Probe Report for Inverse Agonists of Retinoic Acid Receptors (RAR):

The following 2 compounds are identified as probes in this report:

- Compound 14: SR-03000000057, SID = 46499846)
- Compound 22: SR-03000000065, SID = 46499854)

Additional data not published in PubChem but presented in this probe report may be found in the following publications:

[1] Synthesis of Small Molecule Inhibitors of the Orphan Nuclear Receptor Steroidogenic Factor-1 (NR5A-1) Based on Isoquinolinone Scaffolds. Joshua Roth, Franck Madoux, Peter Hodder, and William R. Roush (in preparation)

[2] Discovery of Isoquinolinone Derivatives as Selective Inhibitors of Steroidogenic Factor 1 (NR5A1) Activity via Cell-based High-Throughput Screening Assays. Franck Madoux, Xiaolin Li, Pierre Baillargeon, Peter Chase, Gina Zastrow, Michael Cameron, Patrick Griffin, Scott Thacher and Peter Hodder (in preparation) and

[3] Selective Retinoic Acid Receptor Inverse Agonists Revealed from Profiling a Novel Nuclear Receptor Library. Gina Zastrow, Joshua Roth, Kevin Hayes, Franck Madoux, Scott Busby, Peter Hodder, William Roush, Juliana Conkright and Patrick R. Griffin (in preparation)

Project Title: Selective Inverse Agonists of the Retinoic Acid Receptor (NR1B)
Center Based Initiative: Profiling NR Panel and Probe Optimization
Center Based Initiative Investigators: Griffin, Hodder, and Roush
Target or Pathway: retinoic acid receptor (RAR) alpha, beta, gamma (RARs; systematic nomenclature NR1B)
Screening Center Name: The Scripps Research Institute Molecular Screening Center
Principal Investigator of Screening Center: Hugh Rosen
Probe PubChem Compound Identifier: SID = 46499846, SID = 46499854

Original Grant Number: 1 X01 MH077624-01
Original Assay Provider & Institution: Xiaolin Li, PhD, Orphagen Pharmaceuticals
Original Assay or Pathway Target: SF-1 and ROR

Assay Provider Information

Specific Aim: To identify modular, chemically tractable, selective, and cell-permeable inverse agonists of the nuclear receptor RAR.

Significance: The retinoic acid receptors (RARs) are ligand-dependent transcription factors that belong to the nuclear receptors (NRs) superfamily and have broad roles in development, cell growth and survival, vision [4], spermatogenesis [5], inflammation [6], and neural patterning [7]. These receptors act in-*trans* as homodimers or heterodimers with retinoid X receptors (RXRs). The actions of RARs are stimulated by the binding of cognate natural ligands (all *trans* retinoic acid and 9-*cis* retinoic acid) and synthetic ligands. Ligand activation drives physical interactions with coregulatory proteins (co-repressors and co-activators) and other molecular targets such as p21 and AP1/JUN [8], as well as DNA or chromatin of target genes [9]. Due to their broad action in diverse cell and tissue populations, RARs are essential signaling proteins for basic and clinical research.

Rationale: Small molecules targeting RAR action have demonstrated some success as therapeutic targets for a wide range of diseases [10]. As a result of the role of RARs in cell differentiation and apoptosis, several studies suggest that modulating the activity of RARs and RXRs may have potent anticancer effects, as seen in trials for acute promyelocytic leukemia (APL) [8], and may possess therapeutic potential for metabolic disorders such as diabetes and obesity [11]. Unfortunately, the use of RAR modulators has been associated with cellular toxicities, including teratogenicity and mucocutaneous toxicity [12], which typically result from the nonselective action of these compounds at nontarget receptor isotypes and/or cell populations. Thus, the design of receptor-selective compounds may provide additional avenues for understanding the biological roles of these receptors and further uncover the therapeutic potential of these proteins in metabolic diseases and cancer.

Screening Center Information

Center Based Initiative: We have developed a novel Gal4 screen containing all 48 human nuclear receptors for selectivity profiling of probe molecules targeting nuclear receptors. The screen was designed to facilitate rapid profiling of probe compounds resulting from a HTS screen or medicinal chemistry efforts over other nuclear receptors. The utility of this profiling assay is demonstrated here as potent and selective RAR inverse agonists were identified in this profiling assay when applied to probe optimization of SF-1 inverse agonists.

