Probe Report

Project Title: High-Throughput Screening of Select Orphan Nuclear Receptors

Grant number: 1X01-MH077624-01

Screening Center Name: The Scripps Research Institute Molecular Screening Center

Principal Investigator of Screening Center: Hugh Rosen, PhD

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Assay or Pathway Target: NR5A1 (steroidogenic factor 1)

Target Aliases: SF-1; FTZ1; ELP; AD4BP; nuclear receptor subfamily 5, group A, member 1

Probe PubChem Compound Identifier (CID): 4076092, 4289057

Specific aim: To identify selective cell-permeable inhibitors of the steroidogenic factor 1

Significance: Nuclear receptors (NRs) have proven to be successful therapeutic targets for a wide range of diseases. Evidence from deletion studies performed *in vivo* suggests that NR5A1 is implicated in cancer and obesity. No NR5A1 inhibitor has been reported so far. The identification of specific chemical probes may provide valuable tools to decipher NR5A1 mode of action and further investigate its therapeutic potential.

Rationale:

The nuclear receptor SF-1 plays a central role in sex determination and the formation of steroidogenic tissues during development, and is involved in endocrine function throughout life (Luo, Ikeda et al. 1995; Parker, Rice et al. 2002; Val, Lefrancois-Martinez et al. 2003). It is expressed in the pituitary, testes, ovaries, and adrenal gland (Val, Lefrancois-Martinez et al. 2003). SF-1-deficient mice exhibit male-to-female sex reversal (Luo, Ikeda et al. 1994), impaired development of adrenals and gonads (Luo, Ikeda et al. 1995; Sadovsky, Crawford et al. 1995), defective pituitary gonadotroph, and an agenesis of the ventromedial hypothalamic nucleus (Ikeda, Luo et al. 1995; Shinoda, Lei et al. 1995).

SF-1 regulates steroid hormone production at many levels, including direct regulation of expression of major P450 enzymes involved in steroid hormone synthesis. Hence, ligands for SF-1 may have clinical applications as modulators of adrenal steroid synthesis. In particular, a properly designed inhibitor of SF-1 is predicted to have therapeutic utility in the treatment of metastatic prostate cancer through suppression of both adrenal androgen and gonadal testosterone synthesis.

Although SF-1 has been shown to be rarely associated with clinical disorders of sexual differentiation so far (Parker, Rice et al. 2002), it has been proposed to cause obesity (Majdic, Young et al. 2002), and more recently to be involved in adrenocortical cell proliferation and cancer (Doghman, Karpova et al. 2007). Hence, it is hypothesized that SF-1 ligands could become a novel class of centrally acting small molecules that regulate energy metabolism and body weight. The ventromedial hypothalamus (VMH) within the brain processes peripheral metabolic input and regulates energy homeostasis. Experimental lesions to the VMH cause obesity in rodents with no change in overall food consumption. SF-1 is a potential therapeutic target for regulation of VMH activity and treatment of obesity. Indeed, knockout of SF-1 disrupts normal embryonic development of the VMH, and the resulting lesion is also indicated to cause obesity in mice.

Taken together, these observations highlight the need for a selective probe that will help decipher SF-1 functions and further uncover its therapeutic potential in obesity, cancer and other diseases.

PubChem Bioassays Names and Identifiers (AID):

Table 1 : Summary of PubChem assay names and identifiers performed to screen for SF-1 inhibitor probes

AID	PubChem Name	Assay Type
525	Primary Cell-based High Throughput Screening assay for inhibitors of the nuclear receptor Steroidogenic Factor 1 (SF-1)	Primary Screen
600	Dose-response cell-based assay for inhibitors of the nuclear receptor Steroidogenic Factor 1 (SF-1)	Confirmation/Titration
599	Counterscreen for inhibitors of the nuclear receptor Steroidogenic Factor 1 (SF-1): A cell-based dose-response assay for inhibition of the RAR-related orphan receptor A (RORA)	Counterscreen

