguing, several problems remain. First, the functional activity of these compounds was determined using a FRET-based biochemical coactivator recruitment assay using small peptides representing the LXXLL motifs of the cofactor; for LRH1 a peptide derived from TIF2 (SRC2) was used and for SF-1 a 23 amino acid peptide fragment of DAX-1 was used. The complimentary cellular activity of these compounds was described for only one compound. Second, the most potent and efficacious agonists lacked functional selectivity over SF-1. The paper describes two partial agonists of LRH1 that are devoid of SF-1 activity showing promise for the ability to obtain functional selectivity. Perhaps more important was the discovery of a potent partial agonist of SF-1 that was devoid of LRH1 activity, strongly suggesting the ability to discover potent and selective agonists within the NR5A subfamily. To test the utility of these compounds, we synthesized two of the most potent compounds described in the Whitby manuscript and evaluated these molecules for their ability to modulate LRH1 in a cell-based assay. Prior to testing, the structures of these compounds were confirmed by NMR and their high purity was confirmed by LC-MS. Unfortunately, these compounds, although potent in a biochemical assay, appear to be cytotoxic even at modest concentrations. Therefore, the discovery of potent, cell active LRH1 modulators remains a high priority in elucidating the tissue-specific functions of LRH1 and its role in mammalian physiology.

Overall the goals of the LRH1 modulator program are to identify potent and selective *in vivo* active LRH1 agonists and inverse agonists. Obviously, we need to better understand the function of this important receptor and its role in diseases like breast cancer if we are going to development new treatments. To do that, first we need to accelerate the identification of chemical probes. Prior to the work described in this Probe Report, there were no potent *in vivo* active LRH1 agonists and no reports of any inverse agonists. Thus, probes ML180 and ML179 are *first in class* LRH1 inverse agonists and these probes constitute significant contributions to the field.

Additional mechanism of action (MOA) studies are required to determine if inverse agonists of LRH1 function by modulating the receptors activity directly by either displacing co-activators or by recruiting co-repressors. It is also possible that these compounds modulate the post-translational status of the receptor directly by impacting recruitment of transcriptional machinery by affecting the receptors' occupancy at promoters of LRH1 target genes. PTMs could also modulate the receptor such that it trans-represses target genes in a DNA binding independent fashion.

• **What will the probe be used for?** Probes ML180 and ML179 can be used in cell biological studies to elucidate the role of LRH1 in metabolic diseases, and tumorigenesis. Recently, LRH1 has been shown to play a role in the transcriptional regulation of pathways involved in cancer. Simpson and coworkers were the first to report high levels of LRH1 expression in preadipocytes and they correlated the expression of LRH1 with transcriptional activation of the aromatase cytochrome p450 (CYP19) gene [1]. Aromatase expression has been shown to be highly upregulated in breast tumors as well as breast adipose tissue surrounding tumors. The hallmark of upregulation of CYP19 expression is a switch in promoters from the normal adipose-specific promoter I.4 to the gonadal type PII promoter on the CYP19 gene [2-4]. The Cyp19PII promoter is cAMP-dependent and stimulators such as prostaglandin E2, forskolin, and PMA dramatically induce LRH1-dependent Cyp19PII expression up to, in some reports, 500 fold. This activity was further induced in a synergistic fashion by coexpressing LRH1 and the transcription factors GATA3 and GATA4 in the presence of a CYP19PII luciferase reporter plasmid following stimulators of the cAMP pathway [5]. Moreover, this increase in aromatase expression is completely abrogated following overexpression of SHP which is a negative regulator of LRH1 activity [6, 7]. It has been shown that upregulation of aromatase expression in breast adipose tissue surrounding tumors is responsible for the 10-50 fold increase in estrogen produced in these cells [8]. Unfortunately, this provides fertile ground for the growth and spread of estrogen-dependent tumors which comprise nearly 75% of all breast cancer occurrences in post-menopausal women [1, 9]. Aromatase inhibitors are used clinically in breast cancer treatment but unfortunately these compounds reduce estrogen production in other tissues giving rise to significant side effects such as bone loss. Therefore, inhibitors or inverse agonists of LRH1-dependent activation of aromatase expression could represent a strategy for the development of novel breast-specific tumor therapies.

In addition to modulating expression of the aromatase gene in breast tumors and surrounding tissues, LRH1 has been implicated in cell proliferation and intestinal cancer. Murine hepatic and pancreatic cells overexpressing LRH1 more than doubled in growth rate and formed colonies in soft agar [10]. LRH1 was shown to mediate these effects by acting in synergy with β-catenin to transactivate CyclinD1 and CyclinE1. Furthermore, these effects were reversed by introducing LRH1 siRNA or by overexpression of SHP demonstrating the functionally specific role of LRH1 in cell proliferation. These studies were further extended to examine the effects of LRH1 expression on intestinal tumorigenesis as LRH1 is highly expressed in rapidly dividing intestinal crypt cells. Auwerx and coworkers examined the effects of reduced LRH1 expression on intestinal tumorigenesis using two independent mouse models of the disease [10]. APCmin/- mice that were made haploinsufficient for LRH1 showed lower LRH1 expression and dramatically less tumor formation than mice with wild-type levels

of LRH1 expression. In addition, mice lacking one LRH1 allele that were treated with AOM, a chemical inducer of colon cancer, had significantly reduced aberrant crypt foci than mice that expressed normal levels of LRH1.

More recently, overexpression of LRH1 in both ER positive and ER negative cell lines has been shown to promote motility and cell invasiveness in both ER-positive as well as ER-negative breast cancer cells (MDA-MB-231), with remodeling of the actin cytoskeleton and E-cadherin processing observed [11]. This observation suggests that inverse agonists of LRH1 would also be useful in treating ER negative breast cancer. We present data showing the ability of ML180 to inhibit the growth of MDA-MB-231 cells as well as other ER negative and ER positive cancer cell lines.

- **Who in the research community will use the probe?** Probes ML180 and ML179 can be used by academic researchers studying cell biology, molecular biology, and tumor biology. In addition, the two identified probes will be useful for any researcher working in the field of metabolism, cancer biology, APR and drug discovery.

What is the relevant biology to which the probe can be applied? Most nuclear receptors function as homodimers or as heterodimers with the retinoid X receptor (RXR) to bind their cognate DNA response elements in promoter regions of target genes [12-14]. However, members of the NR5A subfamily such as SF-1 and LRH1 bind DNA with high affinity as monomers through interactions between the Ftz-F1-consensus binding site on target genes and the Ftz-F1 box, which is a 26 amino acid stretch at the C-terminus of the DNA binding domain (DBD) of these receptors [15]. Additionally, most nuclear receptors require binding of ligand to become transcriptionally active. The model of activation for most nuclear receptors suggests that ligand binding induces conformational changes in the LBD of the receptor that leads to recruitment of coactivators and basal transcriptional machinery with subsequent activation of target genes. Interestingly, members of the NR5A subfamily appear to be constitutively active when expressed in cells. Recently a number of laboratories have identified the presence of phospholipids in the ligand binding pockets of both SF-1 and LRH1 and their presence leads to the recruitment of coactivators *in vitro* [16-18]. Whether phospholipids are in fact endogenous ligands of NR5A subfamily receptors remains undetermined. The possibility that the NR5A family represent ligand-independent transcription factors does exist. However, a recent publication by Whitby and coworkers [19] revealed small molecule agonists for both LRH1 and SF-1. These compounds were characterized in a biochemical assay utilizing purified protein. The most potent LRH1 agonist described lacked functional selectivity over SF-1 although a potent partial agonist of SF-1 lacking LRH1 activity was discovered. Partial agonists of LRH1 (EC_{50} 1.2 μM, 45% relative efficacy) were also described that lacked affinity for SF-1. More importantly, we synthesized and characterized several examples from the Whitby manuscript and these

compounds, although potent in a biochemical assay, were cytotoxic even at modest concentrations (~10 μM).

Perhaps more important is a recent publication resulting from our MLPCN screening center describing a HTS campaign for SF-1 [20]. This screen revealed that chemically tractable small molecule inverse agonists of SF-1 can be discovered directly from an HTS campaign. These compounds were inactive in the RORα counterscreen indicating that they do not inhibit luciferase and they are not potent cytotoxic agents. These studies, combined with the Whitby publication, support the notion that potent and selective small molecule agonist and inverse agonists of LRH1 can be discovered.

Crystal structures for both SF-1 and LRH1 have been solved and these structures have provided some clues to the constitutive activity of these receptors [17, 21, 22]. Most nuclear receptors undergo changes in the conformational dynamics of the ligand binding pocket upon ligand binding resulting in stabilization of the AF2 (activation function 2) surface of the coactivator binding interface of the receptors creating a charge clamp to facilitate recruitment and binding of coactivators [12, 14]. In contrast, the crystal structures of SF-1 and LRH1 show that this stabilization of the coactivator interacting region of the LBD's may occur as a result of a unique fourth sandwich layer formed by helix 2 (H2) [17, 22]. This region of H2 comes into close proximity with H12 in the absence of ligand to constitutively stabilizing AF2 facilitating coactivator binding. This structural feature of the receptor is also present in the murine receptor structure that was obtained with an empty ligand binding pocket. As mentioned above, the crystal structures of both human and mouse SF-1 as well as human LRH1 revealed the presence of bacterial phospholipids in the ligand binding pockets of these receptors. While the exact role of phospholipids in modulating the activity of these receptors is controversial, these findings further confirm that the NR5A receptors are capable of binding ligands that could potentially modulate their activity *in vivo*.

Relevance to Human Health: In addition to the role of LRH1 in tumorigenesis discussed above, LRH1 has been shown to play a pivotal role in cholesterol metabolism and bile acid homeostasis in the liver [23]. LRH1 transcriptionally regulates cholesterol 7α-hydroxylase (CYP7A1) [24], the rate-limiting enzyme of the bile acid biosynthetic pathway along with sterol 12 α-hydroxylase (CYP8B1) [25], which is required for cholic acid production. Moreover, LRH1 regulates transcription of a number of key enzymes involved in the uptake of cholesterol from tissues and plasma. Cholesterol ester transfer protein (CETP) is an LRH1 target gene that catalyzes the transfer of cholesterol esters from HDL particles to lipoproteins that can be extracted from serum by the hepatic low density lipoprotein receptor [12, 26]. CETP is currently targeted by several pharmaceutical companies for treatment of hypercholesterolemia, although Pfizer recently withdrew its late stage clinical candidate. It is unclear at this point in time if this

is a problem with the specific compound or a problem with the mechanism. Perhaps modulation of the pathway instead of inhibition of one step might provide an approach that is better tolerated. In addition, apolipoprotein A1 is transcriptionally regulated by LRH1 and acts as an acceptor molecule for phospholipids and cholesterol coming from peripheral tissues forming pre-HDL particles. These particles mature and are transferred into hepatocytes by the scavenger receptor class B type I cell surface receptor (SR-BI) which is also transcriptionally regulated by LRH1 [27, 28]. Thus, it is clear that LRH1 modulation could play an important role in cholesterol homeostasis.

LRH1 also activates genes involved in inflammatory responses. Delerive and coworkers, were the first to identify LRH1 as a negative regulator of the hepatic acute phase response [29]. Ectopic expression of LRH1 led to reduced expression of pro-inflammatory genes such as haptoglobin, serum amyloid A and C-reactive protein following stimulation of hepatocytes with proinflammatory cytokines IL-1b and IL-6. In addition, LRH1 activated expression of interleukin-1 receptor antagonist (IL-1RA), a potent anti-inflammatory molecule, following induction by IL-1b and IL-6 as well as intraperitoneal injection of lipopolysaccharide [30]. Moreover, partial deficiencies in LRH1 expression in hepatocytes resulted in decreases in expression of IL-1RA and an exaggerated inflammatory response *in vitro* and *in vivo* indicating that LRH1 expression leads to the modulation of anti-inflammatory responses. More recently Venteclef and coworkers reported that the GSK selective synthetic agonist induces sumoylation-dependent recruitment of LRH1 to hepatic acute phase response (APR) promoters and this prevents clearance of the corepressor N-CoR resulting in repression of APR genes [31]. Preliminary studies in our lab using Huh7 cells stimulated with IL1β and IL6 results in significant increase in expression of LRH1, haptoglobin (Hp), serum amyloid A-4 (SAA1), and serum amyloid A-4 (SAA4) as determined by qPCR. Surprisingly and in contrast to the anticipated outcome, treatment of these cells with the inverse agonists ML180 and ML179 represses the expression of three of these genes (Hp, SAA1, SAA4) to the level of control cells (unstimulated) and LRH1 to levels below that in control cells. This finding confirms that our inverse agonists can repress the expression of LRH1 and more importantly, suggests that LRH1 inverse agonists can repress APR genes in similar fashion to LRH1 agonists. Clearly the mechanism of action of inverse agonists in this model is likely different than that of agonists. This finding warrants further mechanistic studies which will be carried out as part of this extended probe development project.

1 Introduction

The goal of this project is to identify modulators (agonists and inverse agonists) of the orphan nuclear receptor LRH1, which has been implicated in cancer by enhancing proliferation and cell cycle progression and metabolic disorders through its regulation of genes involved in cholesterol and bile acid homeostasis. In this specific Probe Report we focus on the discovery and characterization of inverse agonists of LRH1.

NR5A2 or Liver receptor homologue-1 (LRH1) is a member of the NR5A, or Ftz-F1, subfamily V nuclear receptors of which there are four members [12]. Murine LRH1 was originally identified due to its sequence homology to the Drosophila Fushi tarazu factor-1 but orthologs have been subsequently identified in several other species including rat, chicken, horse, zebrafish and human [32-37]. LRH1, and its closest family member steroidogenic factor-1 (SF-1, NR5A1), bind to identical DNA consensus sequences (response elements or REs) and both have the ability to bind phospholipids in their ligand binding domains (LBDs) [16-18]. However, LRH1 and SF-1 are expressed in different tissues and thus are considered likely to have non-overlapping, non-redundant functions. SF-1 expression is confined to steroidogenic tissues and adrenals where it regulates development, differentiation, steroidogenesis and sexual determination [13, 35, 37]. LRH1 is highly expressed in tissues of endodermal origin and its expression is essential for normal liver, intestine, and pancreas function. LRH1 has also been shown to be expressed in the ovary and adipose tissue.

In a very recent report, Chand and colleagues investigated the mechanism of action of LRH1 in invasive breast cancer cells [38]. They found that LRH1 promotes motility and cell invasiveness in both ER-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cells and similar effects were observed in non-tumorigenic mammary epithelial cells. Interestingly, both remodeling of the actin cytoskeleton and E-cadherin processing were observed when LRH1 was over-expressed. These findings implicate LRH1 in promotion of migration and invasion in breast cancer independent of estrogen sensitivity. Together these findings provided strong evidence that LRH1 plays a significant role in tumor formation both *in vitro* and *in vivo*. Therefore, the identification of potent and selective LRH1 inverse agonists may provide new approaches for the treatment of cancer.

2 Materials and Methods

The SRIMSC Center Driven Research Project (CDRP) titled “Functional genomics approaches to small molecule discovery” was recently renewed. This project is focused on High throughput Functional Genomics and our overall goal is to continue to apply this platform towards pathway discovery and selectivity profiling of chemical probes emerging from the MLPCN network. During Years 1 and 2 of this MLPCN

Center Driven Research Project we designed and constructed a nuclear receptor (NR) library using resources within the TSRI high-throughput cDNA screening platform. Using a combination of small molecule libraries and these novel GAL4 tagged libraries, we profiled signaling pathways of several orphan nuclear receptors. During this profiling, we discovered activity on RORA, RORG, and LRH1. These orphan NRs have been implicated in a wide range of diseases and syndromes including metabolic and immune disorders, cancer, and neurological dysfunction. One of these discoveries resulted in submission and approval of a MLPCN probe report (AIDs 1901, 1954, 2117, 2139) describing the first synthetic modulators of RORA and RORG. We demonstrated that these compounds can repress glucose production in hepatocytes, and expression of IL17 from TH17 cells, which clearly demonstrated the utility of these chemical probes in models of diabetes and autoimmune disease. A second project within the CDRP was to characterize synthetic inverse agonists of LRH1. The significance of these compounds is described in detail above. As a result, several assays were performed in order to identify novel inverse agonists of LRH1. The specific assays are listed in **Table 1**.

2.1 Assays

Table 1. Summary of Performed Assays

PubChem AID	Assay Name	Tested/ Active	Non-MLSMR Compounds Tested	Purchased Compounds Tested/ Active	Synthesized Compounds Tested/ Active
485348	Primary LRH1 3X %INH	60/19	60	19/5	41/14
488782	LRH1 Dose Response (Aromatase)	16/13	16	5/5	11/8
488779	SF1 3X %INH (Lumi Reporter)	60/3	60	19/2	41/1
488780	SF1 Dose Response	16/0	16	5/0	11/0
488775	VP16 Fold Change	59/9	59	19/1	40/8
488769	QPCR Assay (mRNA Fold change: Haptoglobin; SAA1; SAA4)	2/2	2	1/1	1/1
504928	MTT Assay (Breast Cancer Cell Lines)	1/1	1	1	0
504933	Star INH Assay	2/2	2	2	0
504934	Nuclear Receptor Profiling	1/1	1	1	0
488781	Summary AID	-	-	-	-

The specific descriptions and protocols for each assay can be found in this section and in PubChem.