Assay Implementation and Screening

PubChem bioassay names and identifiers: This report describes syntheses and initial structure activity relationship (SAR) studies of RAR isoform inverse agonists based on isoquinolinone scaffolds. One of the RAR selective probes characterized here (SR-03000000057, SID = 46499846) is a novel analog of compounds originally identified as active against SF-1 in PubChem BioAssays AID 525, 599, and 600. However, not all assays for the RAR campaign are found in PubChem. These assays, including analog synthesis, nuclear receptor (NR) library profiling assay, and cytotoxicity (CellTiter-Glo) assays are detailed here. Please refer to the original SF-1 and SF-1 optimization probe reports for details http://molscreen.florida.scripps.edu/probes.html

List of Relevant AIDs that may be used as counterscreen information: AID 599 (inhibitors of RORα), and AID 675 (dose response of inhibitors of RORα). Data from the counterscreens below will be uploaded to PubChem in the near future.

***Identification of SF-1 inhibitors that serve as scaffolds for RAR probes. Primary and Counterscreen Summary (AID 525, AID 599*)**: Approximately 65,000 compounds were screened for SF-1 inhibition by the Molecular Library Screening Centers Network (MLSCN) at The Scripps Research Institute [2]. All initial hits were counter screened against the retinoic acid receptor-related orphan receptor α (RORα), a phylogenetically distant nuclear receptor, in order to identify and eliminate promiscuous as well as non-selective compounds. This led to the identification of two mid-nanomolar SF-1 selective inhibitors, compounds SIDs 7970631 and 7969543 [2]. While both compounds meet selectivity and cytotoxicity criteria for chemical probes set by the MLSCN, and their selectivity in the counterscreen was acceptable [2], it was believed that their selectivity and cytotoxicity could be improved.

Accordingly, compounds SIDs 7970631 and 7969543, referred to here as compounds 1 and 2, respectively, were selected as starting points for the development of RAR biological probes. After synthesis and selectivity testing of numerous analogs, two compounds (SIDs 46499846 and 46499854) were identified as novel RAR probes with lower cellular toxicity and improved RAR selectivity compared to the initial two leads (**Table 1)**. These two compounds are the focus of the current report.

SR Number	SID	IC_{50} (μM)						
		RARα	RARβ	RARγ	SF-1*	ROR α*	VP16*	CellTiter-Glo*
SR-03000000057	46499846	0.156	0.0603	0.0386	13.67	21.5	24.12	69.42
SR-03000000065	46499854	0.362	0.452	0.698	>99	>99	>99	>99

Table 1. RAR probes. IC_{50} data for probes are shown for cytotoxicity, SF-1, RORα, VP16 assays. *indicates CHO-K1 cells. Other assays used HEK293T cells. Reference ID is the compound ID used in the manuscript (reference 1).

Probe Selectivity Profiling Assays

Confirmation/ Titration Assays. These assays were performed using the same type of assay as described in PubChem AID 600. Details can be found in [2] and in PubChem

at http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=600. Additional AIDs related to probe development efforts will be uploaded to PubChem.

Percent inhibition was calculated as in the primary screen. The confirmation/ titration assays were performed by testing compounds in 10-point 1:3 serial dilution starting at a nominal concentration of 99 μM. Percent inhibition was plotted against compound concentration. A four parameter equation describing a sigmoidal dose-response curve was then fitted with adjustable baseline using Assay Explorer software (MDL Information Systems). The reported IC_{50} values were generated from fitted curves by solving for X-intercept at the 50% inhibition level of Y-intercept. In cases where the highest concentration tested (99 μM) did not result in > 50% inhibition or where no curve fit was achieved, the IC_{50} was determined manually depending on the observed inhibition at the individual concentrations. Compounds with IC_{50} values of greater than 10 μM were considered inactive. Compounds with IC_{50} values equal to or less than 10 μM were considered active.

RORα Counterscreen

This assay was performed using the same assay as in PubChem AID 599, which can be found at http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=599. Additional AIDs related to probe development efforts will be uploaded to PubChem.

Specifically, the RORα counterscreen was performed using the same conditions as the titration assay, except that cells were cotransfected with pFA-hRORα plasmid (Orphagen Pharmaceuticals), which encodes the DNA binding domain (DBD) of the yeast transcription factor GAL4 fused to the ligand binding domain (LBD) of RORa. Each fusion construct was co-transfected into CHO-K1 cells with a plasmid expressing a GAL4 recognition sequence used to drive expression of a luciferase reporter gene. These assays revealed that compounds 1 and 2 did not have activity against RORα.

