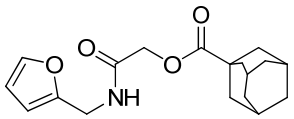
Project: High Throughput Screen for Agonists of the Thyroid Stimulating Hormone Receptor

Probe: [2-(furan-2-ylmethylamino)-2-oxoethyl] adamantane-1-carboxylate



SID: 3714076
MLS000098157
Internal IDs: NCGC00039523

PubChem Primary Bioassay Identifier (AID): 926

SID	EC50 (μM)	Antitarget	Selectivity*
3714076	2.00	FSH, LH	FSH, LH: Inactive

*Selectivity = Inactive in FSH, LH, EC50 >100 uM

Assigned Assay Grant #: X01-MH080680-01

Screening Center Name: NIH Chemical Genomics Center
Principal Investigator of Screening Center: Christopher Austin

Assay Submitter & Institution: Marvin Gershengorn, NIDDK

Assay or Pathway Target: *Thyroid Stimulating Hormone Receptor (G-protein coupled receptor)*

Assay provider information

Specific Aim: Identify small-molecule agonists of the Thyroid Stimulating Hormone Receptor.

Significance: Thyroid Stimulating Hormone (TSH) is an α/β heterodimeric glycoprotein hormone secreted from the anterior pituitary gland which belongs to the glycoprotein hormone family, including Chorionic Gonadotropin (CG), Luteinizing Hormone (LH), and Follicle Stimulating Hormone (FSH) (1,2). TSH binds to the TSH receptor (TSHR), which couples preferentially to the G-alpha (s) (Gs) protein, resulting in activation of adenylate cyclase and increase in cyclic adenosine 3′, 5′ monophosphate (cAMP). THSR is mainly expressed in thyroid follicular cells (3) and regulates their growth and function (4). Recombinant TSH is currently used for the diagnosis in patients with thyroid cancer receiving thyroid hormone suppression therapy, and for screening of residual tumor after surgery (5), but it is expensive and must be administered intramuscularly. An orally active small molecule TSHR agonist would serve as a valuable research tool for studying TSHR pharmacology and physiology, and would have multiple advantages for therapeutic applications. However, no selective small molecule agonist of the TSHR exists, and no small molecule screen for TSHR agonists has ever been reported.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: qHTS Assay for Agonists of the Thyroid Stimulating Hormone Receptor

List of PubChem bioassay identifiers generated for this screening project (AIDs):

AID	Target	Concentration	Bioassay type
1401	TSHR Summary Assay	N/A	Summary
926	TSHR cell line	57μM – 0.7 nM	Primary qHTS (membrane potential measurement)
938	Parental cell line counterscreen	57μM – 0.7 nM	Primary qHTS (membrane potential measurement)
939	TSHR cell line	57μM – 0.7 nM	Confirmation qHTS (membrane potential measurement)
953	Parental counterscreen	57μM – 0.7 nM	Confirmation qHTS (membrane potential measurement)
933	TSHR cell line	92μM – 0.1 nM	Secondary assay (HTRF bioassay)
1402	LHR cell line	92μM – 0.1 nM	Counterscreen (HTRF bioassay)
1403	FSHR cell line	92μM – 0.1 nM	Counterscreen (HTRF bioassay)

Primary Assay Description as defined in PubChem:

Overview:

TSHR is a seven-transmembrane receptor which couples to the Gs protein, resulting in activation of adenylate cyclase and increase in intracellular cAMP level. This cell-based assay utilized a cyclic nucleotide gated ion channel (CNG) as a biosensor for measurement of increase in intracellular cAMP level stimulated by TSHR agonists. The HEK293 cell line expressing human TSHR and a modified CNG was used as the primary screen assay (6,7,8). The membrane potential dye was used to detect membrane depolarization upon the activation of CNG by increased cAMP level. This assay was optimized in a homogenous 1536-well plate format.

