Probe Report for Inhibitors of SF-1/NR5A1:

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

- CID 4329551, SID 26945581: ethyl 2-[1-oxo-2-[2-oxo-2-[[4-(trifluoromethoxy)phenyl]amino]ethyl]isoquinolin-5-yl]oxypropanoate

- CID 4131581, SID 46499821: ethyl 2-[2-[2-[(4-methoxyphenyl)amino]-2-oxoethyl]-1-oxoisoquinolin-5-yl]oxypropanoate

Additional data not published in PubChem but presented here may be found in the following publications:

(1) Synthesis of Small Molecule Inhibitors of the Orphan Nuclear Receptor Steroidogenic Factor-1 (NR5A1) Based on Isoquinolinone Scaffolds. Joshua Roth, Franck Madoux, Peter Hodder, and William R. Roush (submitted) and

(2) Potent, selective and cell penetrant inhibitors of SF-1 by functional uHTS Franck Madoux, Xiaolin Li, Peter Chase, Gina Zastrow, Michael D. Cameron, Juliana J. Conkright, Patrick R. Griffin, Scott Thacher and Peter S. Hodder (submitted).

Project Title: Chemical Optimization of Selective Inhibitors of SF-1
Grant Number: 1 X01 MH077624-01
Screening Center Name: The Scripps Research Institute Molecular Screening Center
Principal Investigator of Screening Center: Hugh Rosen
Assay Provider & Institution: Xiaolin Li, PhD, Orphagen Pharmaceuticals
Assay or Pathway Target: nuclear receptor subfamily 5, group A, member 1 (NR5A1), steroidogenic factor-1 (SF-1)
Probe PubChem Compound Identifier: SIDs 46499821 and 26945581

Assay Provider Information

Specific Aim: To identify selective cell-permeable inhibitors of the steroidogenic factor 1 (orphan nuclear receptor NR5A1)

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

Rationale: The nuclear receptor NR5A1 plays a central role in sex determination and the formation of steroidogenic tissues during development, and is involved in endocrine function throughout life (3-5). It is expressed in the pituitary, testes, ovaries, and adrenal gland (5). NR5A1-deficient mice exhibit male-to-female sex reversal (6), an impaired development of adrenals and gonads (3, 7), defective pituitary gonadotroph, and an agenesis of the ventromedial hypothalamus nucleus (8, 9). NR5A1 regulates steroid hormone production at many levels, including direct regulation of expression of major P450 enzymes involved in steroid hormone synthesis. Hence, ligands for NR5A1 may have clinical applications as modulators of adrenal steroid synthesis. In particular, a properly designed inhibitor of NR5A1 is predicted to have therapeutic utility in the treatment of metastatic prostate cancer though suppression of both adrenal, androgen, and gonadal testosterone synthesis. Although NR5A1 has been shown to be rarely associated with clinical disorders of sexual differentiation so far (4), it has been proposed to cause obesity (10), and more recently to be involved in adrenocortical cell proliferation and cancer (11). Hence, it is hypothesized that SF-1 ligands could become a novel class of centrally acting small molecules that regulates energy metabolism and obesity. 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. NR5A1 is a potential therapeutic target for regulation of VMH activity and treatment of obesity. Indeed, knockout of NR5A1 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 of a selective probe that will help deciphering NR5A1 functions and further uncovering its therapeutic potential in obesity and cancer.

Screening Center Information

Assay Implementation and Screening

PubChem bioassay names and identifiers: This report describes synthetic analogs and initial structure activity relationship (SAR) studies of SF-1 inhibitors based on isoquinolinone scaffolds. The two SF-1 selective probes characterized here are novel analogs of compounds originally identified as active against SF-1 in PubChem BioAssays AID 525, 599, and 600. Analogs described herein have lower cellular toxicity and improved selectivity compared to the initial HTS-derived leads in the original probe report. Please refer to the original SF-1 probe report for details http://molscreen.florida.scripps.edu/probes.html

List of Relevant AIDs that may be used as counterscreen information: Because the probes identified in this report are novel and have not been tested in any BioAssays, no counterscreen data are currently available in PubChem. Data from relevant counterscreens below (RORA, VP16, and cytotoxicity) will be uploaded to PubChem in the near future.