Cell Viability Assays

AIDs related to probe development efforts will be uploaded to PubChem. CHO-K1 cells were plated at 500 cells per well in 1536-well plates in 5 μL of media (F12 supplemented with 10% FBS and 1% Pen/Strep/Neo). Compounds (50 nL of 100X DMSO solution per well) were prepared as 10-point, 1:3 serial dilutions starting at 10 mM, then added to the cells using the pin tool. Plates were then incubated 48 hours at 37°C, 5% CO_2 and 95% relative humidity. After incubation, 5 μL of CellTiter-Glo® reagent (Promega, Madison, WI) were added to each well, and plates were allowed to incubate for 15 minutes at room temperature. Luminescence was recorded for 30 seconds per well using the ViewLux™ reader (PerkinElmer, Turku, Finland). Viability was expressed as a percentage relative to wells containing media only (0%) and wells containing cells treated with DMSO only (100%).

Gal4 NR Library Profiling: Compounds 1 and 2 (Figure 1)

The Gal4 NR library facilitates profiling of a probe compound's reactivity not only with closely related family members of the target but with all known human nuclear receptors.

The power of this assay is demonstrated here where ligands for SF-1 were found to have potent inhibitory activity on RAR, although these two receptors have relatively low homology. Surprisingly, these compounds had no activity on LRH-1, the closest family member to SF-1 (see **Table 2**).

Description of GAL4 NR library: The Gal4 NR library was built by replacing the endogenous N-terminus and DBD of all 48 receptors with a Gal4 DNA binding domain (DBD). The fusion constructs consist of the Gal4 DBD, the hinge domain and LBD (and F domain if applicable) of the human receptors. At the time of these experiments only 79% (38/48) of the Gal4 NR library was complete. The library was plated in triplicate on 384-well plates and HEK293 cells were co-transfected with the well-specific construct and the UAS luciferase reporter with a final volume of 40 µl. Control wells contained constructs encoding for the Gal4 DBD alone (pBind) or Gal4 fused to VP16 were also analyzed. After 24 hours, optimized compounds or DMSO were added to the plates and allowed to incubate for 20 hours prior to addition of 40 ml BriteLite (Perkin Elmer, Waltham, MA) to measure luciferase activity. Compounds that attenuate the Gal4VP16-dependent luciferase activity in the positive control were considered promiscuous or cytotoxic (see cell viability assays described below). Each compound was evaluated using two plates of the Gal4 NR library providing six replicate experiments with replicate DMSO-only plates as negative controls. The luciferase signals for each compound was averaged over the six replicates and compared to DMSO only controls. Compounds with mean signals three standard deviations from the DMSO controls were considered hits in this assay.

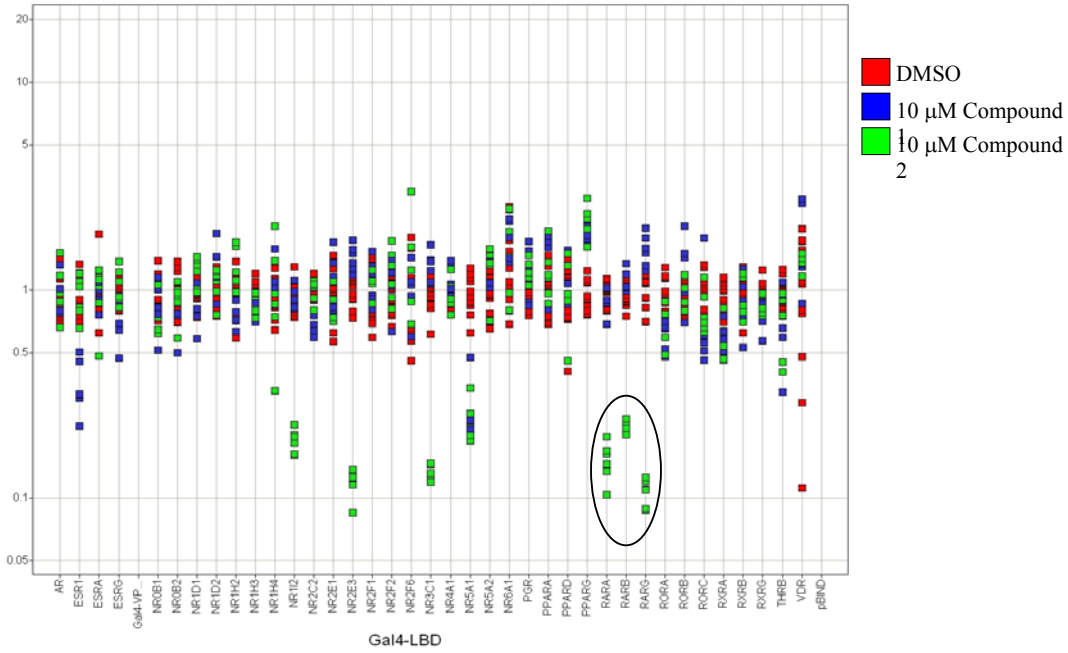


Figure 1. Scattergram of activity of a panel of NHRs following treatment with compounds 1 or 2. The oval highlights that at 10 µM, compound 2 inhibits activity of the three RAR isoforms. The Y axis indicates fold receptor activity normalized to control, and the X axis indicates the GAL4 NR tested.