AID 485348 and 488782: LRH1 Inhibition Assays (single point and dose response)
Name: Center Based Initiative to identify novel inverse agonists of the liver receptor homolog-1 (LRH1; NR5A2): Luminescence-based single point and dose response assays to identify LRH1 inhibitors (Cyp19 aromatase-luciferase reporter 3X%INH and 3XIC50).

Assay Overview:
The purpose of these assays is to determine whether powder samples of possible LRH1 inverse agonist probe candidates can inhibit the activity of LRH1. In these assays, HEK293T cells, co-transfected with a full length LRH1 construct in a pSport6 vector backbone (pS6-LRH1) and a Cyp19-Aromatase-luciferase reporter construct, are incubated for 20 hours with test compound. Tissue-specific expression of Cyp19 is regulated by hormones (specifically estrogens) and is increased in response to breast tumor derived factors. LRH1 has been shown to bind to the Cyp19 promoter and regulates its expression in adipose tissue. As designed, a compound that inhibits LRH1 activity will prevent activation of the pS6-LRH1 construct, thereby preventing LRH1-mediated activation of the Cyp19-Aromatase-luciferase reporter, leading to a decrease in well luminescence. Compounds were tested in triplicate at a nominal concentration of 10 micromolar, and in triplicate using a 9-point dose response series starting at a nominal concentration of 10 micromolar.

Protocol Summary:
Luciferase reporter assays were conducted using a pSport6 full-length LRH1 construct and Cyp19 aromatase luciferase reporter cotransfected into HEK293T cells. Reverse transfections were performed in bulk using $3x10^6$ cells in 10 cm plates,7μg of total DNA and FuGene6 (Roche) in a 1:3 DNA: lipid ratio. Following 24 hour bulk transfection, cells from were counted and re-plated in 384-well plates at a density of 10,000 cells/well. Following 4 hour incubation, cells were treated with DMSO/compounds for 20 hours. The luciferase levels were measured by addition of BriteLite Plus (Perkin Elmer). Data was normalized to luciferase signal from DMSO treated cells. The fold-change inhibition for each compound was calculated as follows:
Cells_treated_with_Test_Compound / Cells_treated_with_Vehicle (DMSO).

The average fold-change of each compound tested was calculated.

PubChem Activity Outcome and Score:
Any compound that exhibited a fold-change inhibition greater than the hit cutoff calculated (> 25%Inhibition) was declared active.

For dose response assays, each test compound's percent inhibition was plotted against compound concentration. A four-parameter equation describing a sigmoidal dose-response curve was then fitted with adjustable baseline using GraphPad Prism software. The reported IC_{50} values were generated from fitted curves by solving for the X-intercept value at the 50% inhibition level of the Y-intercept value. In cases where the

highest concentration tested (i.e. 10 micromolar) did not result in greater than 50% inhibition, the IC_{50} value was determined manually as greater than 10 micromolar. Compounds with an IC_{50} value greater than 5 micromolar were considered inactive. Compounds with an IC_{50} value equal to or less than 5 micromolar were considered active. Activity score was ranked by the potency of the compounds, with the most potent compounds assigned the highest activity scores.

List of Reagents:
LRH1 and Cyp19 plasmid DNAs (assay provider lab)
384-well plates (PerkinElmer, part 6007688)
Britelite Plus (PerkinElmer, part 6016767)
DMEM (Mediatech Inc, Part 10 013 CV)
Fugene 6 (Roche Applied Science, part 11814443001).

AID 488779 and 488780: SF1 Inhibition Counterscreens (single point and dose response)

Name: Center Based Initiative to identify novel inverse agonists of the liver receptor homolog-1 (LRH1; NR5A2): Luminescence-based dose response and single point counterscreen assays to identify inhibitors of the steroidogenic factor-1 (SF1) (3X%INH and 3XIC50).

Assay Overview:
The purpose of this assay is to determine whether powder samples of possible LRH1 inverse agonist probe candidates are nonselective due to inhibition of another nuclear receptor, SF-1. This assay also serves to determine SF-1 inhibitory dose response curves for compounds In this assay, HEK293T cells, co-transfected with a full length SF-1 construct in a pSport6 vector backbone (pS6-SF-1) and a 5xSFRE -luciferase reporter construct, are incubated for 20 hours with test compound. As designed, a compound that inhibits SF-1 activity will prevent activation of the pS6 SF-1 construct, thereby preventing SF-1-mediated activation of the 5xSFRE-luciferase reporter, leading to a decrease in well luminescence. Compounds were tested in triplicate using a 9-point dose response series starting at a nominal concentration of 10 micromolar.

Protocol Summary:
Luciferase reporter assays were conducted using a pSport6 full-length SF-1 construct and 5xSFRE luciferase reporter cotransfected into HEK293T cells. Reverse transfections were performed in bulk using $3x10^6$ cells in 10 cm plates,7μg of total DNA and FuGene6 (Roche) in a 1:3 DNA: lipid ratio. Following 24 hour bulk transfection, cells from were counted and re-plated in 384-well plates at a density of 10,000 cells/well. Following 4 hour incubation, cells were treated with DMSO/compounds for 20 hours. The luciferase levels were measured by addition of BriteLite Plus (Perkin

Elmer). Data was normalized to luciferase signal from DMSO treated cells. The percent inhibition for each compound was calculated as follows:

Cells_treated_with_Test_Compound / Cells_treated_with_Vehicle (DMSO).

PubChem Activity Outcome and Score:
Any compound that exhibited a fold-change inhibition less than the hit cutoff calculated (> 25 %Inhibition) was declared active. For dose response assays, each test compound, percent inhibition was plotted against compound concentration. A four-parameter equation describing a sigmoidal dose-response curve was then fitted with adjustable baseline using GraphPad Prism software. The reported IC_{50} values were generated from fitted curves by solving for the X-intercept value at the 50% inhibition level of the Y-intercept value. In cases where the highest concentration tested (i.e. 10 micromolar) did not result in greater than 50% inhibition, the IC_{50} value was determined manually as greater than 10 micromolar. Compounds with an IC_{50} value greater than 5 micromolar were considered inactive. Compounds with an IC_{50} value equal to or less than 5 micromolar were considered active.

List of Reagents:
SF-1 and SFRE plasmid DNAs (assay provider lab)
384-well plates (PerkinElmer, part 6007688)
Britelite Plus (PerkinElmer, part 6016767)
DMEM (Mediatech Inc, Part 10 013 CV)
Fugene 6 (Roche Applied Science, part 11814443001).

AID 488775: VP16 Inhibition Counterscreen (Single Point Fold Change)
Name: Center Based Initiative to identify novel inverse agonists of the liver receptor homolog-1 (LRH1; NR5A2): Luminescence-based counterscreen assay to identify inhibitors of the human herpes virus VP16 transcriptional activator protein (VP16) (3X%INH).

Assay Overview:
The purpose of this assay is to determine whether powder samples of possible LRH1 inverse agonist probe candidates are nonselective due to inhibition of VP16. In this counterscreen assay the nuclear receptor plasmid was replaced by the $GAL4_{DBD}$-$VP16_{LBD}$ plasmid, which expresses the strong transactivation domain of the herpes simplex virus Virion Protein 16 (VP16) fused to the GAL4 DBD. Cells are co-transfected with the 5xGAL4 response element (UAS) luciferase reporter to monitor $GAL4_{DBD}$-$VP16_{LBD}$ activity, followed by incubation with test compounds for 18-24 hours. As designed, compounds that inhibit VP16 activity will decrease p$GAL4_{DBD}$-$VP16_{LBD}$ activity, leading to reduced activation of the pG5-luc and decreased well luminescence.

These compounds are likely to be nonselective inhibitors or cytotoxic. Compounds were tested in triplicate at a nominal concentration of 5 micromolar.

Protocol Summary:
Luciferase reporter assays were conducted using a pBind $GAL4_{DBD}$-$VP16_{LBD}$ construct and UAS luciferase reporter cotransfected into HEK293T cells. Reverse transfections were performed in bulk using $4x10^6$ cells in 10 cm plates, 9μg of total DNA and FuGene6 (Roche) in a 1:3 DNA: lipid ratio. Following 24 hour bulk transfection, cells from were counted and replated in 384-well plates at a density of 10,000 cells/well. Following 4 hour incubation, cells were treated with DMSO/compounds for 20 hours. The luciferase levels were measured by addition of BriteLite Plus (Perkin Elmer). Data was normalized to luciferase signal from DMSO treated cells. The fold-change inhibition for each compound was calculated as follows:
Cells_treated_with_Test_Compound / Cells_treated_with_Vehicle (DMSO).

PubChem Activity Outcome and Score:
Any compound that exhibited a fold-change inhibition less than the hit cutoff calculated (> 10 %Inhibition) was declared active.

List of Reagents:
VP16 and GAL4 UAS plasmid DNAs (assay provider lab)
384-well plates (PerkinElmer, part 6007688)
Britelite Plus (PerkinElmer, part 6016767)
DMEM (Mediatech Inc, Part 10 013 CV)
Fugene 6 (Roche Applied Science, part 11814443001).

AID 488769: QPCR mRNA Fold Change Assay (Haptoglobin, SAA1, SAA4 gene targets)

Name: Center Based Initiative to identify novel inverse agonists of the liver receptor homolog-1 (LRH1; NR5A2): fluorescence-based cell-based quantitative PCR assay to identify inhibitors of LRH1 target gene expression.

Assay Overview:
The purpose of this assay is to determine whether powder samples of possible LRH1 inverse agonist probe candidates are able to block the expression of pro-inflammatory target genes haptoglobin, SAA1, and SAA4. LRH1 activation has been shown to prevent the cytokine induced stimulation of certain proinflammatory genes from the liver that are activated in the acute phase response. The genes are Haptoglobin, SAA1 and SAA4. In these assays huh7 cells endogenously expressing LRH1 were treated with 10micromolar compound or DMSO vehicle for 18 hours. Following compound incubation, 3nM IL1β and IL6 inflammatory cytokines were added to cells and incubated

for a further 3 hours followed by isolation of RNA, conversion to cDNA, and Taqman-based QPCR. As designed, a compound that inhibits LRH1 activity will reduce target gene expression following addition of cytokines, leading to decreased production of the PCR amplicon, thereby reducing fluorescence, and increasing Ct.

Protocol Summary:

Huh7 cells endogenously expressing LRH1 were plated in 6 well plates at a density of 200,000 cells/well and after 18 hour incubation, treated with 10micromolar compound or DMSO vehicle for additional 18 hours. Following compound incubation, 3nM IL1b and IL6 inflammatory cytokines were added to cells and incubated for a further 3 hours. Then cells were lysed and RNA was isolated using the RNeasy kit (QIAGEN, Valencia, CA). DNA was generated using the Taqman reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (PCR) was performed in triplicate using LightCycler RNA Amplification Kit HybProbe master mix (Roche) with Taqman MGB Probe 6FAM--MGBNFQ on a model LightCycler480 real time PCR system (Roche). Data are expressed as the mean percent inhibition plus or minus SD of 3 replicates normalized to 100μg total RNA. The percent inhibition was normalized based on measurement of total RNA.

PubChem Activity Outcome and Score:

Any compound that exhibited a fold-change inhibition less than the hit cutoff calculated (> 25 %Inhibition) was declared active.

List of Reagents:

384-well plates (PerkinElmer, part 6007688)
Britelite Plus (PerkinElmer, part 6016767)
DMEM (Mediatech Inc, Part 10 013 CV)
Fugene 6 (Roche Applied Science, part 11814443001).
QiaShredder (Qiagen, 79656)
RNeasy mini kit (Qiagen, 74104)
LightCycler RNA Amplification Kit HybProbe (Roche, 12015145001)
Forward primer (Lifetech Applied Biosystems)
Reverse primer (Lifetech Applied Biosystems)
MGBProbe (Lifetech Applied Biosystems, 4304971, 6FAM)
LightCycler480 multiwell plate 96 (Roche, 04729692001)
LightCycler480 sealing foil (Roche, 04729757001)
Specific Primer Sequences are as follows:
Haptoglobin-For- 5’-AATGTGAAGCAGATGACG-3’
Haptoglobin-Rev-5’-GGGCAATGTCTTTCGCTGT-3’
SAA1-For-5’-CTGCAGAAGTGATCAGCG-3’

SAA1-Rev-5'-ATTGTGTACCCTCTCCCC-3'
SAA4-For-5'-CCAGTGAAAGCTGGCGTT-3'
SAA4-Rev-5'-GAGAAGTGTGTGGCTCACAGCC-3'

AID 504933: Star Inhibition Counterscreen (dose response)

Name: Late stage assay provider results from the probe development effort to identify inverse agonists of the liver receptor homolog-1 (LRH-1; NR5A2): luminescence-based cell-based assay to identify inhibitors of the StAR (Steroidogenic acute regulatory protein).

Assay Overview:

The purpose of this assay is to determine whether powder samples of possible LRH1 inverse agonist probe candidates can inhibit the activity of LRH1, as measure by inhibition of promoter activity of the Steroidogenic acute regulatory protein (Star). In this assay, HEK293T cells, co-transfected with a full length LRH1 construct in a pSport6 vector backbone (pS6-LRH1) and a Star-luciferase reporter construct are incubated for 20 hours with test compound. StAR is a transport protein that regulates cholesterol transfer within the mitochondria, which is the rate-limiting step in the production of steroid hormones. It is primarily present in steroid-producing cells, including theca cells and luteal cells in the ovary, Leydig cells in the testis and cell types in the adrenal cortex. Compounds were tested in triplicate at a nominal concentration of 10 micromolar, and in triplicate using a 9-point dose response series starting at a nominal concentration of 10 micromolar.

Protocol Summary:

Luciferase reporter assays were conducted using a pSport6 full-length LRH1 construct and StAR luciferase reporter cotransfected into HEK293T cells. Reverse transfections were performed in bulk using $3x10^6$ cells in 10 cm plates, 9µg of total DNA and X-tremeGENE 9 DNA Transfection Reagent in a 1:3 DNA: lipid ratio. Following 24 hour bulk transfection, cells from were counted and re-plated in 384-well plates at a density of 8,000 cells/well. Following 4 hour incubation, cells were treated with DMSO/compounds for 20 hours. The luciferase levels were measured by addition of BriteLite Plus Reagent. Data was normalized to luciferase signal from DMSO treated cells. The fold-change inhibition for each compound was calculated as follows:

Cells_treated_with_Test_Compound / Cells_treated_with_Vehicle (DMSO).

Then the average percent maximal response and IC_{50} of each compound tested were calculated.

PubChem Activity Outcome and Score:

Any compound that exhibited a fold-change inhibition greater than the hit cutoff calculated (> 25%Inhibition) was declared active. For dose response assays, each test compound, percent inhibition was plotted against compound concentration. A four parameter equation describing a sigmoidal dose-response curve was then fitted with adjustable baseline using GraphPad Prism software. The reported IC_{50} values were generated from fitted curves by solving for the X-intercept value at the 50% inhibition level of the Y-intercept value. In cases where the highest concentration tested (i.e. 10 micromolar) did not result in greater than 50% inhibition, the IC_{50} value was determined manually as greater than 10 micromolar. Compounds with an IC_{50} value greater than 5 micromolar were considered inactive. Compounds with an IC_{50} value equal to or less than 5 micromolar were considered active. For PubChem, activity score was ranked by the potency of the compounds, with the most potent compounds assigned the highest activity scores.

List of Reagents:

LRH1 and StAR plasmid DNAs (assay provider lab)
384-well plates (PerkinElmer, part 6007688)
Britelite Plus (PerkinElmer, part 6016767)
DMEM (Mediatech Inc, Part 10 013 CV)
X-tremeGENE 9 DNA Transfection Reagent (Roche Applied Science, part 06365809001).

504934: Nuclear Receptor Profiling Counterscreen

Name: Late stage assay provider results from the probe development effort to identify inverse agonists of the liver receptor homolog-1 (LRH-1; NR5A2): luminescence-based high throughput cell-based assay to identify modulators of human nuclear receptors.

Assay Overview:

The purpose of this assay is to identify compounds that act as modulators of human nuclear receptors and to demonstrate the utility of the GAL4 nuclear receptor library (13). This assay screens endogenous and synthetic ligands against a GAL4 nuclear receptor library which was built by replacing the endogenous N-terminus and DNA-binding domain (DBD) of all 48 receptors with a GAL4 DBD. The fusion constructs consist of the GAL4 DBD, the hinge domain, ligand binding domain (LBD), and F domain if applicable, of the human receptors. Plasmids coding for full-length receptors were also included for some receptors. In this assay HEK293T cells are co-transfected with a single GAL4 receptor and a luciferase reporter containing an upstream activating sequence (UAS) recognized by the GAL4 DBD, followed by treatment with test compounds. As designed, compounds that modulate activity of a particular nuclear receptor will modulate the binding of the GAL4 DBD to the UAS, thereby modulating

luciferase production, resulting in an increase or decrease in well luminescence. Compounds are tested in triplicate at a nominal test concentration of 2 micromolar.