***Identification of SF-1 inhibitors that serve as scaffolds for new SF-1 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 359 initial hits were confirmed in a SF-1 titration assay (AID 600), and further 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 (AID 599). These efforts 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 (see Table 1) in the counterscreen was acceptable (2), it was believed that their selectivity and cytotoxicity could be improved.

Accordingly, these two compounds were selected as starting points for the development of SF-1 chemical probes. After purchase and/or synthesis and selectivity testing of numerous analogs, two compounds (SIDs 46499821 and 26945581) were identified as novel probes with lower cellular toxicity and improved SF-1 selectivity compared to the initial two leads **(Table 1).** These two compounds are the focus of the current report.

Reference ID	SR Number	SID	IC_{50} (μM)			
			SF-1	RORα	VP16	Cytotoxicity
1	SR-01000622779	7970631	0.26	>33	>33	>33
2	SR-01000622833	7969543	0.76	>33	>33	>99
31	SR-03000000023	46499821	0.20	>99	>99	>99
32	SR-03000000030	26945581	0.20	>99	>99	>99

Table 1. SF-1 probes. IC_{50} data for previous probes (SIDs 7970631 and 7969543) and probe analogs (SIDs 46499821 and 26945581) synthesized during the current probe optimization campaign are shown for SF-1, RORα, VP16, and cytotoxicity (CellTiter-Glo) assays. Reference ID is the compound ID used in the synthesis manuscript (reference 1).

Probe Selectivity Profiling Assays

SF-1 Confirmation/ Titration Assay

These assays were performed using the same protocol 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.

Percent inhibition was calculated as in AID 600. 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.

RORA Counterscreen

This assay was performed using the same protocol as in PubChem AID 599, which can be found at http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=599. Specifically, cells were cotransfected with pFA-hRORA plasmid (Orphagen Pharmaceuticals), which encodes a Gal4 DNA binding domain (DBD) fused to the ligand binding domain (LBD) of RORA. Compounds that inhibited RORA activity were identified as nonselective.

VP16 Promiscuity Assay

This assay was performed using the same protocol as in PubChem AID 600, except that 125 ng of pGal4DBD_VP16LBD plasmid (a kind gift from Dr. Michael Conkright, Department of Cancer Biology, Scripps Florida) was used for transfections instead of the pFA-hSF-1 plasmid. Compounds that inhibited VP16 activity were identified as nonselective.

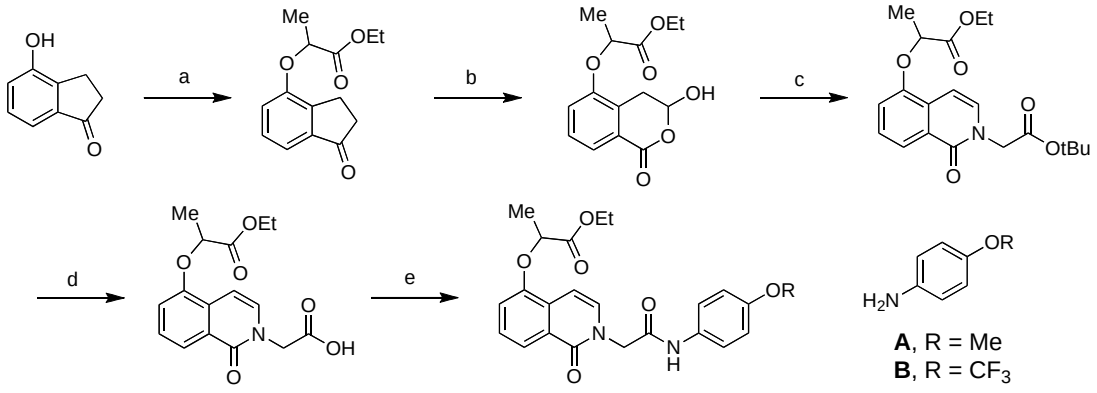
Cell Viability Assay

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 24 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%).