Profiling Results: The two original SF-1 probes that were shown to be devoid of activity towards RORa and VP16, were profiled in the GAL4-NR library. Compounds that inhibit activity (inverse agonism) of a particular NR will demonstrate lower luminescence compared to the DMSO control group, and activators will show an increase**.** These assays revealed that at 10 μM**,** that compound 1 was highly selective for SF-1 while compound 2 inhibited all three RAR isoforms (**Figure 1)**, suggesting that analogs of compound 2 could be designed with improved RAR potency and selectivity. The most interesting aspect of these compounds is their absence of activity on LRH-1, the closest family member of SF-1.

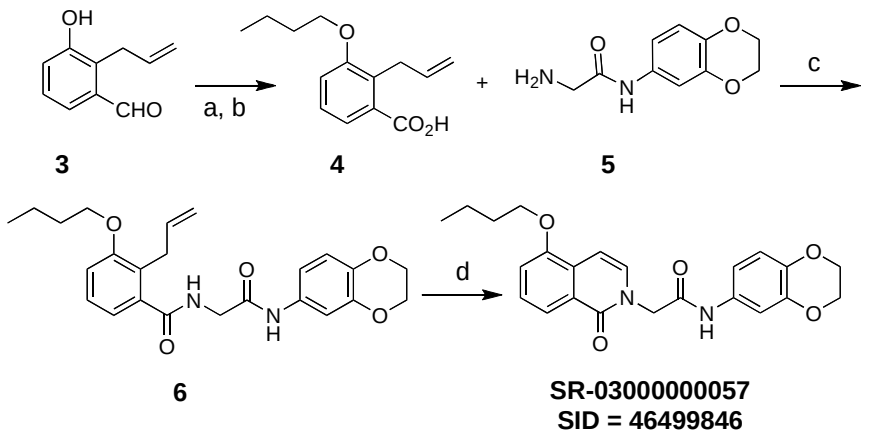
SF1_LBD	RORa_LBD	RARg_LBD	LRH1_LBD	RARa_LBD	RARb_LBD	
***	15.8	22.7	64.5	21	22.1	SF1_LBD
	***	26.5	15.3	24.3	26.5	RORa_LBD
		***	22.1	86.7	89.5	RARg_LBD
			***	21	21.5	LRH1_LBD
				***	92.8	RARa_LBD
					***	RARb_LBD

Table 2. Ligand binding domains, as determined by the Refseq entry, for SF1, LRH1, RORα, RARα, RARβ, and RARγ where aligned using ClustalW. Percent identities are presented as a matrix of associations.

Synthesis of analogs of compound 2 with improved RAR selectivity

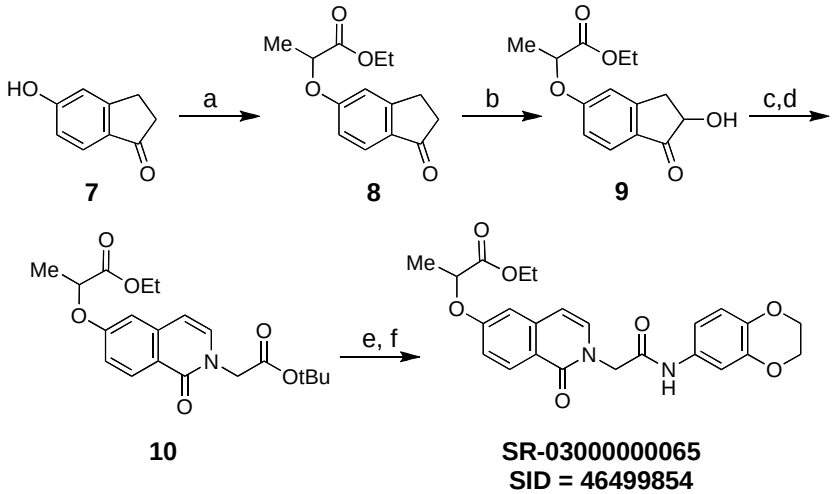
In an effort to optimize potency of HTS-identified probes for SF-1, a modular approach was taken to identify key SAR elements for SF-1 interaction as described below. We have previously developed three distinct routes for the synthesis of analogs of compounds 1 and 2 that enable different aspects of the SAR of this series to be examined (please refer the publication on SF-1 analog synthesis that has been submitted and to SRIMSC SF-1 chemistry optimization probe report for information regarding analog synthesis, found at http://molscreen.florida.scripps.edu/probes.html). Details of the syntheses of the two RAR probes are discussed below.