Protocol Summary:
The nuclear receptor library was plated into 384-well plates. HEK293T cells were reverse transfected with the well-specific construct and the UAS luciferase reporter pGL4.31 using Fugene6 transfection reagent in a final volume of 40 microliters. Control wells containing constructs encoding for the GAL4 DBD alone (pBind) or GAL4 fused to VP16 were also analyzed. After 24 hours, optimized compounds (2 µM final concentration) or DMSO was added to the plates and allowed to incubate for 20 hours. Next, 40 microliters of BriteLite was added to all wells and luciferase activity was measured on the PerkinElmer Envision 2104. Compounds that attenuate the GAL4-VP16-dependent luciferase activity in the positive control are considered promiscuous or cytotoxic. Each compound was evaluated using two plates of the GAL4 NR library providing six replicates and was normalized to DMSO and VP16 well controls. Compounds with mean signals three standard deviations from the DMSO controls were considered hits in this assay.

List of Reagents:
Fugene6 transfection reagent (Roche Applied Sciences)
BriteLite reagent (Perkin Elmer)
pGL4.31 construct (Promega)
384-well plates (Greiner, part 789176)

504928: Breast Cancer Cell Cytotoxicity Counterscreen (MTT)
Name: Late stage assay provider results from the probe development effort to identify inverse agonists of the liver receptor homolog-1 (LRH-1; NR5A2): absorbance-based cell-based assay to identify cytotoxic compounds in various cell types.

Assay Overview:
The purpose of this assay is to determine cytotoxicity dose response curves for powder samples of possible LRH1 inverse agonist probes candidates, using a variety of tumor cell lines. This assay is based upon the reduction of the yellow tetrazolium salt (MTT) in metabolically active cells to form insoluble purple-blue formazan crystals, which are solubilized by the addition of a detergent. MTT reduction occurs inside cells via the action of mitochondrial dehydrogenases. Formazan production is directly proportional to cell number, and metabolically inactive cells produce low levels of formazan. All values were normalized to DMSO negative control. As designed, a compound that inhibits cell growth or proliferation or is directly cytotoxic, will lead to decreased production of purple formazan crystals, thereby reducing well absorbance. Some compounds were tested in duplicate using a 5-point dilution series starting at a nominal concentration of 10 micromolar. For some assays, doses tested were 10.0, 3.3, 1.1,

0.37, 0.123, 0.041, 0.0137, and 0.0045 micromolar. Control treatment of cells was media alone.

Protocol Summary:
A selection of some of the possible cell types tested in this assays are listed here: Human B lymphocyte (Lymphoma) Raji cells, Mouse B lymphocyte EuMyc Lymphoma cells, Mouse Pre-B lymphocyte (cancer) 70Z/3 cells, $9x10^{10}$ cells, Mouse T lymphocyte (Leukemia) Jurkat cells, Human Breast Cancer MCF7 cells, Human Breast Cancer T47D cells, Human Breast Cancer MD-MDA-231 cells, Human breast (non-cancer) HS578 cells, Human Breast Cancer SKBR cells.

Cells were seeded in 96-well plate based on the standard cell concentrations, 50 µL per well. Cells were cultured in 37°C, 5%CO_2 for 3-5 days. Add MTT (Chemicon International) 10 µL per well, continue culturing for 4 hours. Add isopropanol/0.04N HCL, 100 µL per well, pipit up and down to dissolve the formazan into a homogeneous blue solution. Read at 570nm and 630nm. Use dual wavelength of 570nm subtracted from reference 630 nm. The percent maximal response (inhibition) for each replicate well of each compound was calculated as follows:
[Cells_treated_with_Test_Compound] / [Cells_treated_with_Vehicle(DMSO)]

Then the average percent maximal response and standard deviation of each compound tested were calculated. For selected test compounds, fold inhibition was plotted against compound concentration. Either the reported IC_{50} or fold change values were calculated from GraphPad Prism software. Compounds with an IC_{50} greater than 10 µM or a fold change value greater than 0.6 were considered inactive. Compounds with an IC_{50} equal to or less than 10 µM, or a fold change value less than 0.6 were considered active.

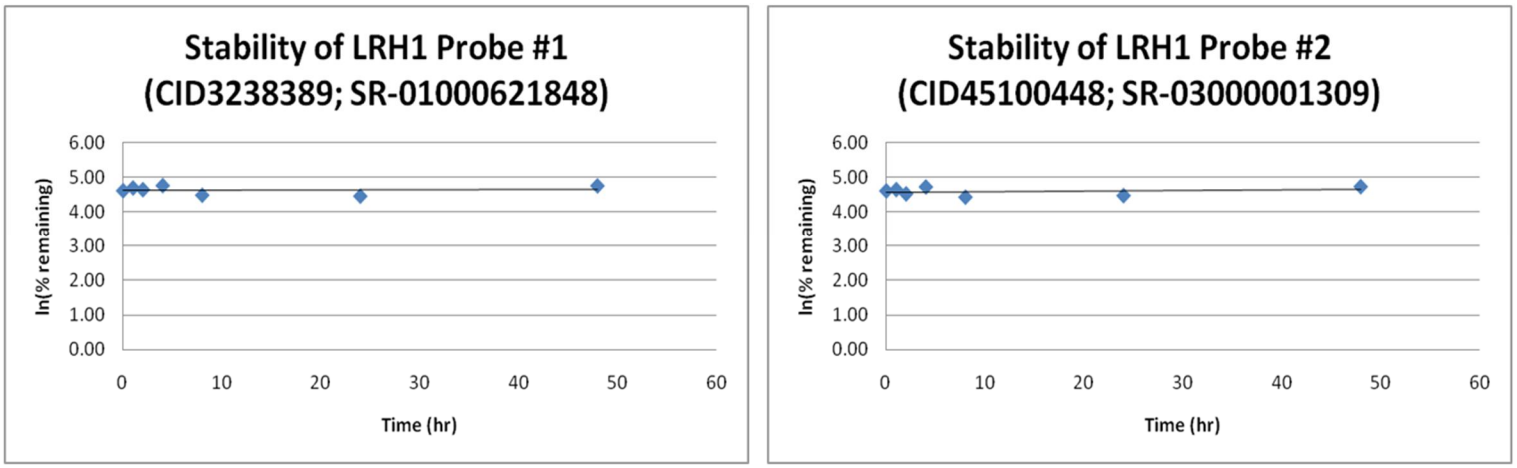
List of Reagents: MTT (purchased from Chemicon International)

2.2 Probe Chemical Characterization

Compound	SR Number	MLS	CID	SID	Solubility in PBS (µM)	Michael Acceptor 100 µM GSH trap	Stability in PBS (t1/2 (hr)
PROBE #1 (ML180)	SR-01000621848-2	MLS003153119	CID 3238389	SID 99344023	*8.5*	No	> 48 hr
PROBE #2 (ML179)	SR-03000001309-1	MLS003153122	CID 45100448	SID 92092843	*3.9*	No	> 48 hr

Graphical representations of the stability of the new probe compounds are shown in **Figure 1**. The new probes have been tested in numerous cell-based assays, demonstrating their cellular permeability, solubility, and stability.

Figure 1. Probe Stability



LC-MS/MS

All analytical methods were in MRM mode where the parent ion was selected in Q1 of the mass spectrometer. The parent ion was fragmented and a characteristic fragment ion monitored in Q3. MRM mass spectroscopy methods are particularly sensitive because additional time is spent monitoring the desired ions and not sweeping a large mass range. Methods were rapidly set up using Automaton® (Applied Biosystems), where the compounds were listed with their name and mass in an Excel datasheet. Compounds were submitted in a 96-well plate to the HPLC autosampler and slowly injected without a column present. A narrow range centered on the indicated mass was scanned to detect the parent ion. The software then evaluated a few pre-selected parameters to determine conditions that maximized the signal for the parent ion. The molecule was then fragmented in the collision cell of the mass spectrometer and fragments with m/z larger than 70 but smaller than the parent mass were determined. Three separate collision energies were evaluated to fragment the parent ion and the

largest three ions were selected. Each of these three fragment ions was further optimized and the best fragment was chosen. The software then inserted the optimized masses and parameters into a template method and saved it with a unique name that indicated the individual compound being optimized. Spectra for the parent ion and the fragmentation pattern were saved and reviewable later.

Solubility

The solubility of compounds was tested in phosphate buffered saline, pH 7.4. Compounds were inverted for 24 hours in test tubes containing 1-2 mg of compound with 1 mL of PBS. The samples were centrifuged and analyzed by HPLC (Agilent 1100 with diode-array detector). Peak area was compared to a standard of known concentration. In cases when the concentration was too low for UV analysis or when the compound did not possess a good chromophore, LC-MS/MS analysis was used.

Stability

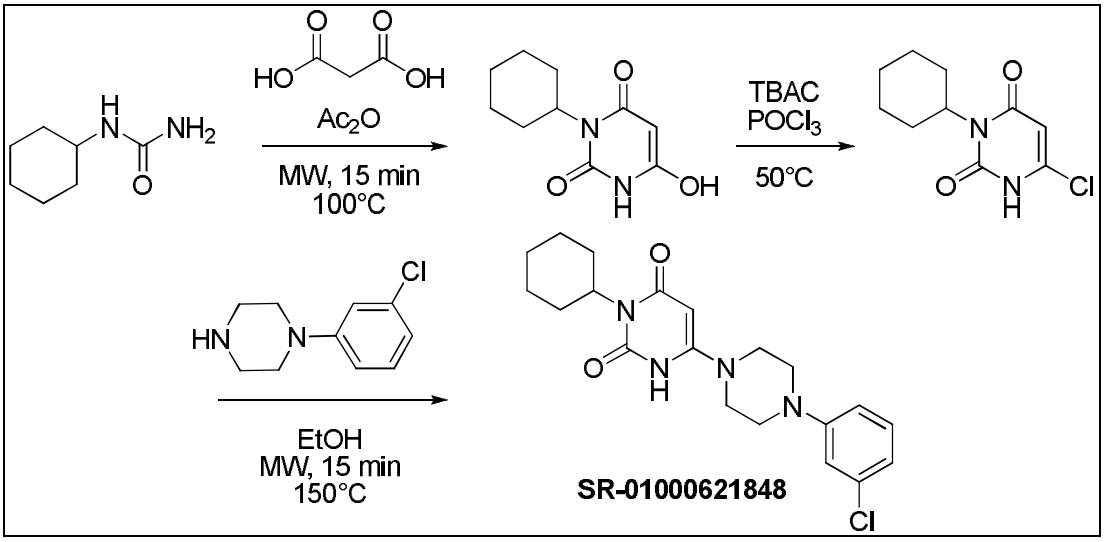
Demonstration of stability in PBS was conducted under conditions likely to be experienced in a laboratory setting. The compound was dissolved in 1 mL of PBS at a concentration of 10 µM, unless its maximum solubility was insufficient to achieve this concentration. Low solubility compounds were tested between ten and fifty percent of their solubility limit. The solution was immediately aliquoted into seven standard polypropylene microcentrifuge tubes which were stored at ambient temperature in a block microcentrifuge tube holder. Individual tubes were frozen at -80°C at 0, 1, 2, 4, 8, 24, and 48 hours. The frozen samples were thawed in a room temperature and an equal volume of acetonitrile was added prior to determination of concentration by LC-MS/MS.

Determination of glutathione reactivity

One µL of a 10 mM compound stock solution was added to 1 mL of a freshly prepared solution of 100 µM reduced glutathione. Final compound concentration was 10 µM unless solubility limited. The solution was allowed to incubate at 37°C for two hours prior to being directly analyzed for glutathione adduct formation. LC-MS/MS analysis of GSH adducts was performed on an API 4000 Q-TrapTM mass spectrometer equipped with a Turboionspray source (Applied Biosystems, Foster City, CA). Two methodologies were utilized—a negative precursor ion (PI) scan of m/z 272, corresponding to GSH fragmenting at the thioether bond, and a neutral loss scan of -129 AMU to detect GSH adducts. This triggered positive ion enhanced resolution and enhanced product ion scans [39, 40].

2.3 Probe Preparation: Synthesis of LRH1 Inverse Agonist Probes and Analogs

Figure 2. Synthesis of SR-01000621848 (Probe 1: ML180) (CID 3238389)



The first reaction was performed using a known method [41]. Cyclohexylurea (510 mg, 3.52 mmol) and malonic acid (370 mg, 3.52 mmol) were weighted into a sealed tube. Acetic anhydride (671 μL, 7.03 mmol) was added and the vial was heated 7 min in a microwave at 100°C. The mixture was then evaporated, EtOH was added and the product precipitated after triturating crude product. The solid was filtrated to provide 300 mg of **3-cyclohexyl-6-hydroxypyrimidine-2,4(1*H*,3*H*)-dione** (41%) as white powder. **^{1}H NMR (400 MHz, $(CD_3)_2SO$):** 1.09 (qt, *J* = 12.9, 3.4 Hz, 1H), 1.25 (qt, *J* = 12.9, 3.4 Hz, 2H), 1.52-1.64 (m, 3H), 1.76 (broad d, *J* = 13.0 Hz, 2H), 2.14 (qd, *J* = 12.5, 3.4 Hz, 2H), 3.58 (s, 2H), 4.41 (tt, *J* = 12.2, 3.5 Hz, 1H), 11.19 (s, 1H).

The chlorination reaction was performed using a known method [42]. 3-Cyclohexyl-6-hydroxypyrimidine-2,4(1*H*,3*H*)-dione (272 mg, 1.29 mmol) and tetrabutylammonium chloride (771 mg, 2.72 mmol) were weighed in a round bottom flask. Phosphorus oxychloride (2.55 mL) was added and the mixture was heated 4h at 50°C, then cooled to ambient temperature and poured in cold water. The aqueous phase was extracted three times by $CHCl_3$. The combined organic extracts were dried over Na_2SO_4, filtrated and evaporated. The crude residue was purified by silica gel column and eluted with hexane-EtOAc (60/40) to obtain 216 mg of **6-chloro-3-cyclohexylpyrimidine-2,4(1*H*,3*H*)-dione** (73%) as a white powder. **^{1}H NMR (400 MHz, $(CD_3)_2SO$)**: 1.10 (qt, *J* = 12.9, 3.2 Hz, 1H), 1.26 (qt, *J* = 12.9, 3.1 Hz, 2H), 1.47-1.65 (m,

3H), 1.76 (broad d, *J* = 12.9 Hz, 2H), 2.25 (qd, *J* = 12.4, 3.3 Hz, 2H), 4.41 (tt, *J* = 12.1, 3.8 Hz, 1H), 5.82 (s, 1H), 12.21 (s, 1H).

6-Chloro-3-cyclohexylpyrimidine-2,4(1*H*,3*H*)-dione (50 mg, 0.22 mmol) and 1-(3-chlorophenyl)piperazine (129 mg, 0.66 mmol) were weighed into a sealed tube. Ethanol (300 μL) was added and the vial was heated 10 min in a microwave at 150°C. The mixture was directly applied to a silica gel column and eluted with CH_2Cl_2-MeOH (95/5) to obtain 51 mg of **SR-01000621848** (61%, purity 99%) as a reddish powder.

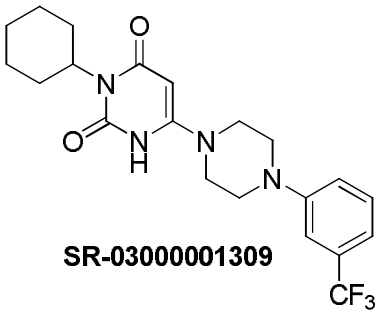
FTIR: 2937, 2828, 1691, 1625, 1592, 1430, 1377, 1234, 1195, 1019, 946, 784, 768, 687, 679 cm^{-1}.

^{1}H NMR (400 MHz, ($CDCl_3$)): 1.17 (qt, *J* = 13.2, 3.4 Hz, 1H), 1.40 (qt, *J* = 13.2, 3.2 Hz, 2H), 1.62-1.76 (m, 3H), 1.85 (broad d, *J* = 13.0 Hz, 2H), 2.31 (qd, *J* = 12.6, 3.1 Hz, 2H), 3.27-3.34 (m, 4H), 3.45-3.53 (m, 4H), 4.78 (bt, *J* = 12.0 Hz, 1H), 4.99 (bs, 1H), 6.80 (dd, *J* = 8.3, 2.1 Hz, 1H), 6.87-6.91(m, 2H), 7.20 (d, *J* = 8.4 Hz, 1H), 10.33 (s, 1H)

^{13}C NMR (100 MHz, ($CDCl_3$)): 25.6, 26.4, 29.0, 46.0, 48.5, 80.2, 114.4, 116.2, 120.6, 130.3, 135.2, 151.5, 153.3, 164.0.

MS (ES-) *m/z* = 387 (found for $C_{20}H_{25}ClN_4O_2$-H^+).

Data for SR-03000001309 (Probe 2: ML179) (CID 45100448)



SR-03000001309

SR-03000001309 (95% last step, purity >98%) obtained as a white powder, was synthesized following the same procedures described for **SR-01000621848**, replacing in the last step 1-(3-chlorophenyl)piperazine by 1-(3-(trifluoromethyl)phenyl)piperazine.

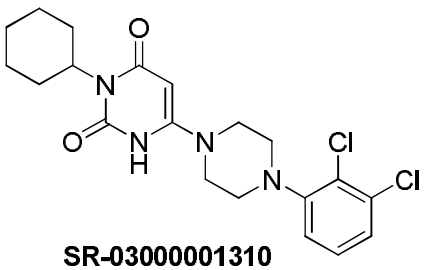
FTIR: 2935, 2854, 1694, 1626, 1450, 1434, 1389, 1353, 1310, 1232, 1161, 1120, 110, 947, 781, 694 cm^{-1}.