TSHR cell line and Parental cell lines used in membrane potential assay:

A HEK293 cell line stably expressing TSHR, as well as a modified CNG, was purchased from BD biosciences (Rockville, MD). When TSHR is stimulated, intracellular cAMP level increases, which activates CNG channels to cause membrane depolarization. A fluorescent membrane potential dye (BD biosciences) was utilized to detect the membrane depolarization in this assay (Fig. 1). The cells were maintained in DMEM medium (Invitrogen) containing 10 % FBS (Hyclone), 100 units/ml Penicillin, 100 ug/ml Streptomycin (Invitrogen), and 250 ug/ml Geneticin (Invitrogen) and 1 ug/ml Puromycin (Invitrogen) at 37C in 5% CO2. A parental HEK293 cell line expressing CNG without TSHR was used in a parallel screen to eliminate compounds that activate targets other than TSHR.

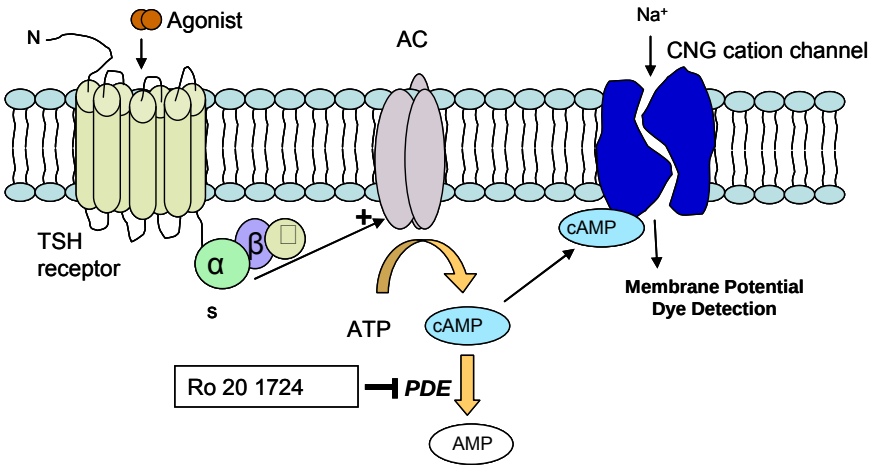


Fig. 1. Schematic illustration of the assay principle of the ACT:One cAMP assay for TSHR. A TSHR was co-expressed in a HEK293 cell line with a modified CNG channel. When TSH binds to TSHR it increases the intracellular cAMP level. The CNG channels are activated by the increased cAMP that results in membrane depolarization after the influx of cations such as sodium and calcium.

Assay protocol:

The suspension cells (TSHR or parental) were dispensed into 1536-well assay plates at 4 ul/well with 1000 cells in DMEM (Invitrogen) containing 10% FCS (Hyclone), 100 units/ml Penicillin, 100 ug/ml Streptomycin followed by culture at 37 °C with 5% CO2 for 20 to 30 hrs. The robotic screen was initiated by an addition of 4 ul/well membrane potential dye containing 100 uM RO 20-1724 (Sigma Aldrich), phosphodiesterase inhibitor. The assay plates were incubated at room temperature for 30 min to allow for dye equilibration across the cell membrane. The compound in DMSO solution or control was then added at 23 nl/well to the assay plates, which were incubated for additional 30 min. The assay plates were detected in an Envision fluorescence plate reader (Perkin Elmer) in bottom-read mode for fluorescence intensity with excitation of 480 and emission at 530 nm.

Table 1. Protocol for the TSHR cAMP assay in Act:One assay format

Step	Parameter	Value	Description
1	Cells	4 µl	1000 cells/well
2	Culture	18-30 hr	37°C, 5% CO2
3	Reagent	4 µl	BD membrane potential dye
4	Incubation	30 min	Room temperature
5	Compound	23 nl	Library Compounds in 0.128 to 10 uM titration series or control
6	Incubation	30 min	Room temperature
7	Detector	Ex 480/Em 530	Envision plate reader

Center Summary of the Primary Screen:

The primary screen was performed with 7-concentration titration for a collection of approximately 73,180 compounds.