Summary of Screen: 64,908 compounds from the 65K version of the MLSMR library were screened in the assay described in the next section (AID 525). 359 compounds that showed greater %inhibition than the hit-cutoff (47.96%, calculated as the average of %inhibition of all compounds tested plus three times their standard deviation) have been designated as primary hits (hit ratio of 0.55%). These compounds were then cherrypicked and retested in dose response using the same assay (AID 600). Among them, 213 compounds showed an IC_{50} inferior at 10 μM (59%). All 359 compounds were titrated in parallel in a cell-based assay targeting another nuclear receptor, the Retinoic Acid Receptor-related orphan receptor A (RORA, see AID 599). Indeed, since SF-1 and RORA are phylogenetically distant (Moore, Collins et al. 2006) and thus hypothesized to be responsive to different ligands, the RORA assay was implemented as a counterscreen to identify both promiscuous and non-selective compounds. As a result, compounds that showed an IC_{50} below 1 μM in the SF-1 assay and that demonstrated selectivity towards SF-1 ($[\log^{10}(IC_{50}$ in RORA)-$\log^{10}(IC_{50}$ in SF-1)]≥1) have been selected. Only compounds CID 4289057 and CID 4076092 passed these criteria. It is worth noting that these compounds are analogs that share the exact same molecular formula and weight (Table 2). Both compounds have been reordered as powder (Life Chemicals), validated by LC-MS and rerun in the SF-1 and RORA assays. CIDs 4289057 and 4076092 consistently gave an IC50 of approximately 200 nM and 600 nM in the SF-1 assay, respectively, with no significant inhibition in the RORA assay at these concentrations (Figure 1).

Primary Assay Description (AID525): The transcriptional cell-based assay utilized a fusion of the DNA-binding domain of the yeast transcription factor Gal4 with the ligand-binding domain of target receptor SF-1 (encoded by the pFA-hSF-1 plasmid, Orphagen Pharmaceuticals) to regulate a luciferase reporter containing 5xGal4 response elements at its promoter region (pG5-luc, Stratagene). Both pFA-hSF-1 and pG5-luc plasmids were transiently cotransfected in CHO-K1 (Chinese Hamster Ovary) cells. The presence in this cell line of required co-activators allows the expression of luciferase driven by activated SF-1 nuclear receptors. Compounds that inhibit basal transcription of luciferase are detected through the suppression of light emission using the SteadyLite luciferase detection kit (Perkin Elmer). Such compounds constitute potential inhibitors of the SF-1 nuclear receptor. The primary HTS assay was conducted in 1536-well format. All compounds were tested once at a 10μM final concentration. Results were expressed as %inhibition relative to DMSO treated wells (High Luminescence Control) and cells transfected with the reporter only (no pFA-hSF-1 cotransfected, Low Luminescence Control).

Confirmation/Titration Assay Description (AID600): The confirmation/ titration screen was performed using the same assay that was used for the primary screening. Compounds were tested in 10-point, 1:3 serial dilution starting at a nominal concentration of 99μM. Percent inhibition was calculated as in the primary screen. Percentage inhibition of each test compound 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 (99μM) did not result in > 50% inhibition or where no curve fit was achieved, the IC_{50} was determined manually depending on the observed

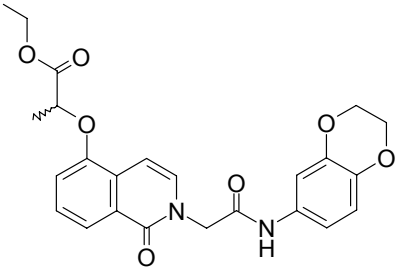
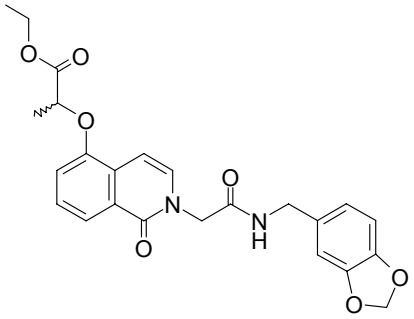
inhibition at the individual concentrations. Compounds with IC_{50} values of greater than 10μM were considered inactive. Compounds with $IC_{50\ values}$ equal to or less than 10μM were considered active.

Counterscreen Assay Description (AID599):

The RORA counterscreen was performed using the same conditions as the titration assay. However, cells were cotransfected with pFA-hRORA plasmid (Orphagen Pharmaceuticals), which encodes a Gal4 DBD fused to the LBD of RORA.