^{1}H NMR (400 MHz, ($CDCl_3$)): 1.16 (qt, *J* = 13.1, 3.3 Hz, 1H), 1.40 (qt, *J* = 13.1, 3.3 Hz, 2H), 1.60-1.76 (m, 3H), 1.83 (broad d, *J* = 13.6 Hz, 2H), 2.31 (qd, *J* = 12.4, 3.3 Hz, 2H), 3.32-3.39 (m, 4H), 3.49-3.56 (m, 4H), 4.79 (bt, *J* = 12.1 Hz, 1H), 5.01 (d, *J* = 2.0 Hz, 1H), 7.08 (dd, *J* = 8.1, 2.3 Hz, 1H), 7.10-7.14 (m, 1H), 7.16 (d, *J* = 7.6 Hz, 1H), 7.39 (d, *J* = 8.0 Hz, 1H), 10.50 (s, 1H)

^{13}C NMR (100 MHz, ($CDCl_3$)): 25.5, 26.3, 28.9, 46.1, 48.4, 80.2, 112.5 (q, *J* = 4.4 Hz), 117.1(q, *J* = 4.1 Hz), 119.2, 124.1 (q, *J* = 272.7 Hz), 129.8, 131.7 (q, *J* = 32.1 Hz), 150.6, 153.4, 164.0.

MS (ES-) *m/z* = 421 (found for $C_{21}H_{25}F_3N_4O_2$-H^+).

Data for SR-1310 (Probe Analog)



SR-03000001310

SR-03000001310 (97% last step, purity >98%) obtained as a white powder, was synthesized following the same procedures described for **SR-01000621848**, replacing in the last step 1-(3-chlorophenyl)piperazine by 1-(3-(trifluoromethyl)phenyl)piperazine.

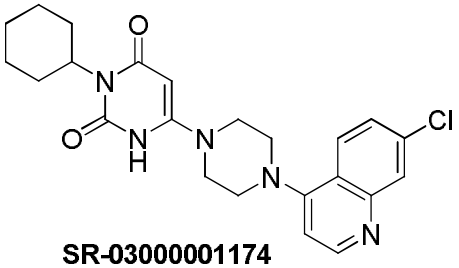
FTIR: 2937, 2856, 1691, 1622, 1575, 1451, 1432, 1423, 1388, 1373, 1241, 1198, 1019, 955, 787 cm^{-1}.

^{1}H NMR (400 MHz, ($CDCl_3$)): 1.12 (qt, *J* = 13.1, 3.1 Hz, 1H), 1.36 (qt, *J* = 13.1, 3.1 Hz, 2H), 1.61-1.71 (m, 3H), 1.80 (broad d, *J* = 13.4 Hz, 2H), 2.30 (qd, *J* = 12.1, 3.2 Hz, 2H), 3.11-3.20 (m, 4H), 3.48-3.59 (m, 4H), 4.77 (bt, *J* = 12.2 Hz, 1H), 5.01 (bs, 1H), 6.93 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.17 (t, *J* = 8.0 Hz, 1H), 7.23 (dd, *J* = 8.1, 1.6 Hz, 1H), 10.29 (s, 1H)

^{13}C NMR (100 MHz, ($CDCl_3$)): 25.5, 26.3, 28.9, 46.5, 50.7, 79.9, 118.5, 125.5, 127.6, 127.7, 134.4, 150.2, 153.2, 153.3, 164.1.

MS (ES-) *m/z* = 421 (found for $C_{20}H_{24}Cl_2N_4O_2$-H^+).

Data for SR-1174 (Probe Analog)



SR-03000001174

SR-03000001174 (97% last step, purity >98%) obtained as a white powder, was synthesized following the same procedures described for **SR-01000621848**, replacing at the last step 1-(3-chlorophenyl)piperazine by 7-chloro-4-piperazin-1-ylquinoline.

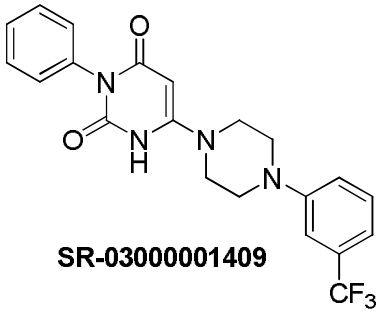
FTIR: 2922, 2848, 1694, 1620, 1575, 1425, 1377, 1232, 1197, 1012, 874, 867, 829, 822, 790, 729 cm^{-1}.

^{1}H NMR (400 MHz, ($CDCl_3$)): 1.05 (qt, *J* = 13.1, 3.6 Hz, 1H), 1.25-1.42 (m, 2H), 1.57-1.70 (m, 3H), 1.76 (broad d, *J* = 13.3 Hz, 2H), 2.14 (qd, *J* = 12.4, 3.1 Hz, 2H), 3.31-3.37 (m, 4H), 3.60-3.67 (m, 4H), 4.77 (bt, *J* = 11.9 Hz, 1H), 5.04 (d, *J* = 1.8 Hz, 1H), 6.86 (d, *J* = 4.9 Hz, 1H), 7.47 (dd, *J* = 9.0, 2.2 Hz, 1H), 7.94 (d, *J* = 9.0 Hz, 1H), 8.09 (d, *J* = 2.1 Hz, 1H), 8.77 (d, *J* = 4.9 Hz, 1H), 10.51 (s, 1H)

^{13}C NMR (100 MHz, ($CDCl_3$)): 25.6, 26.3, 28.9, 46.3, 51.6, 53.4, 80.5, 109.1, 121.6, 124.5, 126.8, 129.1, 135.4, 150.1, 151.8, 153.3, 156.0, 163.9.

MS (ES-) *m/z* = 438 (found for $C_{23}H_{26}ClN_5O_2$-H^+).

Data for SR-1409 (Probe Analog)



SR-03000001409

SR-03000001409 (97% last step, purity >98%) obtained as a white powder, was synthesized following the same procedures described for **SR-01000621848**, replacing at the first stage cyclohexylurea by phenylurea and at the last step 1-(3-chlorophenyl)piperazine by 1-(3-(trifluoromethyl)phenyl)piperazine.

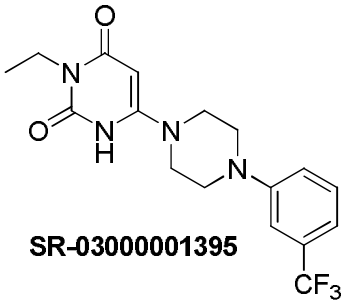
FTIR: 3049, 2844, 1726, 1700, 1606, 1495, 1449, 1314, 1232, 1165, 1112, 1099, 1074, 950, 778, 697, 689 cm^{-1}.

^{1}H NMR (400 MHz, ($CDCl_3$)): 2.99-3.04 (m, 4H), 3.36-3.43 (m, 4H), 5.08 (s, 1H), 6.95-7.01 (m, 2H), 7.18 (d, *J* = 7.8 Hz, 1H), 7.23-7.27 (m, 2H), 7.38-7.44 (m, 2H), 7.44-7.50 (m, 2H), 10.95 (s, 1H).

^{13}C NMR (100 MHz, ($CDCl_3$)): 46.0, 48.0, 79.4, 112.5 (q, *J* = 4 Hz), 116.9 (q, *J* = 4 Hz), 119.2, 124.2 (q, *J* = 272.0 Hz), 128.5, 128.6, 129.1, 129.7, 131.6 (q, *J* = 31.7 Hz), 134.8, 150.6, 153.5, 163.5.

MS (ES-) *m/z* = 415 (found for $C_{21}H_{19}F_3N_4O_2$-H^+).

Data for SR-1395 (Probe Analog)



SR-03000001395

SR-03000001395 (97% last step, purity >98%) obtained as a white powder, was synthesized following the same procedures described for **SR-01000621848**, replacing at the first stage cyclohexylurea by ethylurea and at the last step 1-(3-chlorophenyl) piperazine by 1-(3-(trifluoromethyl)phenyl)piperazine.

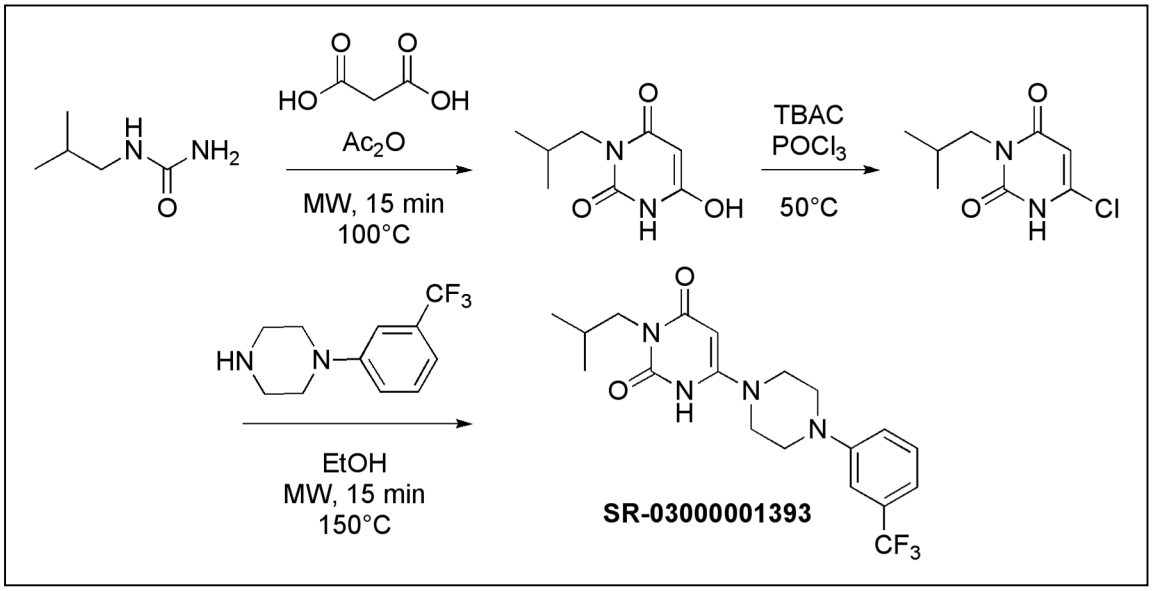
FTIR: 2972, 1718, 1695, 1604, 1585, 1496, 1445, 1311, 1231, 1151, 1122, 1097, 1075, 956, 809, 787, 767, 700 cm^{-1}.

^{1}H NMR (400 MHz, ($CDCl_3$)): 1.23 (t, *J* = 6.9 Hz, 3H), 3.33-3.38 (m, 4H), 3.50-3.56 (m, 4H), 3.95 (q, *J* = 7.1 Hz, 2H), 5.03 (bs, 1H), 7.10 (dd, *J* = 8.4, 2.5 Hz, 1H), 7.12-7.15 (m, 1H), 7.17 (d, *J* = 7.7 Hz, 1H), 7.41 (d, *J* = 8.0 Hz, 1H), 10.43 (s, 1H)

^{13}C NMR (100 MHz, ($CDCl_3$)): 13.1, 35.4, 46.2, 48.3, 80.1, 112.8 (q, *J* = 3.7 Hz), 117.2 (q, *J* = 3.8 Hz), 119.3, 124.0 (q, *J* = 272.7 Hz), 129.9, 131.7 (q, *J* = 31.8 Hz), 150.5, 153.1, 153.3, 163.4.

MS (ES-) *m/z* = 367 (found for $C_{17}H_{19}F_3N_4O_2$-H^+).

Figure 3. Synthesis of SR-1393 (Probe Analog)



Data for 6-chloro-3-isobutylpyrimidine-2,4(1*H*,3*H*)-dione, **^{1}H NMR (400 MHz, ($CDCl_3$))**: 0.92 (d, *J* = 6.8 Hz, 6H), 2.12 (n, *J* = 6.8 Hz, 1H), 3.74 (d, *J* = 7.5 Hz, 2H), 5.86 (s, 1H), 9.41 (bs, 1H).

SR-03000001393 (97% last step, purity >98%) obtained as a white powder, was synthesized following the same procedures described for **SR-01000621848**, replacing at the first stage cyclohexylurea by isobutylurea and at the last step 1-(3-chlorophenyl)piperazine by 1-(3-(trifluoromethyl)phenyl)piperazine.

FTIR: 2959, 1698, 1621, 1494, 1447, 1310, 1229, 1165, 1119, 1098, 1075, 994, 950, 785, 696 cm^{-1}.

^{1}H NMR (400 MHz, ($CDCl_3$)): 0.94 (d, *J* = 6.8 Hz, 6H), 2.15 (n, *J* = 6.8 Hz, 1H), 3.32-3.39 (m, 4H), 3.50-3.56 (m, 4H), 3.73 (d, *J* = 7.5 Hz, 2H), 5.02 (bs, 1H), 7.10 (dd, *J* = 8.4, 2.5 Hz, 1H), 7.12-7.15 (m, 1H), 7.17 (d, *J* = 7.7 Hz, 1H), 7.41 (d, *J* = 8.0 Hz, 1H), 10.53 (s, 1H).

^{13}C NMR (100 MHz, ($CDCl_3$)): 20.2, 27.2, 46.2, 47.2, 48.3, 80.0, 112.60 (q, *J* = 3.8 Hz), 117.1 (q, *J* = 3.8 Hz), 119.3, 124.1 (q, *J* = 272.9 Hz), 129.9, 131.8 (q, *J* = 32.0 Hz), 150.6, 153.3, 153.6, 163.9

MS (ES-) *m/z* = 395 (found for $C_{19}H_{23}F_3N_4O_2$-H^+).

3 Results3.1 Summary of Screening Results This LRH1 probe development effort was a center-based initiative. The MLPCN collection was not screened.

3.2 Dose Response Curves for Probes and Aromatase Assay.

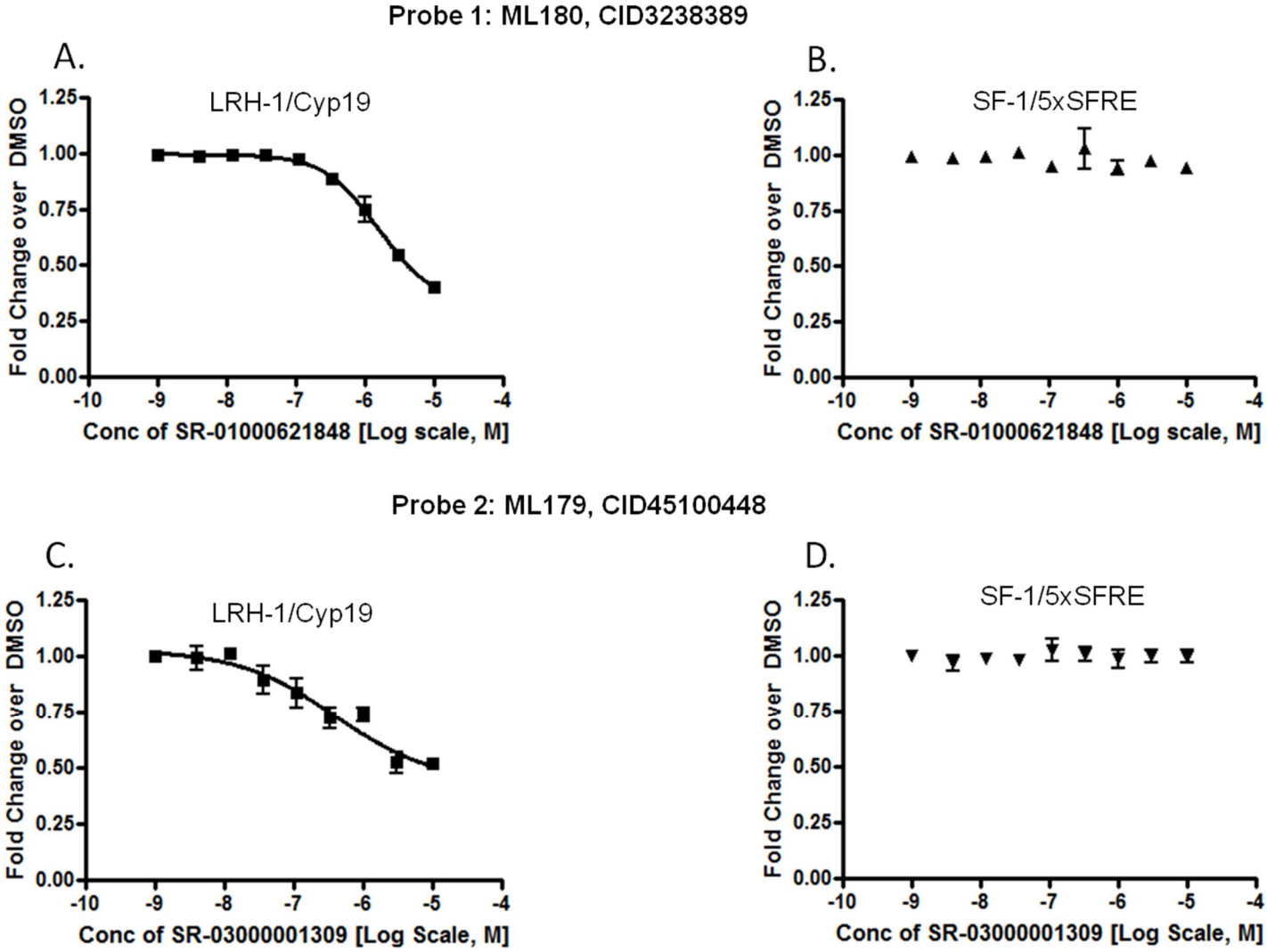


Figure 4.