RAR probe **14** (**SR-03000000057** (**SID 46499846**)) was synthesized as summarized in Scheme 1. Aldehyde **3**, available from the Claisen rearrangement of *m*-salicaldehyde allyl ether (Danishefsky, S. J.; Harrison, P. J.; Webb II, R. R.; O'Neill, B. T. *J. Am. Chem. Soc.* **1985**, *107*, 1421) was alkylated with iodobutane. The resulting product was then oxidized using chromic acid to give carboxylic acid **4**. Standard peptide coupling of **4** with glycinamide **5** provided diamide **6**, which was then converted to RAR probe **SR-03000000057** (**SID 46499846**) via ozonolysis of the vinyl group and dehydration of the hemiaminal that is formed *in situ*.



Scheme 1. (a) iodobutane, acetone, K_2CO_3, , 60°C, 4 h (60-85%); (b) 1.5 equiv H_2CrO_4, acetone, 1 h (98%); (c) **5**, EDC, DMAP, CH_2Cl_2 (83%); (d). (i). O_3, CH_2Cl_2, –78°C, (ii) PPh_3, (iii). catalytic I_2, CH_2Cl_2 (45%).

RAR probe 22 (SR-**03000000065**, **SID = 46499854**) was synthesized as summarized in Scheme 2. Alkylation of commercially available 5-hydroxytetalone **(7)** with ethyl (±)-2-bromopropionate provided **8**. Treatment of indanone **8** with TBS-OTf and Et_3N gave the silyl enol ether which, without isolation, was treated with catalytic N-methylmorpholine N-oxide (NMO) and catalytic osmium tetroxide. The resulting α-hydroxyindanone **9** was oxidized using $NaIO_4$ to give the δ-hydroxylactone, which was condensed with glycine *tert*-butyl ester. Subsequent deprotection of the t-butyl ester of **10** and then coupling of the carboxylic acid with 1,4-benzodioxan-6-amine provided RAR antagonist probe **SR-03000000065**, **SID = 46499854**.



Scheme 2. (a) *dl*-ethyl-2-bromopropionate, K_2CO_3, acetone, 60°C, 4 h, (72%) (b) (i). TBSOTf, Et_3N, THF (ii) OsO_4 (2%), NMO, *t*-BuOH, acetone. (c) $NaIO_4$, acetone, H_2O, THF, *t*-BuOH. (d) glycine tert-butyl ester hydrochloride, benzene, Et_3N and AcOH until pH 3-5, 100°C sealed tube 12 h. (16%, 3 steps from **8**) (e) 2 M HCl in Et_2O and EtOH, (99%) (f) 1,4-benzodioxan-6-amine, EDC, DMAP, CH_2Cl_2, (55%)

Structure Activity Relationships of the RAR probes

All synthesized SF-1 inhibitor analogs were tested in GAL4 NR assays to determine their ability to inhibit RAR isoforms α, β, and γ or pSPORT6 as a control (Figure 2). A heat map representation of the effect of each compound on the activities of GAL4-RARs, GAL4-SF-1, GAL4-LRH-1 [liver receptor homolog-1 (LRH-1, NR5A2)], GAL4-VP16 control, and empty vector pSPORT6 normalized to DMSO control is shown in **Figure 3**. Together, these assays suggest that analogs **14** and **22** are potent inverse agonists of RAR with significant enhancement in selectivity compared to the SF-1 probe compound 2. Compounds **14** and **22** possess structural features that likely contribute to their ability to antagonize RARs. Both share at least two polar regions comprised of carboxylic acid groups, reported to act as an ionic bridge with an arginine in the LBD of RARs [9]. Typically, adding larger hydrophobic constituents leads to reduced activity of inhibitors [13]. Of particular interest for further follow up is the apparent selectivity of some compounds over RARγ.