Screening results:

Table 2: Features of the two SF-1 selected compounds

CID Formula MW Supplier Cat#	Structure Name	Primary (AID525) Inh. at 10μM	Titration (AID600) SF-1 IC_{50}	Counterscreen (AID599) RORA IC_{50}
4076092 $C_{24}H_{24}N_2O_7$ 452.46 Life Chemicals F1808-0160	 ethyl 2-[2-[2-(2,3-dihydro-1,4-benzodioxin-7-ylamino)-2-oxoethyl]-1-oxoisoquinolin-5-yl]oxypropanoate	81.8%	837 nM	>30 uM
4289057 $C_{24}H_{24}N_2O_7$ 452.46 Life Chemicals F1808-0172	 ethyl 2-[2-[2-(1,3-benzodioxol-5-ylmethylamino)-2-oxoethyl]-1-oxoisoquinolin-5-yl]oxypropanoate	83.1%	315 nM	>30 uM

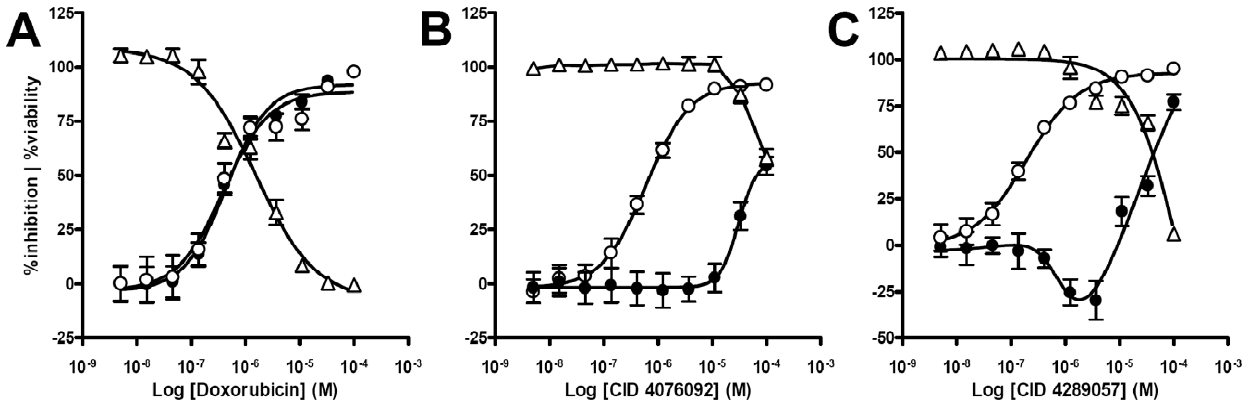
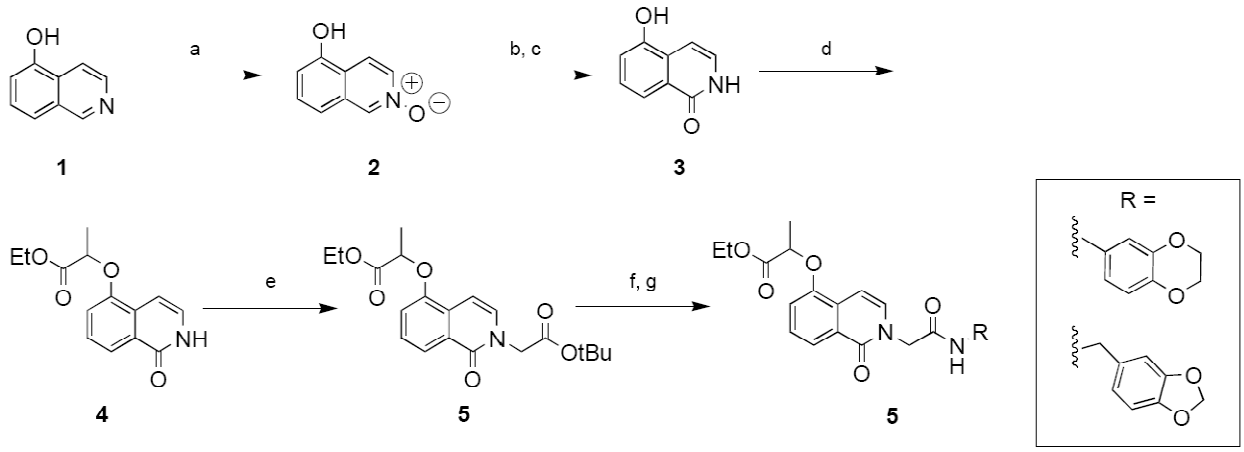


Figure 1: Dose response curves of the cytotoxic compound doxorubicin (A), compound CID 4076092 (B), and CID 4289057 (C) in the SF-1 assay (○), the RORA assay (●) and a cell viability assay (△). CID 4076092 and CID 4289057 gave an IC_{50} in the SF-1 assay of 603nM and 195nM, respectively.