Dose response curves for SR-01000621848 (Probe 1, ML180) and SR-01000001309 (Probe 2, ML179) two LRH-1 modulator probes. 293T cells were cotransfected with (A and C) full length LRH-1 and Cyp19 aromatase reporter, or (B and D) full length SF-1 and 5xSFRE reporter and treated with various concentrations of either SR-01000621848 (A and B) or SR-03000001309 (C and D) for 20 hours prior to luciferase activity measurement. Relative change was determined by normalizing to vehicle treatment. Treatment with both probes, SR-01000621848 (Max Rep=52% ; IC_{50}= 3.7μM) and SR-03000001309 (Max Rep = 46% ; IC_{50} = 320nM) showed selective efficacy against LRH-1 over SF-1.

These aromatase dose response data are available in PubChem as AID488782.

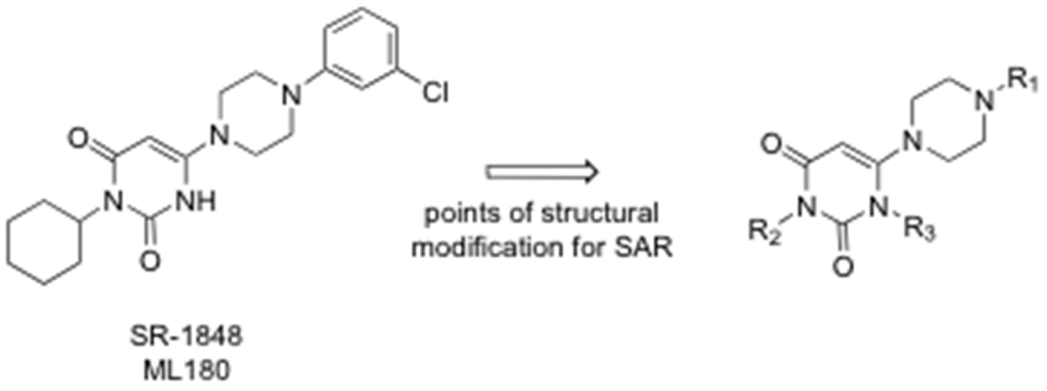
3.3 Scaffold/Moiety Chemical Liabilities

There are no known chemical liabilities associated with the probes **ML180** and **ML179**, or the identified probe analogs.

Discussion of SAR Studies

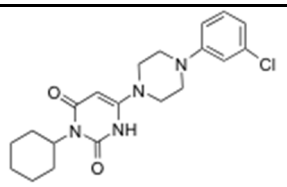
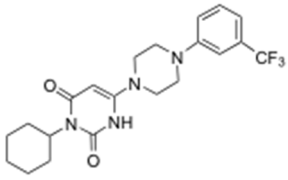
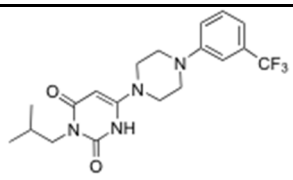
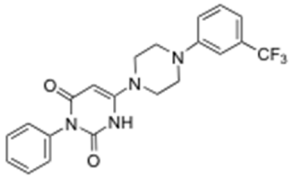
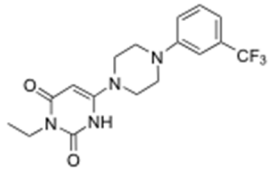
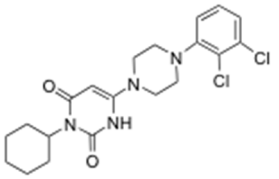
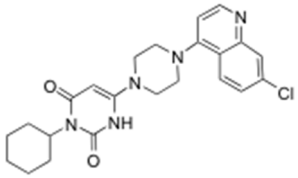
Starting with the initial selective LRH1 inverse agonist hit, SR-1848 (CID 3238389; ML180), we performed an extensive SAR study via analog purchase and synthesis. Three different sites in SR-1848 have been modified. A range of substituted aryl and heteroaryl groups have been introduced in position R_1 (see **Table 4** in **Section 3.4**). In general, aryl units at the R_1 position possessing meta substituents (e.g., meta-Cl as in SR-1848; a CF_3 group at this position in SR-1309, or the substituted quinoline in SR-1174) result in the greatest LRH1 repression. The optimal substituents at the R_2 position—which give rise to the lowest IC_{50} values for LRH1 inverse agonism, are alkyl groups like isobutyl in SR-1393 or cyclohexyl in many other analogs (e.g., SR-1309), or ethyl in SR-135. Introduction of a substituent at R_3 leads to a significant reduction or elimination of LRH1 activity; the results obtained thus far indicate that R_3 = H is required.

Figure 5. Points of Structural Modification for SAR



SR-1848
ML180

Table 2. SAR of Probes and Selected Analogs

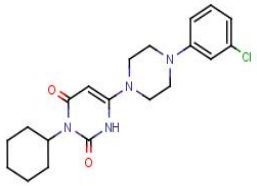
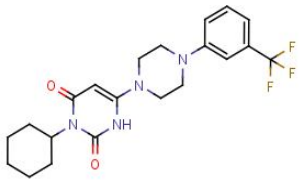
Structure	Identification Numbers	LRH1 IC_{50} (μM)	LRH1 Max Repression
	CID 3238389 (ML180, Probe 1) SR-1848	3.7	64% (10 μM)
	CID 45100448 (ML179, Probe 2) SR-1309	0.281	40% (550 nM)
	CID 45382271 (probe analog) SR-1393	0.061	35% (200 nM)
	CID 45480137 (probe analog) SR-1409	0.65	24% (2 μM)
	CID 45382284 (probe analog) SR-1395	0.378	40% (5 μM)
	CID 45100455 (probe analog) SR-1310	0.285	42% (5 μM)
	CID 44825223 (probe analog) SR-1174	4	75% (10 μM)

We selected SR-1848 (ML180) and SR-1309 (ML179) as the probes because these two compounds give the best results in inhibition of cancer cell growth. While SR-1848 is only 3.7 μM IC_{50} vs. LRH1, it gives the maximum LRH1 repression of all compounds studied to date (64% at 10 μM). Probe ML179 (SR-1309) is 13-fold more

potent (IC_{50}) than ML180 (SR-1848) against LRH1, but demonstrates a lower maximum repression of LRH1 transcription compared to ML180 (40% vs. 64%), albeit at a lower concentration. While SR-1393 (CID 45382271) is the most potent LRH1 inverse agonist discovered to date (IC_{50} = 61 nM), it is weaker in repressing LRH1 transcription (35% at 200 nM) than SR-1309. In tests of these compounds against various cancer cell lines, SR-1309 proved superior to SR-1393 in the majority of cell lines studied, and therefore SR-1309 was declared a probe rather than SR-1393.

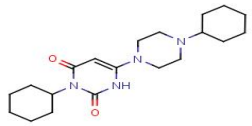
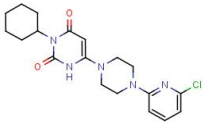
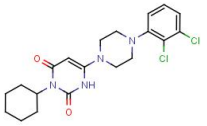
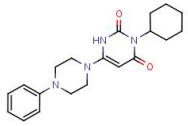
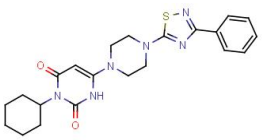
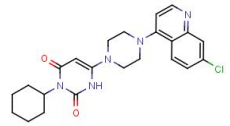
3.4 SAR Tables

Table 3. SAR of Probe Compounds

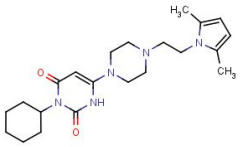
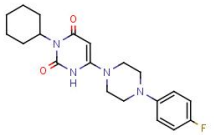
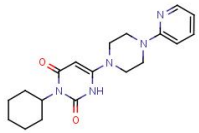
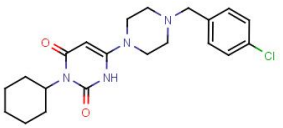
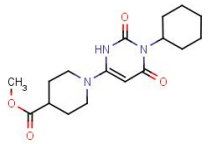
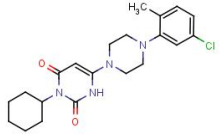
Compound Information						LRH1 % INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)		QPCR mRNA Fold Change (AID488769)					
Sample ID	**Structure**	**Source**	**MLS ID**	**SID**	**CID**	**LRH-1 AVG % Inhibition (at 5 uM)**	**SD**	**LRH-1 IC50**	**SD**	**SF-1 Avg Fold Change (at 5 uM)**	**SD**	**SF-1 IC50**	**SD**	**VP-16 Avg Fold Change (at 5uM)**	**SD**	**Haptoglobin mRNA Avg Fold change (at 10 uM)**	**SD**	**SAA1 mRNA Avg Fold change (at 10 uM)**	**SD**	**SAA4 mRNA Avg Fold change (at 10 uM)**	**SD**
PROBE #1 SR-01000621848-2		P	MLS003153119	99344023	3238389	52.33% (Active)	0.055	3.7uM (Active)	0.031	4.67% (Inactive)	0.13	> 10 uM (Inactive)	0.067	1.3% (Inactive)	0.012	0.87 (Active)	0.04	0.91 (Active)	0.00	0.55 (Active)	0.09
PROBE #2 SR-03000001309-1		S	MLS003153122	92092843	45100448	45.67% (Active)	0.051	320nM (Active)	0.075	-1%	0.01	> 10 uM (Inactive)		-6%	0.049	0.86 (Active)	0.04	0.93 (Active)	0.04	0.55 (Active)	0.10

Analogs with “ND” indicates that their activities for a particular anti-target or mechanism-of-action assay were Not Determined due to lack of LRH1 activity compared to probes. Only the two probes were tested in the LRH1 target gene QPCR assay.

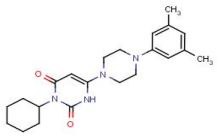
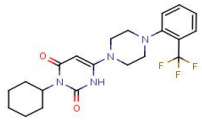
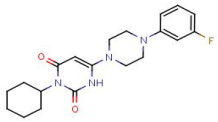
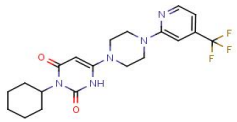
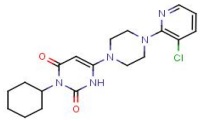
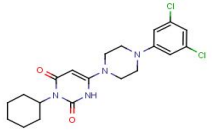
Table 4. SAR of R1 Analogs

Compound Information: R1 Analogs						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	**Structure**	**Source**	**MLS ID**	**SID**	**CID**	**LRH-1 AVG %Inhibition (at 5 uM)**	**SD**	**LRH-1 IC50**	**SD**	**SF-1 Avg Fold Change (at 5 uM)**	**SD**	**SF-1 IC50**	**SD**	**VP-16 Avg Fold Change (at 5uM)**	**SD**
SR-03000001170-1		S		89649733	44825215	35.33% (Active)	0.045	921nM (Active)	0.069	9.33% (Inactive)	0.08	9.2uM (Inactive)	0.06	0%	0.000
SR-03000001307-1		P		92092833	1484049	45% (Active)	0.026	538nM (Active)	0.065	-3%	0.06	N/D		-7%	0.058
SR-03000001310-1		S	MLS003153123	92092844	45100455	42% (Active)	0.147	430nM (Active)	0.100	3.3% (Inactive)	0.08	N/D		-5%	0.050
SR-01000140561-3		P		89650166	3244825	32% (Active)	0.087	2.6uM (Active)	0.087	-3%	0.06	N/D		-16%	0.124
SR-03000001171-1		S		89649734	44825216	42% (Active)	0.053	4.8uM (Active)	0.038	0.67% (Inactive)	0.11	N/D		-27%	0.058
SR-03000001174-1		S	MLS003153125	89649737	44825223	34.667% (Active)	0.042	4.0uM (Active)	0.242	11.33% (Inactive)	0.04	N/D		7% (Inactive)	0.026

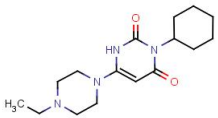
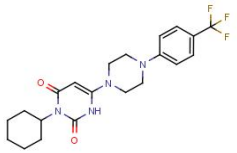
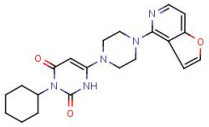
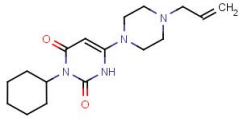
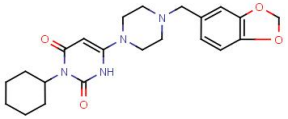
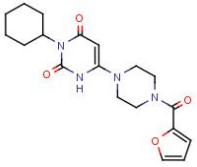
ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

Compound Information: R1 Analogs (continued)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	Structure	Source	MLS ID	SID	CID	LRH-1 AVG %Inhibition (at 5 uM)	SD	LRH-1 IC50	SD	SF-1 Avg Fold Change (at 5 uM)	SD	SF-1 IC50	SD	VP-16 Avg Fold Change (at 5uM)	SD
SR-03000001302-1		S		92092825	45100433	32.33% (Active)	0.045	ND		7% (Inactive)	0.07	ND		17% (Active)	0.044
SR-01000621845-2		P		89650164	3240644	1.33% (Inactive)	0.121	ND		-2%	0.11	ND		-9%	0.017
SR-03000001172-1		S		89649735	44825230	36% (Active)	0.036	20uM (Inactive)	0.017	-7%	0.08	N/D		-8%	0.076
SR-03000001173-1		S		89649736	44825240	30.33% (Active)	0.074	80uM (Inactive)	0.027	-4%	0.05	N/D		-4%	0.066
SR-01000140537-3		P		92092838	3241826	18% (Inactive)	0.156	ND		6%	0.11	ND		-12%	0.072
SR-03000001383-1		S		93375297	45382280	16.33% (Inactive)	0.035	ND		-12%	0.12	ND		0%	0.006

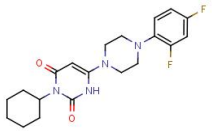
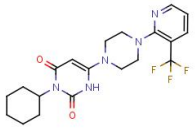
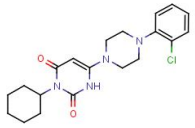
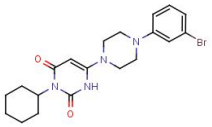
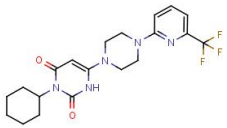
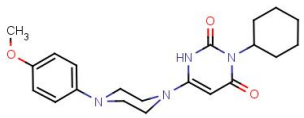
ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

Compound Information: R1 Analogs (continued)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	Structure	Source	MLS ID	SID	CID	LRH-1 AVG %Inhibition (at 5 uM)	SD	LRH-1 IC50	SD	SF-1 Avg Fold Change (at 5 uM)	SD	SF-1 IC50	SD	VP-16 Avg Fold Change (at 5uM)	SD
SR-03000001384-1		S		93375298	45382275	38.333% (Active)	0.040	13uM (Inactive)	0.015	-4%	0.13	N/D	N/D	-6%	0.085
SR-03000001385-1		S		93375299	45382282	16% (Inactive)	0.095	ND		5% (Inactive)	0.09	ND		14.3% (Active)	0.021
SR-03000001386-1		S		93375300	45382276	36.67% (Active)	0.091	ND		-112%	0.19	ND		ND	
SR-03000001387-1		S		93375301	45382268	20.3% (Inactive)	0.091	ND		-83%	0.44	ND		-12%	0.030
SR-03000001388-1		S		93375302	45382285	16% (Inactive)	0.090	ND		13.33% (Inactive)	0.10	ND		16% (Active)	0.090
SR-03000001389-1		S		93375303	45382283	20% (Inactive)	0.046	ND		-9%	0.07	ND		1.3% (Inactive)	0.012

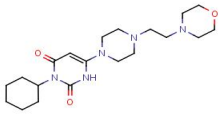
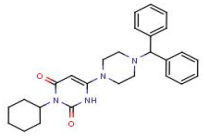
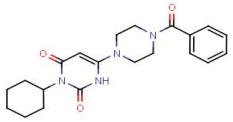
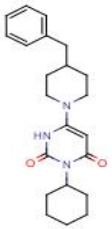
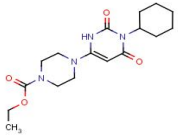
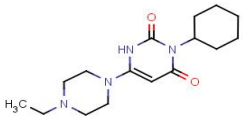
ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

Compound Information: R1 Analogs (continued)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	Structure	Source	MLS ID	SID	CID	LRH-1 AVG %Inhibition (at 5 uM)	SD	LRH-1 IC50	SD	SF-1 Avg Fold Change (at 5 uM)	SD	SF-1 IC50	SD	VP-16 Avg Fold Change (at 5uM)	SD
SR-01000140541-4		P		92092841	3571734	5.33% (Inactive)	0.055	ND		4.33% (Inactive)	0.06	ND		0%	0.100
SR-03000001175-1		S		89649738	44825217	0% (Inactive)	0.121	ND		1% (Inactive)	0.06	ND		-5%	0.084
SR-03000001176-1		S		89649739	44825219	2.67% (Inactive)	0.081	ND		-14%	0.17	ND		-4%	0.105
SR-03000001300-1		S		92092823	45100453	-0.30%	0.173	ND		-13%	0.21	ND		-48%	0.157
SR-03000001301-1		S		92092824	45100462	16.67% (Inactive)	0.055	ND		23.67% (Active)	0.08	ND		-5%	0.031
SR-01000607332-2		S		92092826	3237172	11% (Inactive)	0.046	ND		6% (Inactive)	0.05	ND		16.67% (Active)	0.235