Additional AIDs related to probe development efforts described above will be uploaded to PubChem.

Synthesis of probe compounds 31 and 32 (SIDs 46499821 and 26945581)

Probe compounds **31** and **32** may be synthesized as summarized in Scheme 1, and are also commercially available (LifeChemicals part numbers F1808-0154 and F1808-0165, respectively. Information is available at http://lifechemicals.emolecules.com/). Syntheses of additional analogs of HTS-derived isoquinolinone SF-1 antagonists **1** and **2** (SIDs 7970631 and 7969543) can be found in reference 1.



31 (SID 46499821), R = Me
32 (SID 26945581), R = CF_3

Scheme 1. (a) *dl*-ethyl-2-bromopropionate, K_2CO_3, acetone, 60°C, 4 h (80%). (b) (i) TBSOTf, Et_3N, THF (ii) O_3, CH_2Cl_2, -78°C (iii) 1.5 equiv, dimethyl sulfide, 1 h at 25°C (c) 3 equiv. of glycine *tert*-butyl ester hydrochloride, 3 equiv. Et_3N, AcOH to achieve pH 3 to 5, benzene, 100°C, sealed tube, 12 h (67%, 2 steps). (d) 1:2 TFA:dichloroethane, 2 h (99%). (e) **A** or **B**, HATU, DMAP, CH_2Cl_2, yields: **31** (50%), **32** (27%).

Structure Activity Relationships

Data on cytotoxicity and inhibition of SF-1, RORA, and VP16 activities by the original two probes and related analogs are summarized in Tables 2-4.

Analogs I. Amide Unit Modifications (Table 2)

Initial efforts focused on replacing the oxygenated benzylic amide or anilide units of the original two probes, but introduction of alkyl amides as in compounds **23** and **24** led to significant loss of SF-1 activity (Table 2)**.** Replacement of the highly oxygenated benzylic amide unit of **1** with potentially more metabolically stable benzyl (**25**) or *p*-fluorobenzyl (**26**) amides led to a decreased in SF-1 activity.

Introduction of a phenethyl amide (**28**) yielded a 360 nM SF-1 inhibitor. Better results were obtained by replacing the highly oxygenated and potentially metabolically sensitive anilide unit of compound **2** with other aniline amides, such as the *p*-methylphenyl (**30**), *p*-methoxyphenyl (**31**), *p*-trifluoromethoxyphenyl (**32**) and the *p*-ethoxyphenyl (**33**) amides. The four latter analogs displayed SF-1 inhibitor activity in the range 200-330 nM, at least twice improved over original screening hit compound **2**. In addition, analogs **31** (SID 46499821) and **32** (SID 26945581**)** displayed improved selectivity vs. additional counterscreens (e.g., VP-16 assay) as well as lower cellular toxicity**.** The results support the role of compounds **31** (SID 46499821) and **32** (SID 26945581**)** as novel selective SF-1 probes.

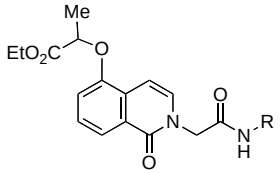
Analogs II. Ether Unit Modifications (Table 3)

Clear evidence of a productive SAR for the isoquinolinone alkoxy substituent has been demonstrated by analogs **14** and **36-39** (Table 3)**.** For most of these analogs, modification of the ether unit (R') reduces or eliminates SF-1 antagonism. An exception is compound **38** (SID 46499849), where modification of the ether unit resulted in improved SF-1 antagonism and

reduced cytotoxicity, compared to original SF-1 probe compound **1**. Compound **38** (SID 46499849) warrants further examination as a possible SF-1 probe.