Probes synthesis:

Compounds CIDs 4289057 and 4076092 may be made through the following synthetic pathway.



a) *m*CPBA b) Acetic anhydride, 140°C, 4 hours c) NaOMe, MeOH, 2 hours d) NaH, DMF, 30 min. then DL-ethyl-2-bromopropionate
e) Cs_2CO_3, *tert*-butyl bromoacetate f) Trifluoroacetic acid g) EDC and 1,4-benzodioxan-6-amine or 1,3-benzodioxol-5-ylmethylamine

Figure 2: Pathway for synthesis of compounds CID 4076092 and CID 4289057

Probe characterization

Selectivity:

The two identified compounds were tested against a panel of nuclear receptors in a reporter cell-based assay relying on Gal4-LBD fusion proteins in HEK 293T cells.

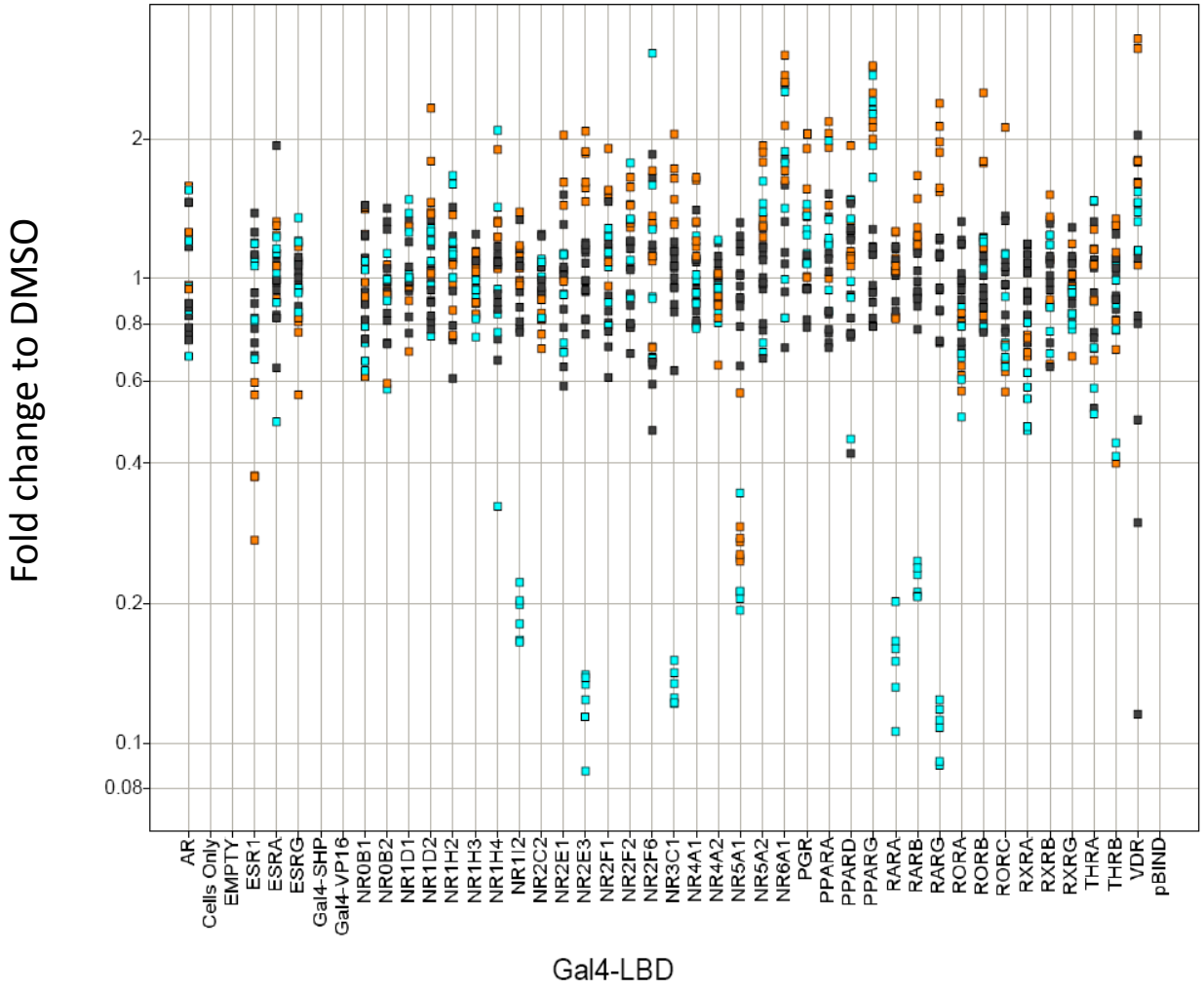


Figure 3: Activity of the proposed SF-1 probes on a panel of nuclear receptors. CID 4289057 (orange squares), CID 4076092 (blue squares) amd DMSO (black squares) have been tested in triplicate at 10 uM in a panel of cell-based assays for nuclear receptors that use Gal4-LBD fusion proteins and a luciferase reporter system.

CID 4289057 exhibited an excellent selectivity towards SF-1 inhibition. CID 4076092, on the other hand, was confirmed as inhibiting SF-1 in this assay, but also inhibited activity of other nuclear receptors, namely NR1I2, NR2E3, NR3C1 and the Retinoic Acid Receptors (RARs) Alpha, Beta and Gamma.

Solubility: Solubility of both compounds in 100mM phosphate buffer was investigated at two different pHs.

CID	Solubility at pH 3.5	Solubility at pH 7.4
4076092	183 μmoles/L	140 μmoles/L
4289057	263 μmoles/L	150 μmoles/L

Cytotoxicity: The cytotoxic effect of both compounds was assessed in CHO-K1 cells using the CellTiter-Glo® kit (Promega). Results are presented Figure 1.

CID	CC_{50}
4076092	> 100 μM
4289057	>30 μM

Permeability: Both compounds were assessed in a Parallel Artificial Membrane Permeation Assay (PAMPA) developed by Beckman Dickinson together with some reference compounds of known permeability. Both compounds demonstrated an excellent artificial membrane permeability (measured logP>-6).