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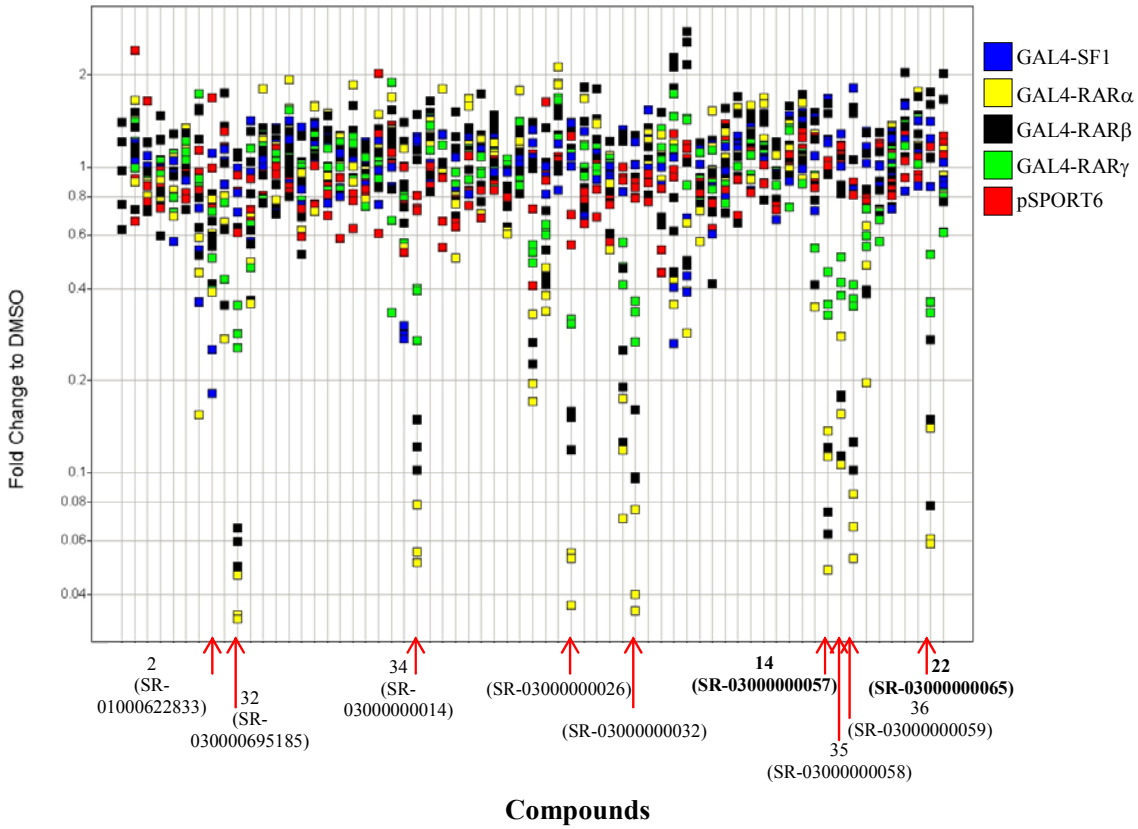


Figure 2. Scattergram of activity of GAL4-SF1, GAL-RARs and pSPORT6 control constructs in the presence of compounds synthesized in the SF-1 chemistry optimization campaign. Compounds 14 and 22 (bold text) are novel RAR probes.

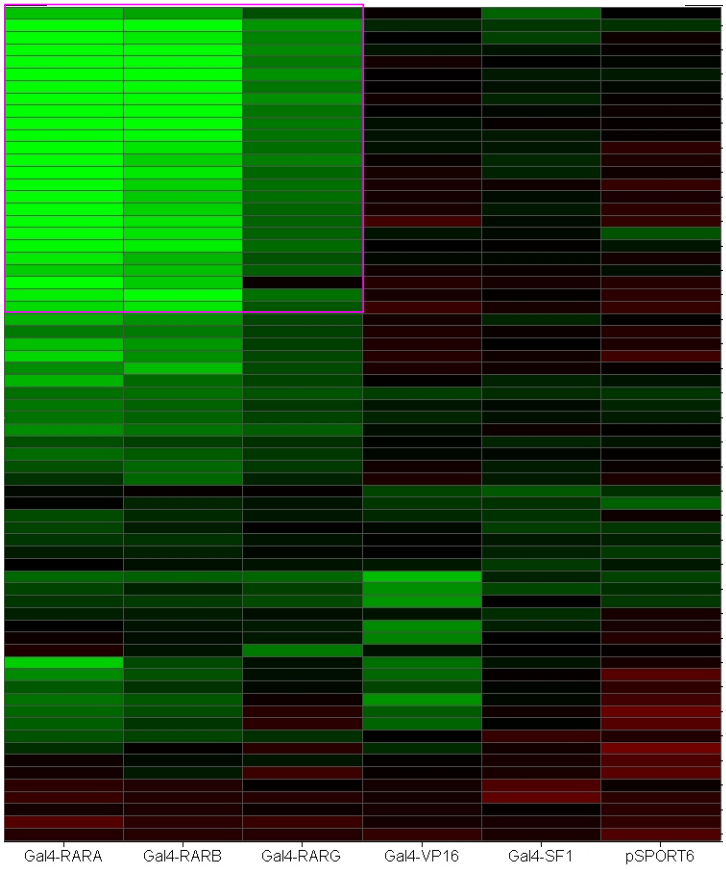
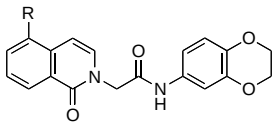