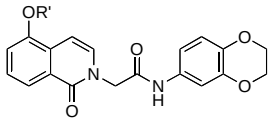
Analogs III. Spacer Unit Modifications (Table 4)

All substitutions of the R" unit spanning the isoquinolinone and amide units led to substantial or complete loss of SF-1 activity (analogs **40-43**) (Table 4).



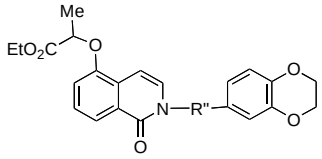
Reference ID	SR Number	SID	Amide R Group	IC_{50} (μM)			
				SF-1	RORα	VP16	Cytotoxicity
1	SR-01000622779	7970631	1,3-benzodioxole-5-methanamine	0.26	>33	>33	>33
2	SR-01000622833	7969543	1,4-benzodioxan-6-amine	0.76	>33	>33	>99
23	SR-03000000063	46499852	$(CH_2)_3CH_3$	9.71	>99	>99	>99
24	SR-03000000034	46499832	cyclohexyl	1.77	>99	>99	>99
25	SR-03000000035	46499833	benzyl	1.06	3.22	>99	>99
26	SR-03000000036	46499834	*p*-fluorobenzyl	0.68	1.68	>99	>99
27	SR-01000607902	7969723	CH_2-2-furanyl	4.71	>33	>99	>99
28	SR-03000000037	46499835	phenylethyl	0.36	>33	>99	>99
29	SR-03000000031	46499829	*p*-nitrophenyl	0.53	>99	>99	>99
30	SR-03000000008	46499806	*p*-methylphenyl	0.33	36.47	>33	40.16
31	SR-03000000023	46499821	*p*-methoxyphenyl	0.20	>99	>99	>99
32	SR-03000000030	26945581	*p*-trifluoromethoxy-phenyl	0.2	>99	>99	>99
33	SR-01000695185	4158022	*p*-ethoxyphenyl	0.20	22.58	24.26	36.99
34	SR-03000000013	46499811	*p*-ethylphenyl	0.11	24.99	85.43	51.69
35	SR-03000000014	46499812	*p*-n-butylphenyl	0.36	30.66	>99	>99

Table 2. IC_{50} data for original probes and synthesized analogs. Top image shows the scaffold. The SF-1 selective probes presented in this report are highlighted yellow. Note that compounds **23**, **24** and **29** also meet the NIH's probe criteria.



Reference ID	SR Number	SID	Ether R' Group	IC_{50} (μM)			
				SF-1	RORα	VP16	Cytotoxicity
14	SR-03000000057	46499846	$CH_2(CH_2)_2CH_3$	13.67	21.5	24.12	69.42
36	SR-03000000058	46499847	$CH_2CH_3CH_2CH_3$	7.86	16.5	17.53	>99
37	SR-03000000059	46499848	$CH_2CH_3CH_2(CH_2)_3CH_3$	0.88	23.1	20.7	>99
38	SR-03000000060	46499849	$C(CH_3)_2CO_2CH_2CH_3$	0.59	>99	>99	>99
39	SR-01000015684	27025589	$CH_2CO_2CH_2CH_3$	inactive	--	--	--

Table 3. IC_{50} data for compounds **14** and **36-39**. Top image shows the scaffold. Note that compound **38** meets the NIH's probe criteria.



Reference ID	SR Number	SID	Spacer R'' Group	IC_{50} (μM)			
				SF-1	RORα	VP16	Cytotoxicity
40	SR-03000000062	46499851	CH_2CH_2CONH	8.46	>99	>99	>99
41	SR-03000000066	46499855	CH(Me)CONH	>99	>99	>99	>99
42	SR-03000000061	46499850	$CH(CH_2Ph)CONH$	>99	>99	>99	>99
43	SR-03000000064	46499853	$CH_2CON(Me)$	3.57	>99	>99	>99

Table 4. IC_{50} data for compounds **40-43**. Top image shows the scaffold. Note that compounds **40** and **43** meet the NIH's probe criteria.