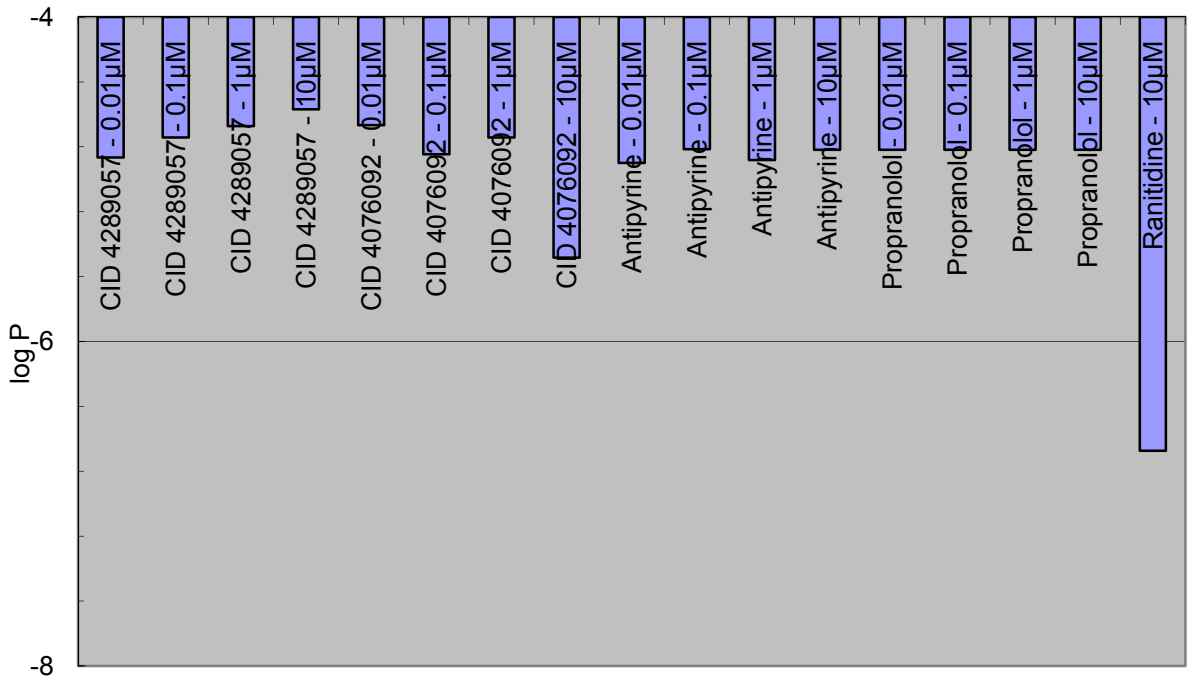


Figure 4: Permeability results of CIDs 4289057 and 4076092

Microsomal stability:

The stability of CIDs 4289057 and 4076092 was investigated in human, monkey, dog, rat and mouse microsomes. Dapsone, tolbutamide, verapamil, and sunitinib were run as reference compounds in the same assay.

Table 3: Microsomal stability results of compounds CID 4076092 and 4289057
Results of reference compounds are given in italic.

Compound	Half-life (minutes)				
	Dog	Monkey	Mouse	Human	Rat
4076092	1.12	≤1	≤1	1.24	≤1
4289057	≤1	≤1	≤1	≤1	1.41
Dapsone	*≥120*	*56.05*	*119.23*	*≥120*	*13.04*
Sunitinib	*30.62*	*27.06*	*13.21*	*54.57*	*32.80*