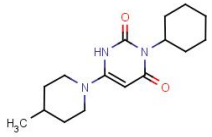
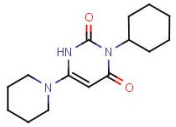
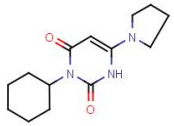
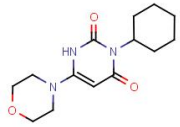
ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

Compound Information: R1 Analogs (continued)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	Structure	Source	MLS ID	SID	CID	LRH-1 AVG %Inhibition (at 5 uM)	SD	LRH-1 IC50	SD	SF-1 Avg Fold Change (at 5 uM)	SD	SF-1 IC50	SD	VP-16 Avg Fold Change (at 5uM)	SD
SR-03000001390-1		S		93375304	45382269	3.33% (Inactive)	0.042	ND		-49%	0.01	ND		26% (Active)	0.020
SR-03000001391-1		S		93375305	45382272	11%	0.144	ND		5.33% (Inactive)	0.13	ND		13.3% (Active)	0.050
SR-03000001403-1		S		93577916	45480135	16% (Inactive)	0.050	ND		-19%	0.06	ND		1.3% (Inactive)	0.257
SR-03000001404-1		S		93577917	45480133	17% (Inactive)	0.053	ND		-23%	0.06	ND		-4%	0.035
SR-03000001410-1		S		93577923	45480139	3.333% (Inactive)	0.035	ND		9% (Inactive)	0.04	ND		2.67% (Inactive)	0.032
SR-01000140539-2		P		85786762	1479244	1.667% (Inactive)	0.012	ND		4.67% (Inactive)	0.02	ND		1.33% (Inactive)	0.015

ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

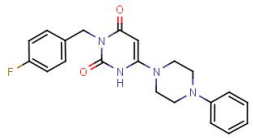
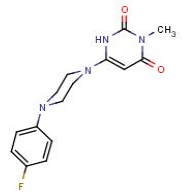
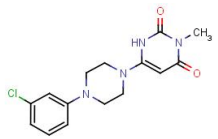
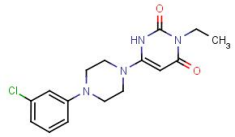
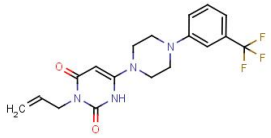
Compound Information: R1 Analogs (continued)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	**Structure**	**Source**	**MLS ID**	**SID**	**CID**	**LRH-1 AVG %Inhibition (at 5 uM)**	**SD**	**LRH-1 IC50**	**SD**	**SF-1 Avg Fold Change (at 5 uM)**	**SD**	**SF-1 IC50**	**SD**	**VP-16 Avg Fold Change (at 5uM)**	**SD**
SR-03000001303-1		S		92092827	45100460	13.33% (Inactive)	0.115	ND		8.67% (Inactive)	0.10	ND		26% (Active)	0.131
SR-03000001304-1		S		92092828	45100430	4% (Inactive)	0.139	ND		-8%	0.03	ND		11.33% (Inactive)	0.045
SR-03000001305-1		S		92092829	45100439	0%	0.006	ND		1.67% (Inactive)	0.08	ND		8% (Inactive)	0.072
SR-01000621846-2		P		92092839	3239085	2.33% (Inactive)	0.045	ND		9% (Inactive)	0.05	ND		26.3% (Active)	0.110
SR-01000140511-3		P		92092840	3245522	16% (Inactive)	0.183	ND		19.3% (Inactive)	0.14	ND		-6%	0.053
SR-01000140541-4		P		92092841	3571734	5.33% (Inactive)	0.061	ND		5.3% (Inactive)	0.08	ND		0%	0.100

ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

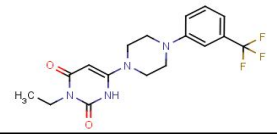
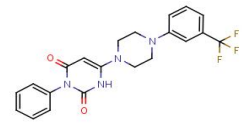
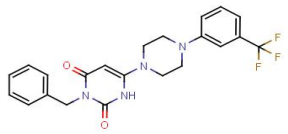
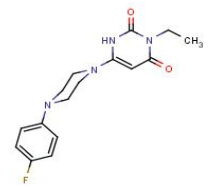
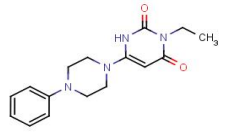
Compound Information: R1 Analogs (continued)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	**Structure**	**Source**	**MLS ID**	**SID**	**CID**	**LRH-1 AVG %Inhibition (at 5 uM)**	**SD**	**LRH-1 IC50**	**SD**	**SF-1 Avg Fold Change (at 5 uM)**	**SD**	**SF-1 IC50**	**SD**	**VP-16 Avg Fold Change (at 5uM)**	**SD**
SR-01000101040-3		P		92092842	3571735	10% (Inactive)	0.020	ND		-5%	0.07	ND		5.67% (Inactive)	0.060
SR-01000140507-3		S		92092831	4141058	20.33% (Inactive)	0.101	ND		23% (Inactive)	0.13	ND		0%	0.006
SR-03000001306-1		S		92092830	45100432	8.67% (Inactive)	0.075	ND		5% (Inactive)	0.17	ND		-6%	0.053
SR-01000101020-3		S		92092832	3242688	5.33% (Inactive)	0.136	ND		0%	0.01	ND		9% (Inactive)	0.052
SR-03000001311-1		S		92092845	45100438	14.33% (Inactive)	0.127	ND		23%	0.08	ND		-11%	0.032

ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay.

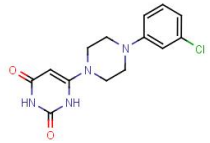
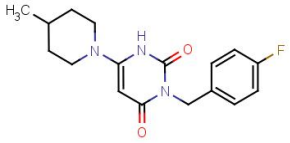
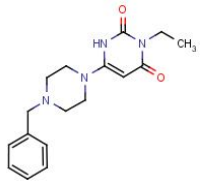
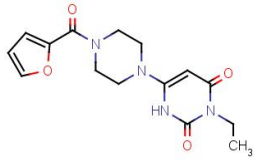
Table 5. SAR of R2 Analogs

Compound Information: R2 Analogs (with variable R1)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	**Structure**	**Source**	**MLS ID**	**SID**	**CID**	**LRH-1 AVG %Inhibition (at 5 uM)**	**SD**	**LRH-1 IC50**	**SD**	**SF-1 Avg Fold Change (at 5 uM)**	**SD**	**SF-1 IC50**	**SD**	**VP-16 Avg Fold Change (at 5uM)**	**SD**
SR-03000001393-1		S	MLS003153124	93375307	45382271	33.33% (Active)	0.098	110nM (Active)	0.023	-95%	0.15	15uM (Inactive)	0.02	8.33% (Inactive)	0.015
SR-01000097977-3		P		92092835	5310579	22.67% (Inactive)	0.140	ND		-18%	0.05	ND		-5%	0.064
SR-01000008560-2		P		85786757	7080816	14% (Inactive)	0.122	ND		-2%	0.07	ND		-13%	0.095
SR-01000008566-2		P		85786758	7080823	40.67% (Active)	0.116	1.1uM (Active)	0.023	-2%	0.07	N/D		-1%	0.091
SR-01000101016-2		P		85786759	7080833	45% (Active)	0.176	4.2uM (Active)	0.036	-10%	0.10	N/D		4.67% (Inactive)	0.031
SR-03000001394-1		S		93375308	45382279	36.67% (Active)	0.012	570nM (Active)	0.052	-164%	0.21	5uM (Inactive)	0.03	1% (Inactive)	0.017

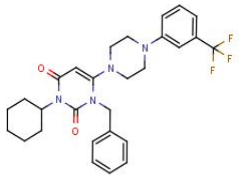
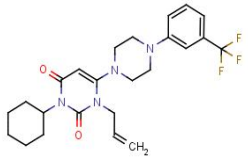
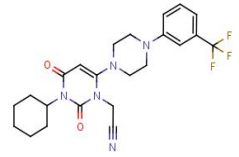
ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay.

Compound Information: R2 Analogs (with variable R1)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	**Structure**	**Source**	**MLS ID**	**SID**	**CID**	**LRH-1 AVG %Inhibition (at 5 uM)**	**SD**	**LRH-1 IC50**	**SD**	**SF-1 Avg Fold Change (at 5 uM)**	**SD**	**SF-1 IC50**	**SD**	**VP-16 Avg Fold Change (at 5uM)**	**SD**
SR-03000001395-1		S	MLS003153121	93375309	45382284	39.33% (Active)	0.049	1uM (Active)	0.183	-81%	0.01	N/D		1.3% (Inactive)	0.045
SR-03000001409-1		S	MLS003153120	93577922	45480137	17.67% (Inactive)	0.040	ND		-3%	0.10	ND		-3%	0.148
SR-03000001392-1		S		93375306	45382277	3% (Inactive)	0.010	ND		1.3% (Inactive)	0.09	ND		6% (Inactive)	0.046
SR-01000101326-2		P		85786761	7080827	2.33% (Inactive)	0.076	ND		10.3% (Inactive)	0.05	ND		-7%	0.040
SR-01000101018-2		P		85786760	6623965	3.67% (Inactive)	0.035	ND		7% (Inactive)	0.03	ND		-7%	0.095

ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

Compound Information: R2 Analogs (with variable R1)						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	**Structure**	**Source**	**MLS ID**	**SID**	**CID**	**LRH-1 AVG %Inhibition (at 5 uM)**	**SD**	**LRH-1 IC50**	**SD**	**SF-1 Avg Fold Change (at 5 uM)**	**SD**	**SF-1 IC50**	**SD**	**VP-16 Avg Fold Change (at 5uM)**	**SD**
SR-03000001149-1		S		89649715	44825226	0%	0.173	ND		2% (Inactive)	0.02	ND		2.67% (Inactive)	0.031
SR-03000001308-1		P		92092834	4621936	5.667% (Inactive)	0.136	ND		0%	0.08	ND		-7%	0.030
SR-01000101008-3		P		92092836	7080829	9.67% (Inactive)	0.087	ND		26.67% (Active)	0.11	ND		-10%	0.100
SR-01000769836-2		P		92092837	7080831	3.33% (Inactive)	0.049	ND		30% (Active)	0.04	ND		0%	0.006

ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

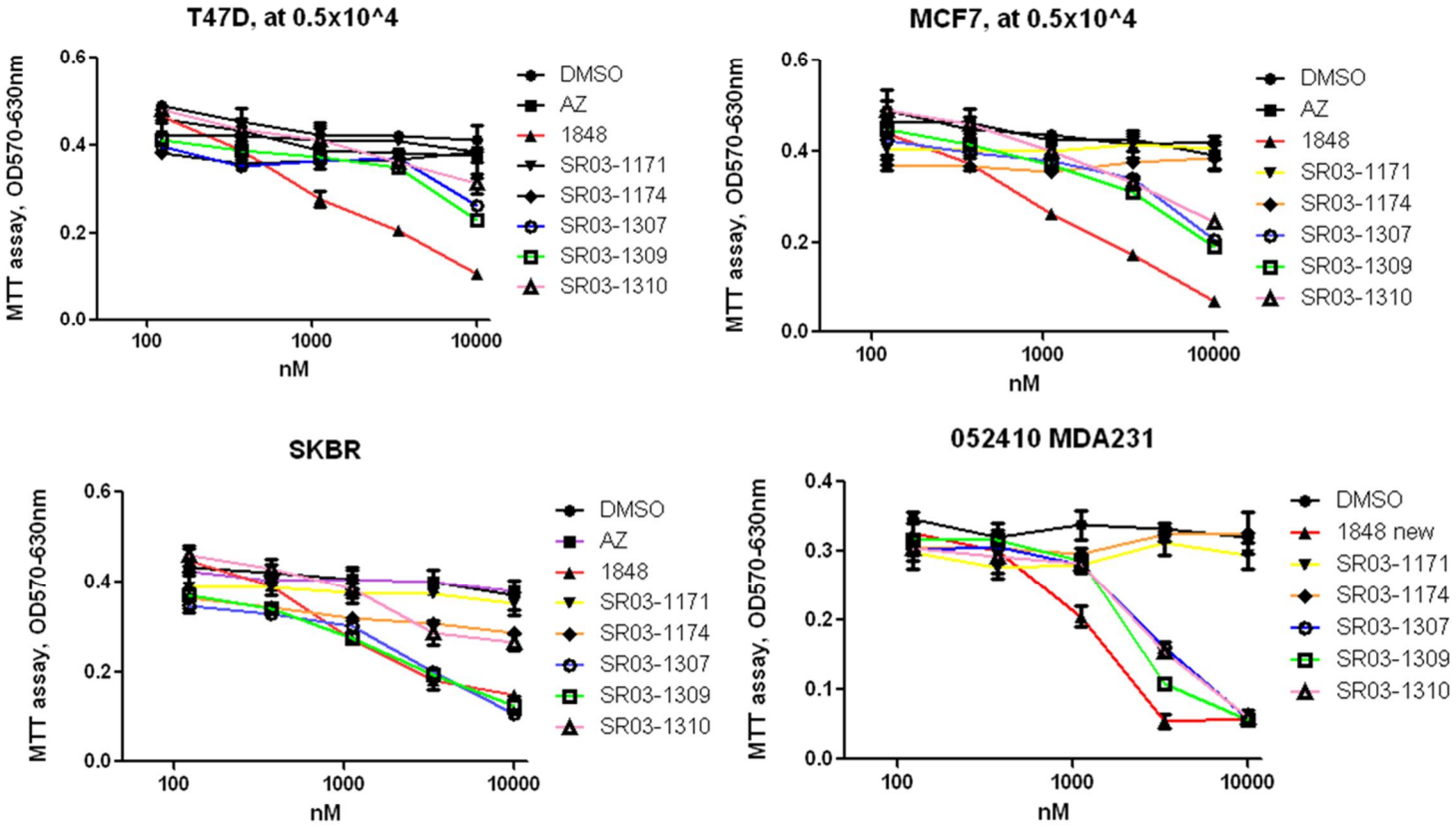
Compound Information: R3 Analogs						LRH1 %INH (AID 485348)		LRH1 Dose Response (AID 488782)		SF1 Fold Change (AID 488779)		SF1 Dose Response (AID 488780)		VP16 Fold Change (AID 488775)	
Sample ID	**Structure**	**Source**	**MLS ID**	**SID**	**CID**	**LRH-1 AVG %Inhibition (at 5 uM)**	**SD**	**LRH-1 IC50**	**SD**	**SF-1 Avg Fold Change (at 5 uM)**	**SD**	**SF-1 IC50**	**SD**	**VP-16 Avg Fold Change (at 5uM)**	**SD**
SR-03000001405-1		S		93577918	45480136	21.3% (Inactive)	0.047	ND		3% (Inactive)	0.06	ND		-1%	0.206
SR-03000001406-1		S		93577919	45480138	29.67% (Active)	0.025	ND		-24%	0.12	ND		20.33% (Active)	0.307
SR-03000001407-1		S		93577920	45480134	2% (Inactive)	0.020	ND		3.67% (Inactive)	0.04	ND		2% (Inactive)	0.062

ND indicates a compound was not tested in the indicated assay due to lack of LRH1 activity compared to probe in the 3X%INH assay

3.5 Cellular Activity

The probe is active in a variety of cell-based assays performed by Dr. Griffin. Importantly, these assays demonstrate that Probe #1 (ML180) and Probe #2 (ML179) are potent in a variety of breast cancer cell lines. These MTT data are available in PubChem as AID 504928.

Figure 6. Probes #1 ML180 (1848) and #2 ML179 (SR03-1309) Potency in 4 Different Breast Cancer Cell Lines (Dose Response Curves)



Function Assays: Star Reporter Assays

LRH1 inverse agonist probe compounds (powder samples) were also tested in assays to monitor their effects on the promoter activity of the Steroidogenic acute regulatory protein (Star). These data are available in PubChem as AID 504933. The dose response curves for each probe are below, in **Figure 7**.