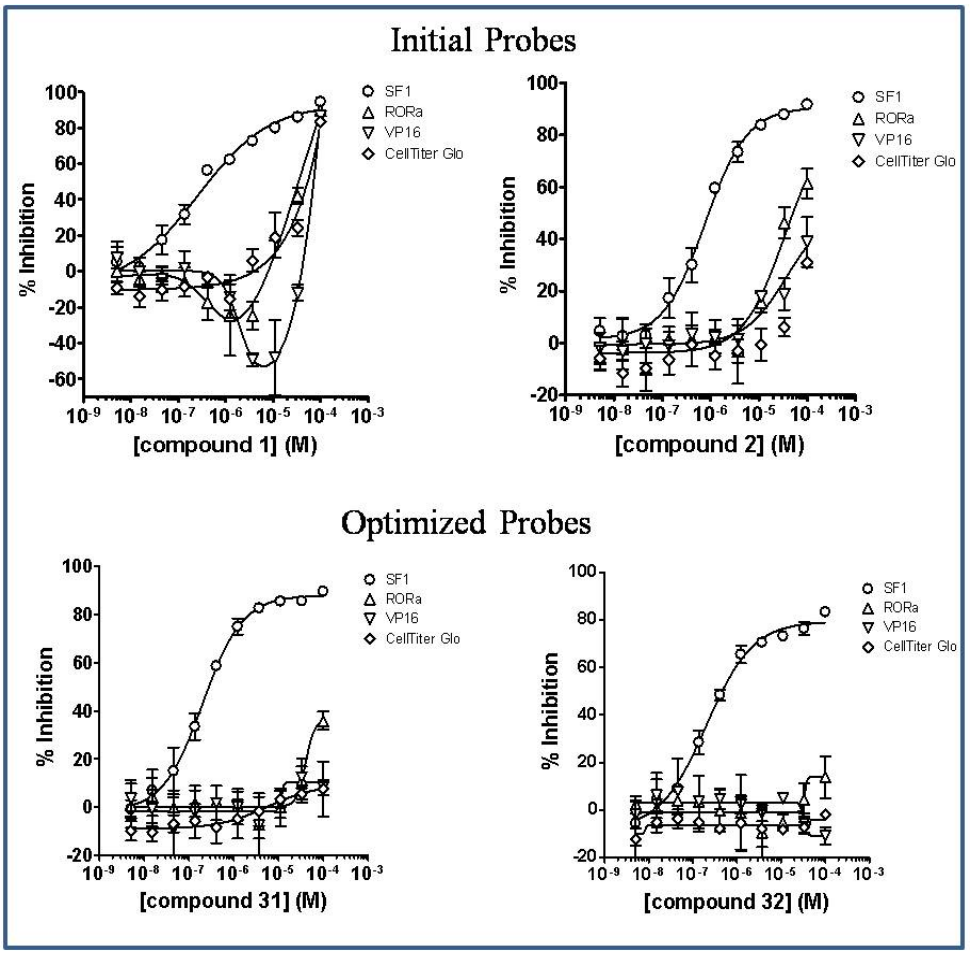


Figure 1: Comparison of inhibition profiles of initial and optimized probes Data represent the mean of three independent experiments plus or minus the calculated standard deviation.

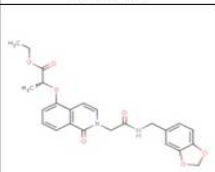
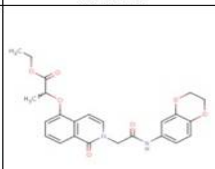
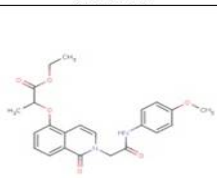
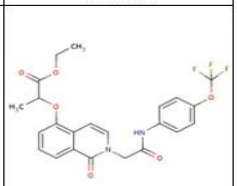
Comparative data on probe, similar compound structures and information on existing probes available to the public: The constitutive activity of SF-1 and lack of information on native ligands creates a challenge to structure-based inhibitor design. The solving of the SF-1 ligand binding domain (LBD) using crystal structure revealed that the large pocket is filled with phospholipids (12). Not surprisingly, sphingosine has been shown to act as an endogenous inhibitor for SF-1 and to inhibit cAMP-dependent CYP17 gene transcription (13). A recent report by ACADIA Pharmaceuticals has shown, using functional cell-based assays, that a selective and potent SF-1 inverse agonist alkyloxyphenol called AC-45594 (4-(heptyloxy)phenol) can down-regulate cAMP-mediated induction of SF-1 target genes by acting as a synthetic ligand (14). The structures of AC-45594 and its analogs are very different from those of the probes characterized in the present report. Details can be found in the reference (14). Unfortunately, it is not possible to directly compare the efficacy of these compounds with those described here, since the assays, detection systems, and cell types vary.