Figure 3. Heat map showing NHR activity in the GAL4-NHR LBD screen following treatment with the indicated compound. Compounds (right axis) were screened at 10 μM in HEK293T cells against a panel of NRs (X axis) to identify potential inhibitor action. The pink box outlines compounds that demonstrate selectivity against one or all RARs, compared to Gal4-SF1, VP16, LRH-1, or pSPORT6 control vector. Green indicates that the compound inhibited receptor activity, while red indicates increased activity, compared to DMSO. Black bars indicate no change in receptor activity.

RAR IC_{50} Titration Assays

Following the identification of analogs that could inhibit one or more RAR isoforms, the compounds were screened in RAR titration assays. The RAR IC_{50} data were then compared against SF-1, RORA, VP16, and CellTiter-Glo (cytotoxicity) IC_{50} data for each compound (**Table 3)**, which confirms that **compounds 14** (SID 46499846) and **22 (SID 46499854)** are novel RAR selective probes, with inhibitory activity that is selective for RARs over SF-1 and RORA. The titration curves are shown in **Figure 4**. None of the compounds produced IC_{50} curves for LRH-1 (maximum LRH-1 inhibition was 20%). These assays will be published to PubChem in the near future.

Table 3. Results of NHR selectivity titration assays. Novel RAR selective probes (compounds **14** and **22**) are highlighted in yellow. *indicates that the assay was performed in CHO-K1 cells. Other assays were performed in HEK293T cells.

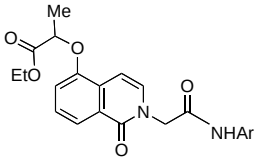


SR-03000000057, R = $O(CH_2)_3CH_3$

SR-03000000058, R = $OCHCH_3CH_2CH_3$

SR-03000000059, R = $OCHCH_3(CH_2)_3CH_3$

			IC_{50} (μM)						
Reference ID	**SR #**	**SID**	**RARα**	**RARβ**	**RARγ**	**SF-1***	**RORα***	**VP16***	**CellTiter-Glo***
14	SR-03000000057	46499846	0.156	0.0603	0.0386	13.67	21.5	24.14	69.42
35	SR-03000000058	46499847	0.161	0.412	0.241	7.86	16.5	17.53	>99
36	SR-03000000059	46499848	3.600	6.9	1.0	0.88	23.1	20.7	>99



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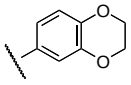
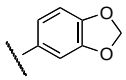
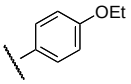


SR-01000622833

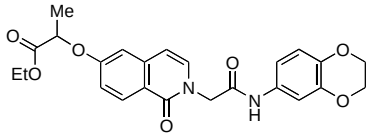


SR-03000000026



SR-01000695185

			IC_{50} (μM)						
Reference ID	**SR #**	**SID**	**RARα**	**RARβ**	**RARγ**	**SF-1***	**RORα***	**VP16***	**CellTiter-Glo***
2	SR-01000622833	7969543	0.200	0.180	0.166	0.76	>33	>33	>99
--	SR-03000000026	27035782	0.774	2.1	0.951	0.51	>99	>99	>99
32	SR-01000695185	4158022	0.133	0.234	0.109	0.2	22.58	24.26	36.99



			IC_{50} (μM)						
Reference ID	**SR #**	**SID**	**RARα**	**RARβ**	**RARγ**	**SF-1***	**RORα***	**VP16***	**CellTiter-Glo***
22	SR-03000000065	46499854	0.362	0.452	0.698	>99	>99	>99	>99

Comparative data on probe, similar compound structures and information on existing probes available to the public: Several currently available RAR inhibitors have been designed which target the activity of an individual RAR isoform selectively. For example, the RARβ selective inhibitor exists: 4-(5H-7,8,9,10-tetrahydro-5,7,7,10,10-pentamethylbenzo[e]naphtho [2,3-b][1,4]diazepin-13-yl)benzoic acid (also called LE135) [14]. Compounds 14 (SID 46499846) and 22 (SID 46499854) in the current report will require testing against other NRs not contained in the original Gal4 NR library (Library contained 38 out of 48 human NRs (79%)).

Recommendations for the scientific use of probe as research tool

The two compounds described herein are selective, potent inverse agonists of RARs. They can be used as research tools for RARα, RARβ, and RARγ. We have developed three routes for the synthesis of substituted isoquinolinones using chemistry that is compatible with a wide variety of functional groups, which has allowed us to explore the SAR of RAR inverse agonists. We have demonstrated that the probes have improved selectivity compared to other NR targets. It may be necessary to modify compound 14 (SID 46499846) to reduce its cytotoxicity.