Tolbutamide	*≥120*	*≥120*	*≥120*	*≥120*	*≥120*
Verapamil	*6.08*	*1.34*	*2.11*	*7.37*	*3.17*

Inhibition of the native promoter:

Both compounds were tested in HEK 293T cells expressing luciferase under control of a multimerized SF-1 response element.

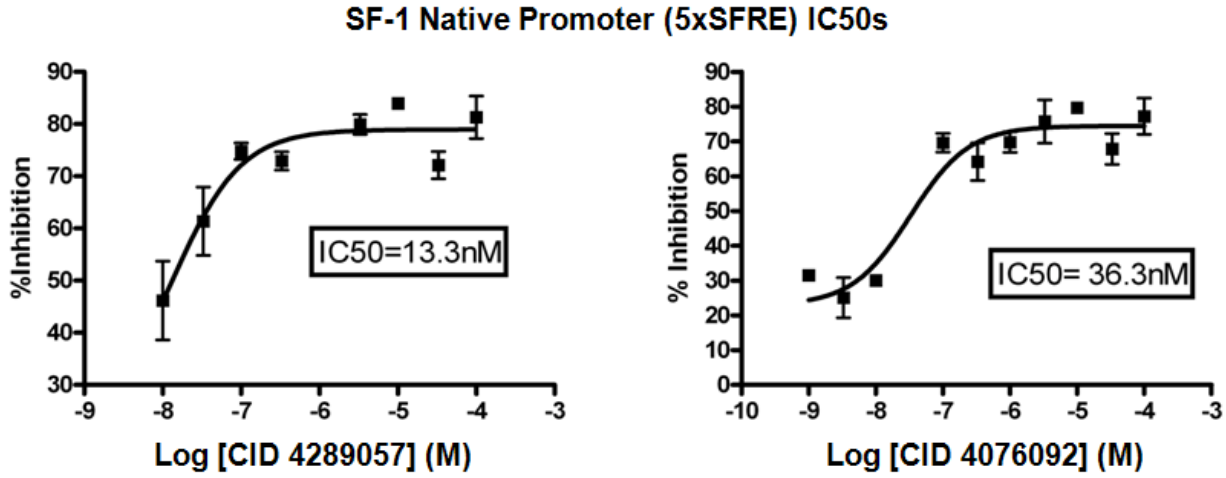
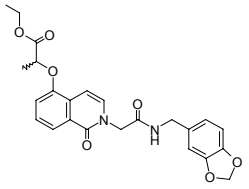
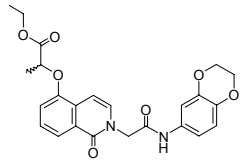
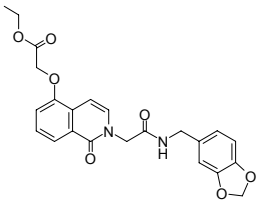
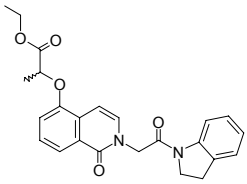
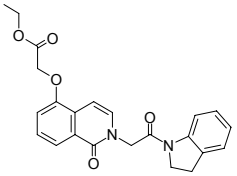
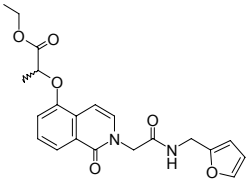
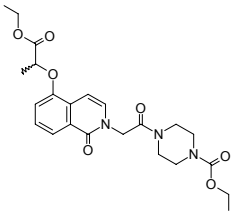
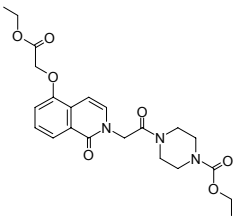