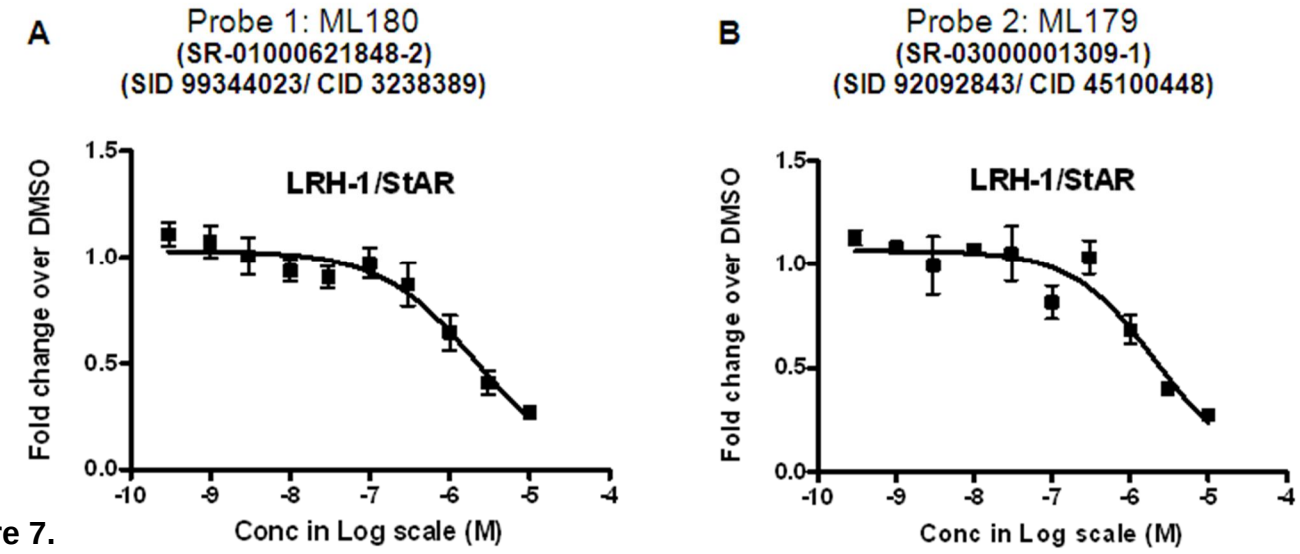


Figure 7.

Dose response curves for SR-01000621848 (Probe 1, ML180) and SR-01000001309 (Probe 2, ML179) two LRH-1 modulator probes. 293T cells were cotransfected with full length **LRH1 and StAR** reporter and were treated with various concentration of SR-01000621848 (A) or SR-01000001309 (B) for 20 hr followed by luciferase activity measurement. Relative change was determined by normalizing to vehicle treatment. Treatment with both probes, SR-01000621848 (Max Rep=74%: IC_{50}= 2.05 µM) and SR-01000001309 (Max Rep=71%: IC_{50}= 2.12µM) showed efficacy against LRH-1.

3.6 Profiling Assays

Selectivity of the probe compounds over the nuclear receptor SF-1 has been monitored and is presented in the previous sections. ML180 did not modulate GAL4-VP16. In addition, probe 1 (ML180; 1848) has been tested against a library of all 48 human nuclear receptors [43]. Preliminary analysis suggests that ML180 has little activity on other nuclear receptors (see **Figure 8**). Note that SR-1848 (ML180) is not active on LRH1 in this assay. We have confirmed that ML180 is active only on full length LRH1 and inactive on the Gal4-LBD-LRH1 construct used in the assay d. These data can also be found in PubChem as AID 504934.

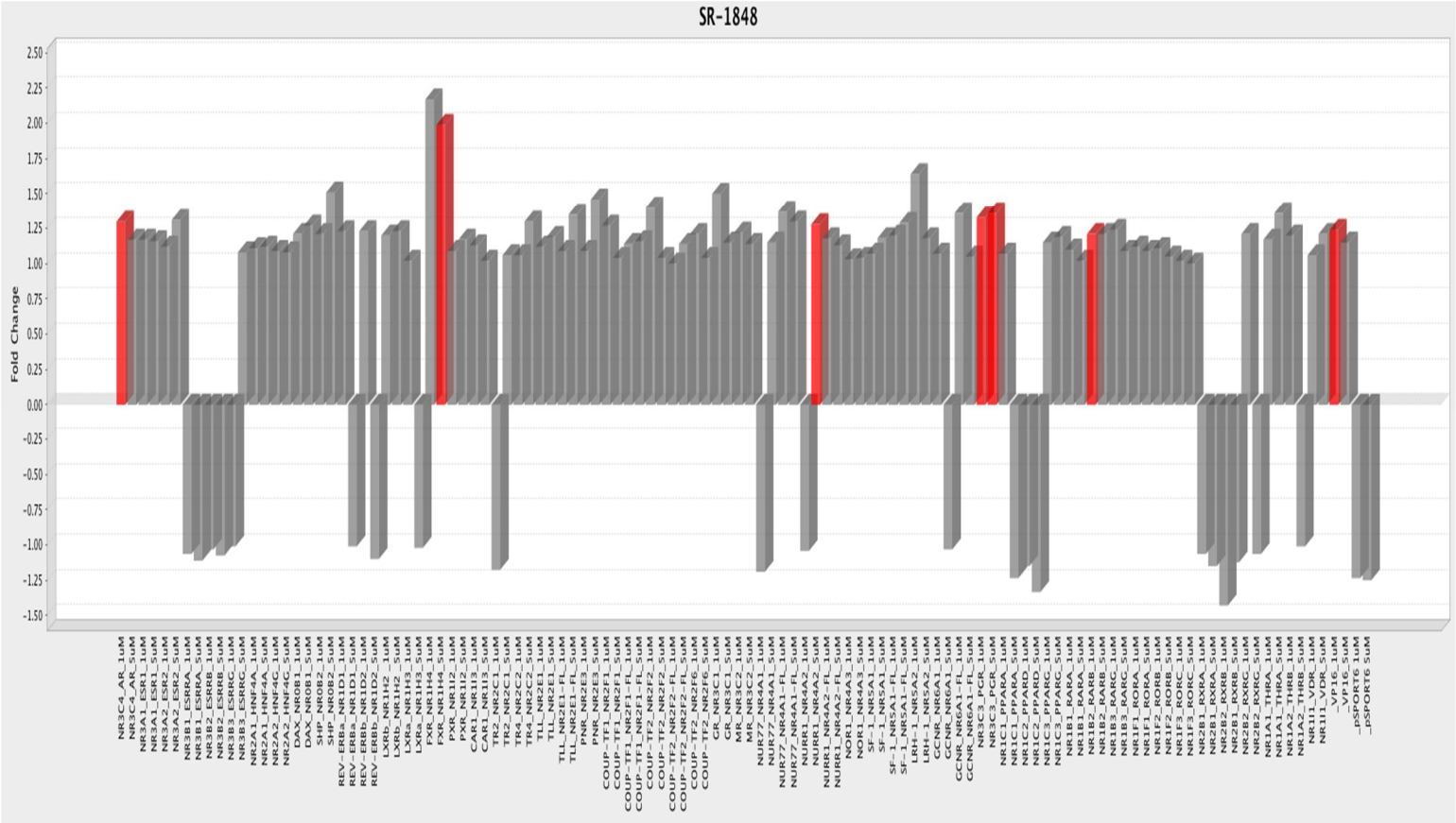


Figure 8. ML180 Nuclear Receptor Promiscuity

ML180 (MLS003153119 = CID 3238389) was tested in 470 assays in PubChem and was active in 15 AIDs (3.19%). Eleven of these are protein targets with five of the 11 being cytochrome P450's. With this knowledge we will closely monitor P450 activity of ML180 analogs. Two other targets were RORA (actual hit is in the SF-1 counterscreen to RORA) and the SF-1 assay. This is the origin of the probes as SF-1 is the closest family member to LRH1. However, as we show above, while ML180 is active on GAL4-SF1, it is not active on full length receptor. The remaining four targets are DNA damage-inducible transcript 3, E3 ubiquitin-protein ligase Mdm2, Mdm4, and centromeric isoform d. Activity is these assays could be indirect and LRH1 dependent.

ML180 was also active in; 1) Luminescence Cell-Based Primary HTS to Identify Inhibitors of Beta Cell Apoptosis. [Primary Screening], 2) Luminescence Cell-Based Dose Retest to Confirm Inhibitors of Beta Cell Apoptosis [Confirmatory], 3) uHTS luminescence assay for the identification of chemical

inhibitors of B-cell specific antigen receptor-induced NF-kB activation [Primary Screening], and 4) HTS to identify inhibitors of zVAD Induced Cell Death in L929 Cells [Primary Screening].

We are confident that ML180 is not an inhibitor of luciferase as we see selectivity over SF-1 in a luminescence-based luciferase reporter assay.

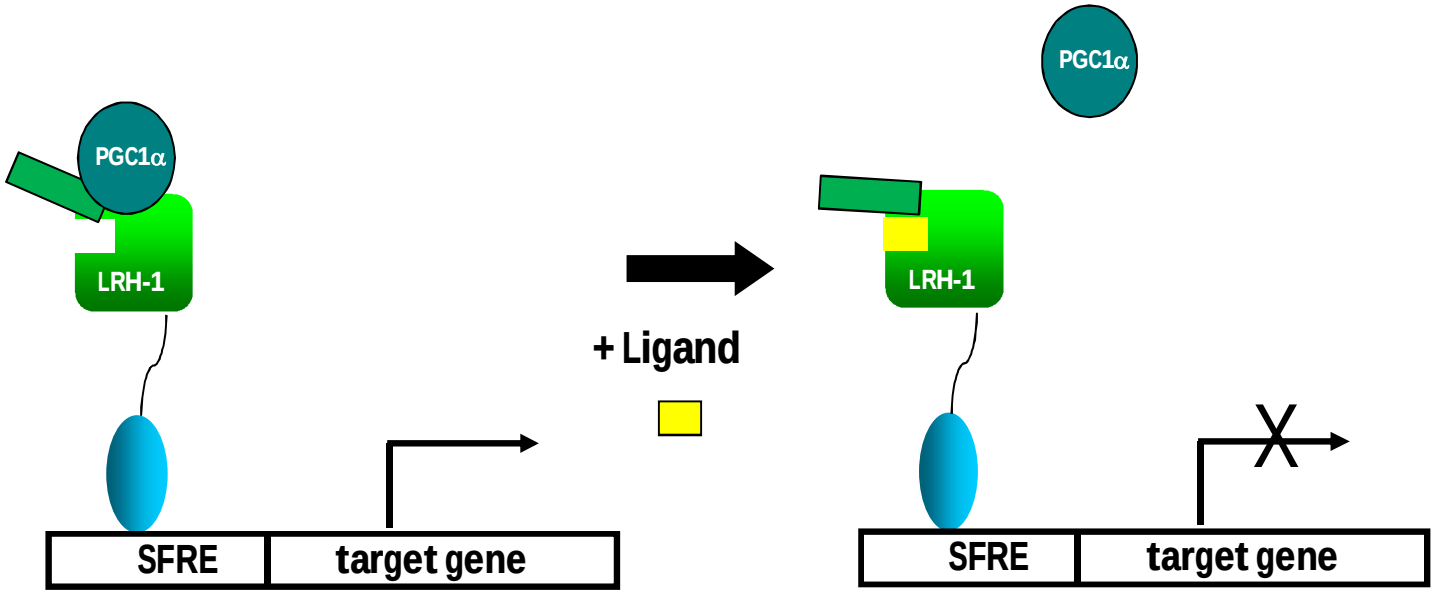
4 Discussion

4.1 Comparison to existing art and how the new probe is an improvement

Currently there are no published inverse agonists of LRH1.

4.2 Mechanism of Action Studies

Figure 9A. Probe MOA (Step 1)



LRH1 is a constitutively active nuclear receptor that binds to identical response elements as SF-1 in promoter regions of LRH1 target genes. The scheme above suggests that LRH1 is associated with NR coactivators such as PGC1a and this interaction drives transactivation of LRH1 target genes. Upon binding to LRH1, inverse agonists such as ML180 alter the conformational dynamics of the receptor causing dissociation of co-activator. It is also likely that inverse agonists enhance LRH1 interaction with NR co-repressors such as SHP.

Figure 9B. Probe MOA (Step 2)

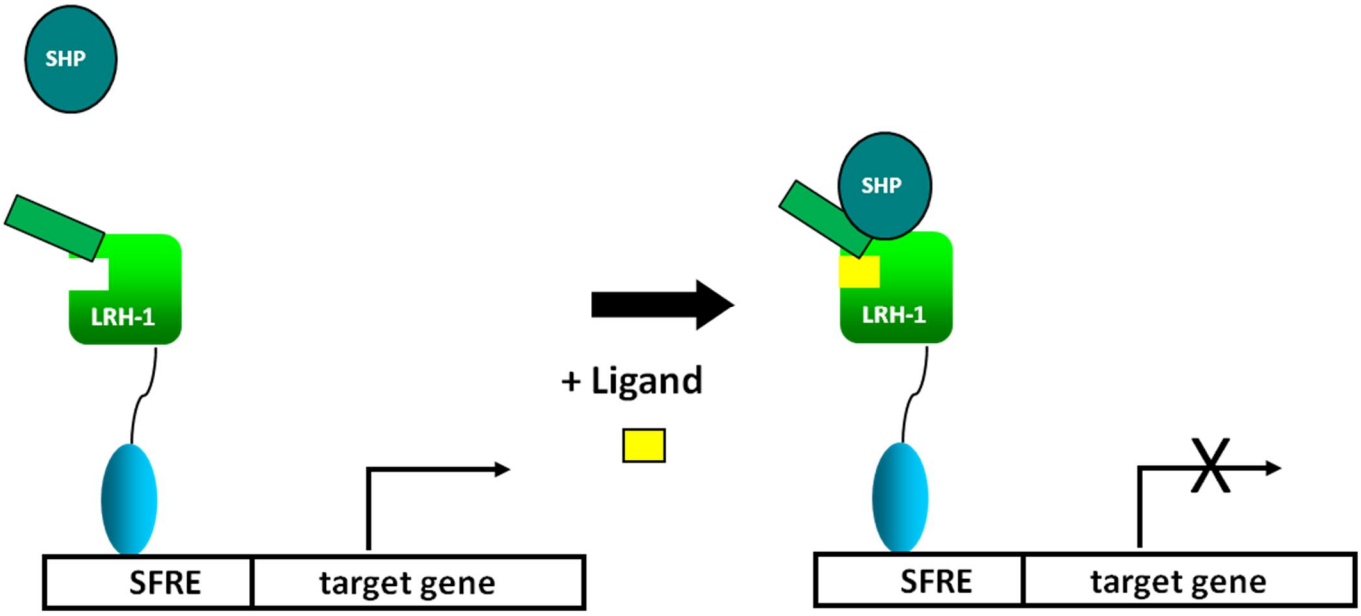
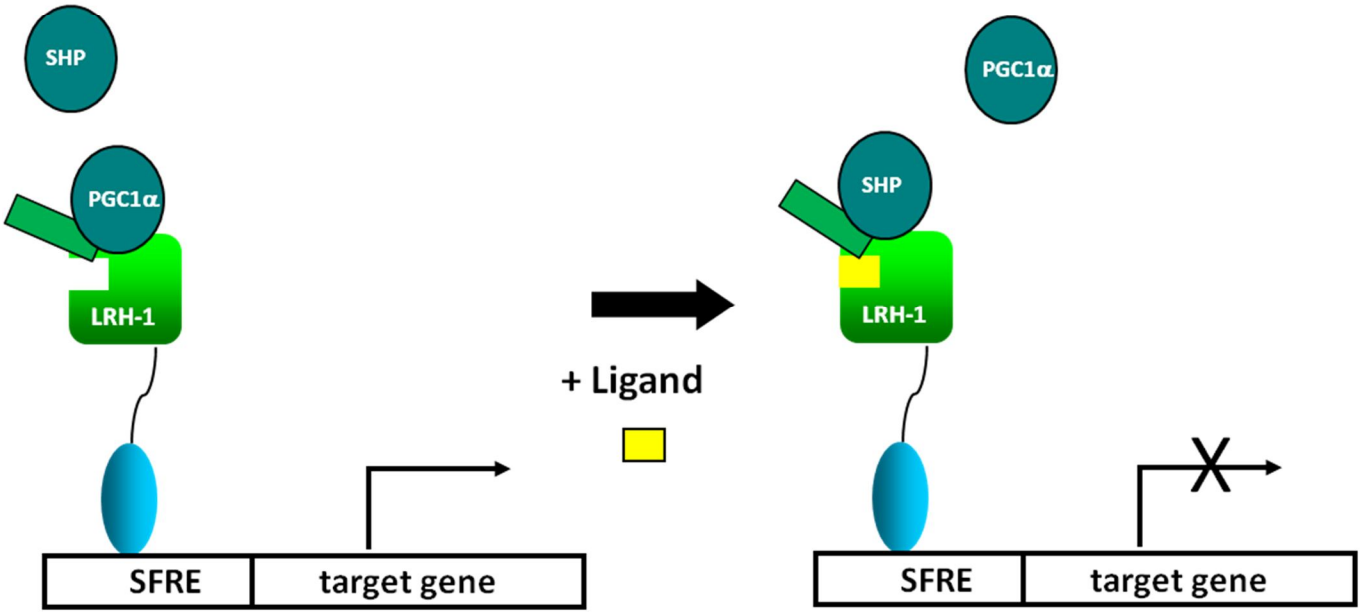
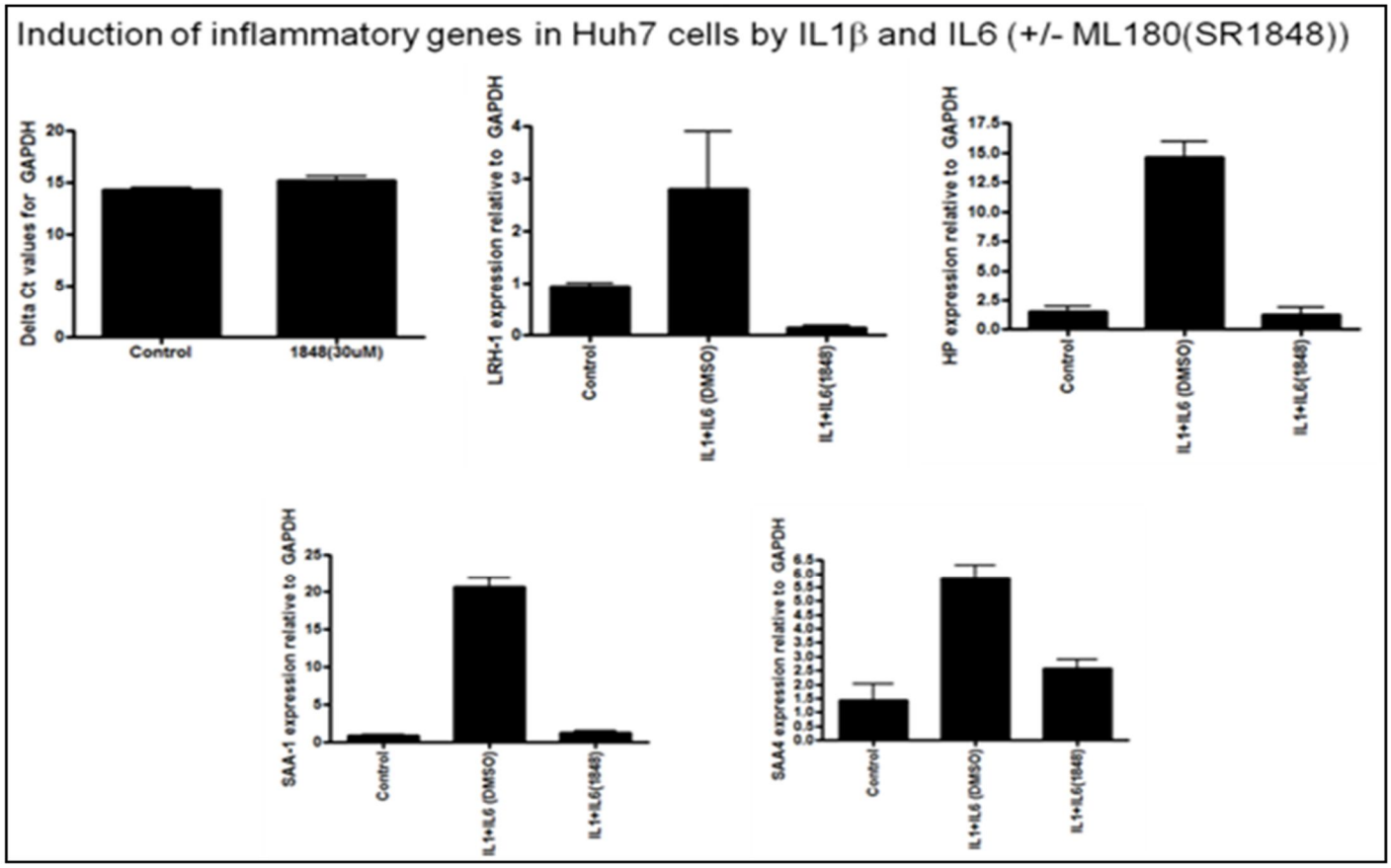