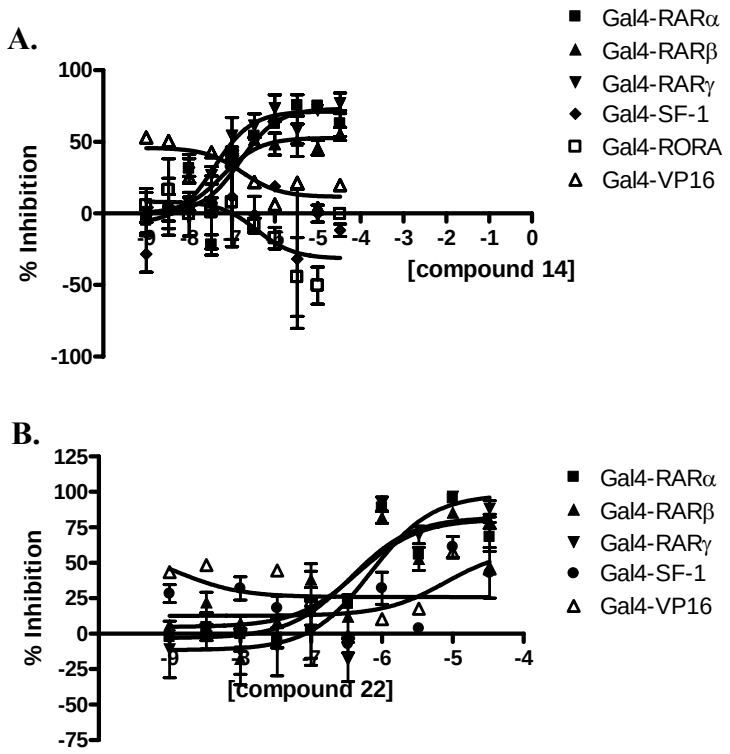


Figure 4. RAR titration curves for probes A) compound 14(SID 46499846) and B) compound 22 (SID 46499854).

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.). Probe structures and calculated chemical properties, determined using Scitegic's PipelinePilot software, are shown in **Table 4**.

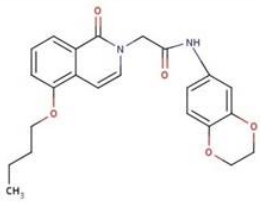
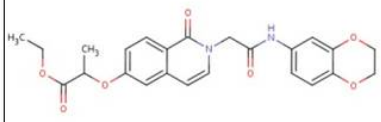
Compound ID	14	22
SR Number	SR-03000000057	SR-03000000065
PubChem SID	46499846	46499854
Structure		
MF	C23H24N2O5	C24H24N2O7
MW	408.447	452.457
Formal Charge	0	0
H Acceptor	5	7
H Donor	1	1
Atom Count	30	33
Rotatable Bonds	7	8
Rings	4	4
Stereoatoms	0	0
AlogP	2.667	1.878
logD	2.667	1.878
Polar surface area	77.1	103.4
Aqueous solubility[a]	-4.871	-4.654
ADMET BBB[b]	-0.557	-1.216
ADMET BBB level[c]	3	3
ADMET absorption level[d]	0	0
ADMET solubility[e]	-3.799	-3.057
ADMETT solubility level[f]	3	3
Vendor	SRIMSC	SRIMSC
Vendor Catalog Number	SR-03000000057	SR-03000000065

Table 4. Calculated chemical properties of RAR probes

SRIMSC indicates the Scripps Research Institute Molecular Screening Center

[a]Aqueous solubility is expressed as logS, where S is the solubility in mol/L. The method used to estimate the solubility is the multiple linear regression model based on Electrotopological State indices published in [15].

[b]ADMET_BBB: Log of Brain/Blood partition coefficient (LogBB).

[c]ADMET_BBB_Level: Ranking of the LogBB values into one of the following levels:
0: Very High 1: High 2: Medium 3: Low 4: Undefined (molecule is outside the confidence area of the regression model used to calculate LogBB).

[d]ADMET Passive Intestinal Absorption properties. A ranking of the molecule into one of the following levels: 0: Good 1: Moderate 2: Poor 3: Very Poor

[e]ADMET_Solubility: Log of the water solubility at 25 degrees, LogSw, in mol/L.

[f]ADMET_Solubility_Level: Ranking of the aqueous solubility values into the following classes:
0: Extremely Low 1: Very Low 2: Low 3: Good 4: Optimal 5: Very Soluble

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