Figure 5: SF-1 native promoter inhibition by compounds CIDs 4076092 and 4289057

Preliminary SAR

A preliminary structure-activity relationship (SAR) study was performed by retrieving primary screening results of analogues found within the library.

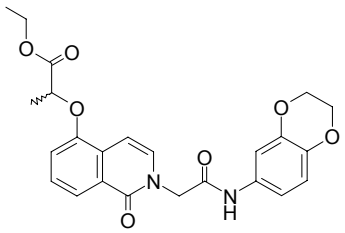
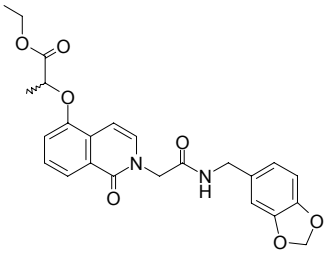
Structure	CID	SF-1 Inh. at 10μM (AID525)	Outcome
	4289057	83.1%	Active
	4076092	81.8%	Active
	2149418	30.6%	Inactive
	4600623	17.6%	Inactive
	2149377	10.8%	Inactive
	4170858	8.4%	Inactive
	3324272	-0.2%	Inactive
	4296362	-12.3%	Inactive

Recommendation of use:

Although the mode of action is not defined yet, both compounds demonstrated their ability to inhibit SF-1. Compound CID4289057 appears to be more active and selective. However, investigators should keep in mind that this compound also exhibits a potential cytotoxic effect. Compound CID4076092, on the other hand, showed a lower cytotoxicity.

Investigators should also note that the poor stability of these compounds in microsomes suggests that they may be rapidly degraded *in vivo*, potentially limiting their efficiency in animal studies.

Summary

CID	4076092	4289057
Structure		
Molecular Weight	452.46 g/mol	452.46 g/mol
Name	ethyl 2-[2-[2-(2,3-dihydro-1,4-benzodioxin-7-ylamino)-2-oxoethyl]-1-oxoisoquinolin-5-yl]oxypropanoate	ethyl 2-[2-[2-(1,3-benzodioxol-5-ylmethylamino)-2-oxoethyl]-1-oxoisoquinolin-5-yl]oxypropanoate
Supplier and Cat#	Life Chemicals F1808-0160	Life Chemicals F1808-0172
Primary Screen (AID525) (%inhibition in the SF-1 assay at 10μM)	81.83%	83.10%
Titration results (AID600) (IC_{50} in the SF-1 assay)	837 nM	315 nM
Titration confirmation from powder (IC_{50} in the SF-1 assay)	600 nM	200 nM
Couterscreen (AID599) (IC_{50} in the RORA assay)	>30 μM	>30 μM
Promiscuity assay (IC_{50} in the VP16 assay)	>30 μM	>30 μM
Inhibition of native promoter (IC_{50})	36.3 nM	13.3 nM
Cytotoxicity (CC_{50})	>100 μM	>30 μM
Solubility at pH 3.5	183 μmoles/L	264 μmoles/L
Solubility at pH 7.4	140 μmoles/L	150 μmoles/L
Permeability (LogP at 1μM)	-4.74	-4.67
Selectivity (names of nuclear receptors inhibited)	SF-1, NR1I2, NR2E3, NR3C1, RARs α β and γ	SF-1, ESR1

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