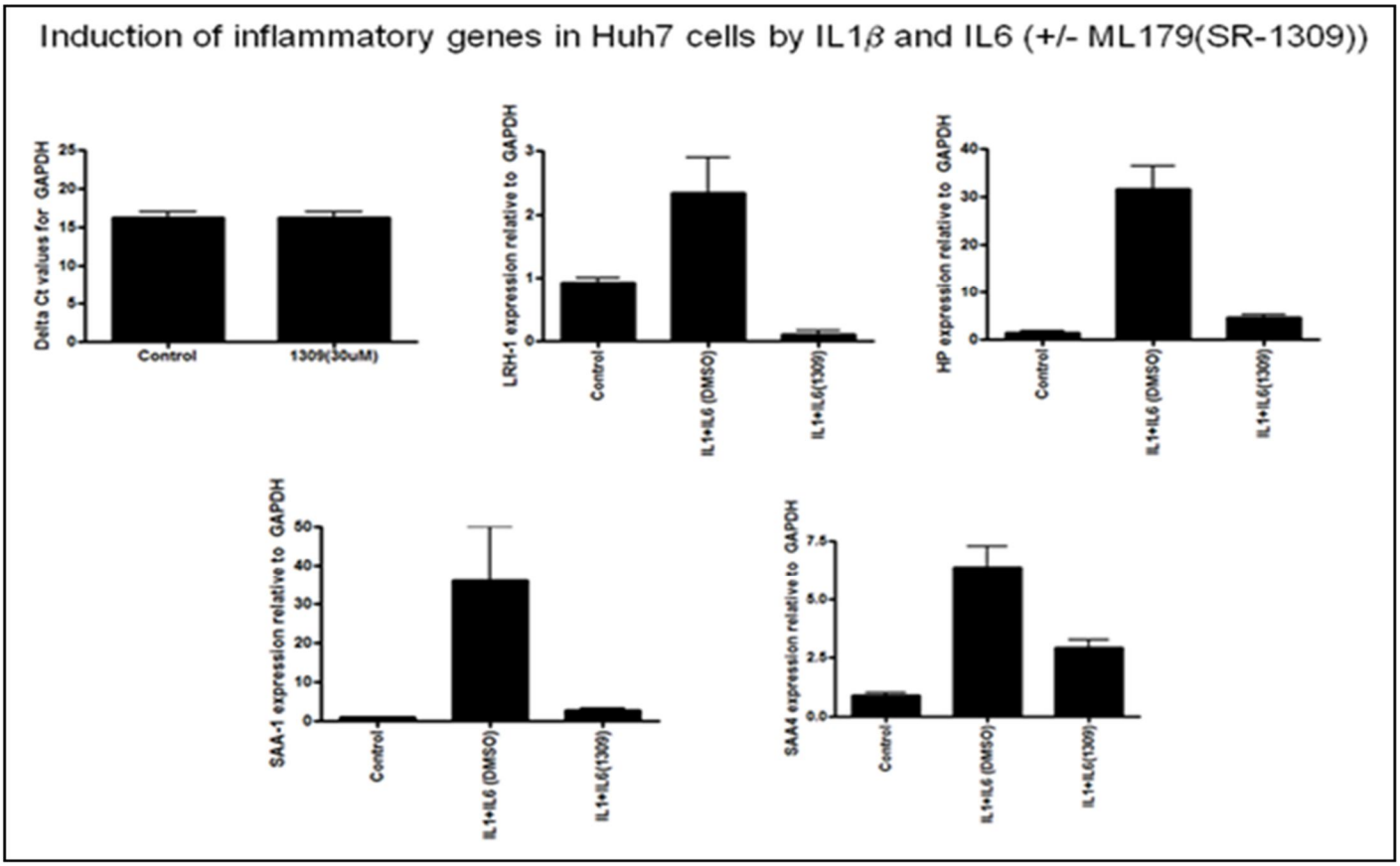
Figure 9C. Probe MOA (Step 3)



Acute Phase Response (APR)- Preliminary studies in our lab using Huh7 cells stimulated with IL1β and IL6 results in significant increase in expression of LRH1, haptoglobin (Hp), serum amyloid A-4 (SAA1), and serum amyloid A-4 (SAA4) as determined by qPCR (see PubChem AID 488769). Surprisingly and opposite of the anticipated outcome, treatment of these cells with the inverse agonists ML180 and ML179 represses the expression of three of these genes (Hp, SAA1, SAA4) to the level of control cells (unstimulated) and LRH1 to levels below that in control cells. **This finding confirms that our inverse agonists can repress the expression of LRH1 and more importantly, suggests that LRH1 inverse agonists can repress APR genes in similar fashion to LRH1 agonists.** Clearly the mechanism of action of inverse agonists in this model is likely different than that of agonists. This finding warrants further mechanistic studies which will be carried out as part of this extended probe development project.

Figure 10. Hp, SAA1 & SAA4 Expression in the Presence and Absence of Probes





The above QPCR data have been submitted to PubChem as AID 488769.

LRH1 Modulation of Aromatase Expression – preliminary studies in our lab show that treatment of hepG2 cells with SR-1848 reduces the expression of aromatase cytochrome p450 gene (Cyp19). Treatment of these cells with a siRNA for LRH1 also reduces the expression of aromatase. Treatment of cells with siRNA for LRH1 blunts the effects of SR-1848 (probe ML180) on aromatase expression. These results have been submitted to PubChem as AID 488782. Optimization of Cyp19 knockdown is ongoing. The relevance of these studies is found in prior discovery that aromatase is highly upregulated in breast cancers as well as breast tissue surrounding tumors.

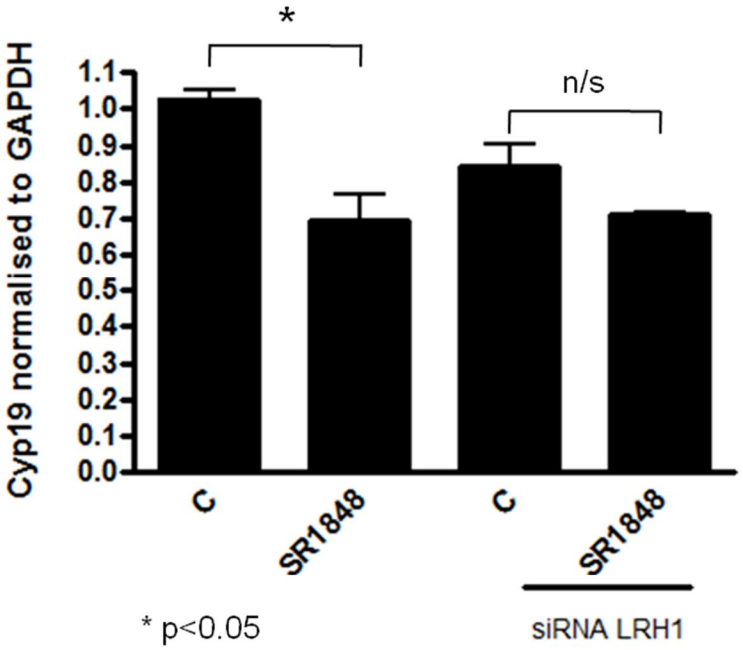


Figure 11.

Reduction of Cyp19 aromatase mRNA expression by ML-180 (SR-1848) is LRH-1 dependent. HepG2 cells treated with ML-180 (SR1848) demonstrate significant reduction in expression of Cyp19 aromatase. This effect is blunted in cells treated with LRH-1 siRNA that have reduced expression of LRH-1.

4.3 Planned Future Studies We intend to continue to explore the SAR in this LRH1 inverse agonist series, in order to identify selective LRH1 probes that have IC_{50}'s <100 nM and >50% maximal repression of LRH1 expression at ≤200 nM. The PK properties of the lead compounds will also be optimized using a series of in vitro and in vivo (mouse) PK studies to identify a compound that is suitable for use in animal studies. In addition to the assays described above, advanced compounds will be tested in the following:

HTRF cell-based aromatase activity assay: LRH1 has been shown to control the expression of Cyp19 (aromatase) and as such inverse agonists should reduce the level of aromatase activity in breast cancer cells. To test this hypothesis, we will use a cell-based HTRF aromatase assay as described here. This method is a homogeneous assay for monitoring the aromatase-mediated conversion of estrone into estradiol in breast cancer cells. The assay is a competitive immunoassay in which estradiol produced by breast cancer cells (e.g. ER-positive MCF7 or ER-negative MDA-MB-

231) and XL665-labeled estradiol compete for binding to an Anti-estradiol MAb labeled with Cryptate. Due to the proximity between the Cryptate and the XL665 molecules, maximum FRET (Fluorescence Resonance Energy Transfer) is generated when the sample does not contain any estradiol. The signal decreases with increasing estradiol concentration present in the breast cancer cell supernatant. The assay has been developed by CisBio and will be run as follows. Briefly, cells (12000-25000 cells/well in 100 µL in 96-well plate) are grown in culture media supplemented with 10% charcoal-depleted FBS for 48h at 37 °C. The cells are washed with PBS and 50 µL of culture media containing various concentrations of the test compound of interest are added to the corresponding wells followed by further incubation for 24h at 37 °C. After this incubation step, the optimal estrone concentration is added to the wells and further incubated for 24h at 37 °C. At the detection step, 10 µL of the cell supernatant are mixed with 5 uL of Anti-estradiol Cryptate conjugate and 5 µL of Estradiol-XL665 and incubated for 2h at RT in 384-well plates (black). Fluorescence signal can be read for instance on a Viewlux ultraHTS microplate imager from PerkinElmer.

Invasion Assay: Compounds that are potent inverse agonists of LRH1 and that are active in the MTT assay will be tested in an invasion assay. We will use the BD BioCoat Tumor Invasion System which is ideal for use to test prospective anti-metastatic compounds. This system is suitable for automation and is compatible with most fluorescence plate readers and liquid handling devices. The plates contain a uniform layer of matrigel matrix which acts as a reconstituted basement membrane providing a barrier to non-invasive cells but providing an appropriate protein structure to study invasion. The coating process occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane. In contrast, invasive cells (malignant and non-malignant cells) are able to detach themselves from and migrate through the coated membrane. The assay will be run as recommended by the manufacturer.

***in vitro* metabolism studies:** *in vitro* metabolic stability studies will be performed by the DMPK laboratory at Scripps Florida, to predict the relative rate of biotransformation to help select candidates that are likely to have favorable pharmacokinetics *in vivo*. Drug candidates are incubated (separately) with pooled human, mouse and rat microsomes and cofactors to determine the rate of metabolism. Incubations will be performed using a 96-well format to increase sample throughput. Typical drug concentrations studied are 1 µM and 10 µM, providing concentrations that are comparable to plasma drug levels that are efficacious. Samples will be collected at multiple time points and the concentration of the parent drug will be determined using HPLC coupled to a triple quadrupole mass spectrometer (LC/MS-MS), allowing the calculation of half-life for each drug. In select cases, we may also proceed with further LC/MS-MS analysis of samples to structurally identify the metabolites. In cases where the most promising compounds have poor metabolic stability, medicinal chemistry will be applied to modify the regions of the compounds undergoing metabolism. Compounds will also be assessed for their (in)ability to inhibit cytochrome P450's. We desire lead compounds that have IC_{50}'s ≥ 10 µM against a battery of six major human CYP's that are run on a weekly basis at Scripps Florida.

***in vivo* mouse PK:** In vivo pharmacokinetics of selected compounds (up to 5 per year) will be assessed in C57Bl6 mice (n = 24). Compounds are dosed intravenously at 1 mg/kg of orally by

gavage at 2 mg/kg. Blood is taken at eight time points and collected into EDTA containing tubes and plasma was generated using standard centrifugation techniques. Because of the small blood volume in mice, each mouse is bled only two times. Plasma proteins are precipitated with acetonitrile and drug concentrations are determined by LC-MS/MS. Data are fit by WinNonLin using a noncompartmental model and basic pharmacokinetic parameters including peak plasma concentration (Cmax), oral bioavailability, exposure (AUC), half life (t1/2), clearance (CL), and volume of distribution (Vd) are calculated. The concentration of drug in the brain will also be measured. All procedures are approved by the Scripps Florida IACUC and the Scripps Florida vivarium is fully AAALAC accredited.

Tumor xenograft in athymic mice: The purpose of these experiments is to study LRH1 inverse agonists for their ability to treat breast cancers, using the standard breast cancer xenograft model in nude mice. This is one of the best studied tumor biology systems for assessing the effects of drugs such as SERMs and aromatase inhibitors, as tumors induced by MCF-7 and T47D breast cancer cells are highly sensitive to hormonal treatment, and also eventually develop acquired resistance as seen in patients. Thus this is an excellent model to test the effects of LRH1 inverse agonists as these compounds should impact levels of estrogen in these animals. Preliminary results suggest that our probe compounds ML179 and ML180 are effective at reducing the proliferation of ER-negative breast cancer cells thus we will also include the use of MDA-MB-231 cells in this model. Briefly, athymic mice will be injected subcutaneously in the lower back with a suspension of MCF-7, T47D, or MDA-MB-231 human breast cancer cells (~10^6 cells) in sterile saline with Matrigel. Compound treatment (10 mg/kg intraperitoneally) and vehicle only controls typically begins approximately 2-4 weeks after implantation when tumors reach ~500 cubic millimeters in size.

DIO mouse model: 4 to 5 week old C57BL/6J mice will be obtained from The Jackson Laboratory. DIO animals (6 animals per group) will be fed a high fat diet for 3 months (Clinton/Cybulsky rodent diet, 40% kcal from fat, devoid of cholesterol; Research Diets, New Brunswick NJ). Lean control mice will receive a standard diet during the same time period. For glucose tolerance tests, mice will be intraperitoneally (i.p.) injected with 10mg/kg of the LRH1 modulator or vehicle only for 6 days, and fasted overnight before i.p. injection of 2 g/kg D-glucose. Blood samples will be collected at time 0, and 15, 30, 60 and 120 min after injection of the glucose Plasma glucose, triglycerides and total lipids will be measured using standard techniques. Body weights and food intake will also be measured.

5 References

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3. Harada, N., *Aberrant expression of aromatase in breast cancer tissues.* J Steroid Biochem Mol Biol, 1997. **61**(3-6): p. 175-84.PMID 9365188.
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