Identification of TSHR agonists: Following the qHTS, the primary screen data was subjected to a classification scheme to rank the quality of the concentration-response curves as described previously (9) (Fig. 2). Briefly, CRCs are placed into four classes. Class 1 contains complete CRCs showing both upper and lower asymptotes and r^2 values > 0.9. Class 2 contains incomplete CRCs lacking the lower asymptote, and shows r^2 values greater than 0.9. Class 3 curves are of the lowest confidence because they are defined by a single concentration point where the minimal acceptable activity is set at 3 SD of the mean activity calculated as described above. Finally, class 4 contains compounds that do not show any CRCs and are therefore classified as inactive. Once an active set of compounds was identified, hierarchical agglomerative clustering with a 0.7 Tanimoto cutoff was performed by using Leadscope (Leadscope Inc., Columbus, OH) fingerprints. For each cluster, maximal common substructures (MCS) were extracted, a manual step of MCS trimming was performed to create a list of scaffolds, and any overlapping scaffolds were abridged to a canonical set. Each scaffold was then represented as a precise definition to indicate descriptors, such as the number of attachment points or the ring size variability. All filters were then relaxed to include the entire negative (class 4) assay data.

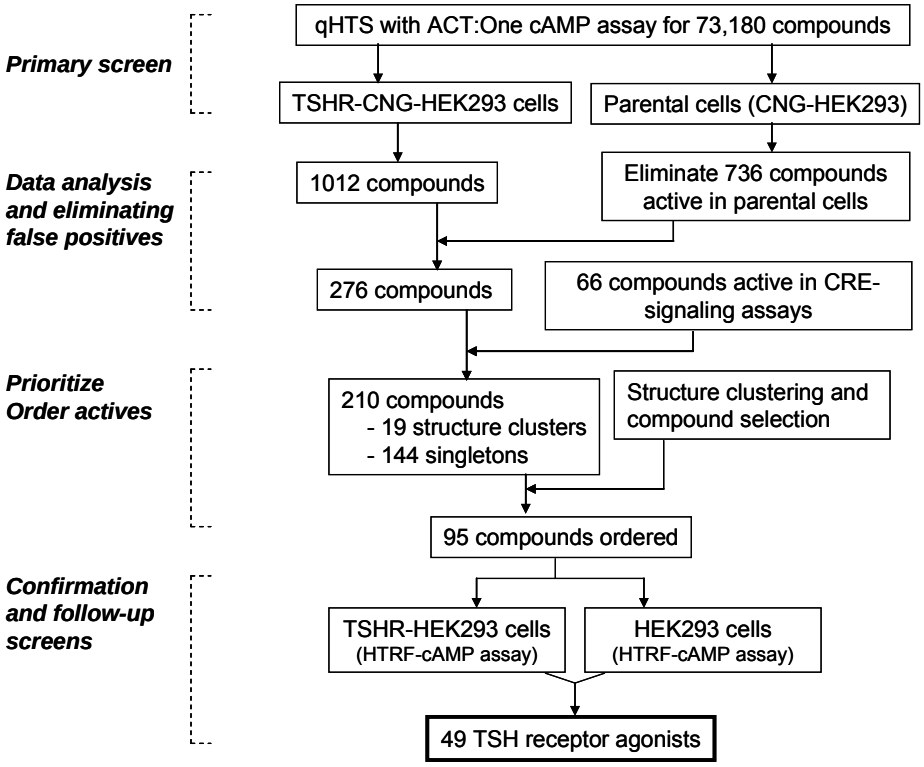
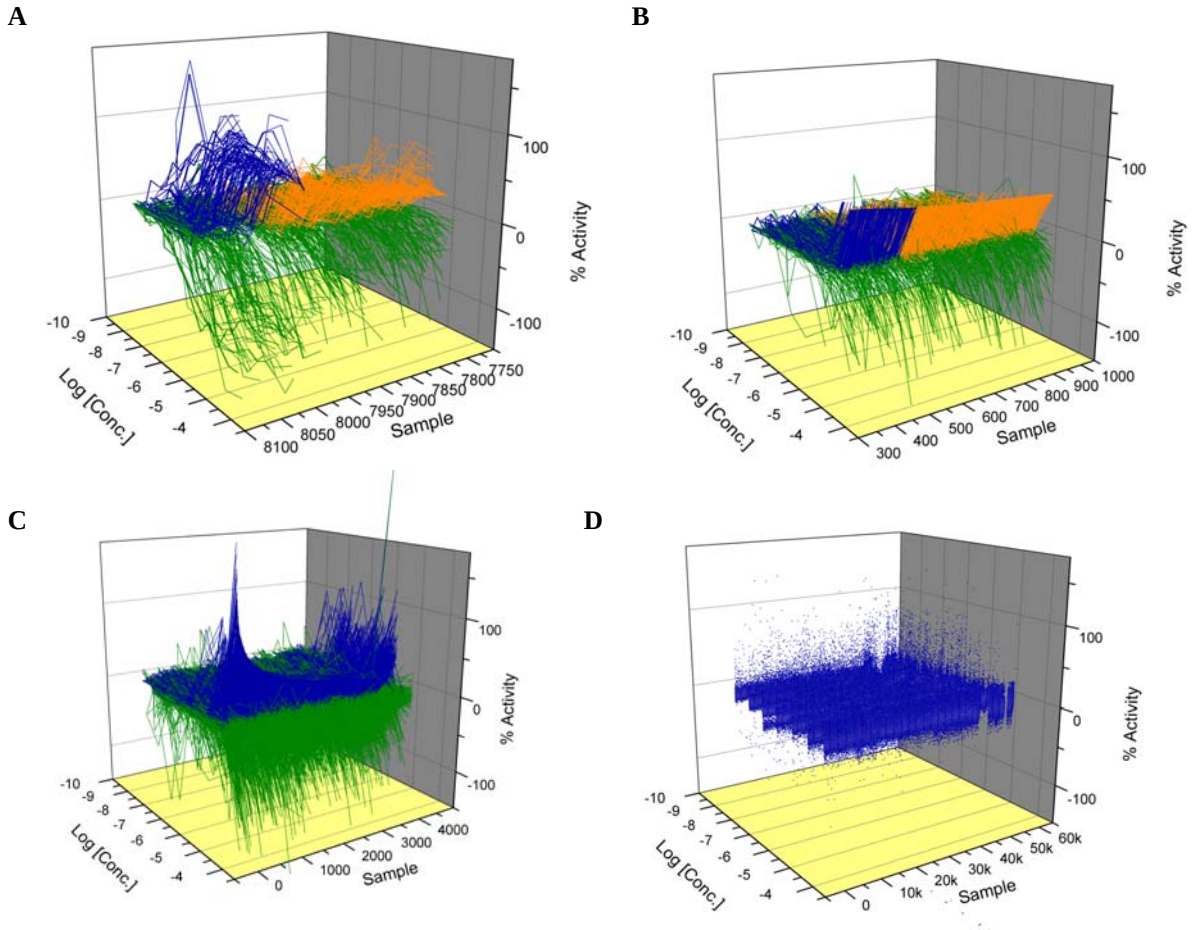