Recommendations for the scientific use of probe as research tool

Compounds **31** and **32** (SIDs 46499821 and 26945581) described here are selective and potent inhibitors of SF-1. They can be used as research tools for SF-1. Three routes for the synthesis of substituted isoquinolinones (reference 1) using chemistry that is compatible with a wide variety of functional groups, have allowed the exploration of SAR of isoquinolinone inhibitors of the orphan nuclear receptor SF-1. This report demonstrates that these probes have improved selectivity vs. other NHR targets, and identifies the aryl carboxamide and isoquinolinone ether units of the two original probes (SIDs 7970631and 7969543) as sites for further optimization. Although several other compounds (**23**, **24**, **29**, **38**, **40** and **43**) were identified as selective and potent against SF-1, and meet the NIH's probe criteria, compounds **31** and **32** are presented here as the superior SF-1 probes.

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.). Probe structures and calculated probe properties, determined using Scitegic's PipelinePilot software, are shown in Table 5.

Table 5. Calculated chemical properties of probes

Reference ID	Previously reported probes		New probes (current report)	
	1	**2**	**31**	**32**
SR Number	SR-01000622779	SR-01000622833	SR-03000000023	SR-03000000030
PubChem SID	7970631	7969543	4131581	4329551
Structure				
MF	C24H24N2O7	C24H24N2O7	C23H24N2O6	C23H21F3N2O6
MW	452.45655	452.45655	424.44645	478.41784
Formal Charge	0	0	0	0
H Acceptor	7	7	6	6
H Donor	1	1	1	1
Atom Count	33	33	31	34
Rotatable Bonds	9	8	9	10
Rings	4	4	3	3
Stereoatoms	1	1	0	0
AlogP	1.867	1.878	2.076	4.212
logD	1.867	1.878	2.076	4.212
Polar surface area	103.4	103.4	94.17	94.17
Aqueous solubility[a]	-4.6027	-4.5953	-5.02714	-6.5890
ADMET BBB[b]	-1.219	-1.216	-1.013	
ADMET BBB level[c]	3	3	3	4
ADMET absorption level[d]	0	0	0	0
ADMET solubility[e]	-2.982	-3.131	-3.035	-5.054
ADMETT solubility level[f]	3	3	3	2
Vendor	Life Chemicals	Life Chemicals	Life Chemicals	Life Chemicals
Vendor Catalog Number	F1808-0172	F1808-0160	F1808-0154	F1808-0165

[a]Aqueous solubility is expressed as logS, where S is solubility in mol/L. The method used is the multiple linear regression model based on Electrotopological State indices published in (15).
[b]ADMET_BBB: Log of Brain/Blood partition coefficient (LogBB). See (16) for details on this method.
[c]ADMET_BBB_Level: Ranking of the LogBB values into one of the following levels (see (16, 17) for details):
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 (see (16, 17) for details):
0: Good
1: Moderate
2: Poor
3: Very Poor
[e]ADMET_Solubility: Log of the water solubility at 25 degrees, LogSw, in mol/L. See (18) for more information.

[f]ADMET_Solubility_Level: Ranking of the aqueous solubility values into the following classes (see (18) for more information):
0: Extremely Low
1: Very Low
2: Low
3: Good
4: Optimal
5: Very Soluble

Bibliography

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