Fig. 2. Flowchart for rapid triaging of primary hits to identify the TSH agonists. The process of TSHR agonist discovery included the qHTS, false positive elimination, active compound selection and confirmation.



Scheme 1. Inhibitors from tsh qHTS data (AID 926) binned into curve classifications 1-4 based on tsh curve-fitting classification criteria. A) Class 1a (navy) and class 1b (orange) curves. Complementary parental data inverted for display purposes (olive). B) Class 2a (navy) and class 2b (orange) curves. Complementary parental data inverted for display purposes (olive). C) Class 3 curves (navy). Complementary parental data inverted for display purposes (olive). D) Data with no apparent dose-response, curve class 4 (navy points).

Hit clustering, SAR analysis and active compound selection:

A total of 1012 active compounds were identified from the primary screen. Among these positives, 736 were also active in the parental cell line that were not TSHR dependent, and thus eliminated. For the remaining 276 active compounds, a further 66 compounds showed activity in a cAMP Response Element (CRE)-signaling pathway assay previously screened in NCGC (PubChem Assay ID: 662), indicating direct activity on the cAMP cascade without TSHR. These compounds were also eliminated. This left 210 active compounds (0.29% of the screened compounds) which comprised 19 structural classes and 144 singletons (see Fig. 3. for a representative compound). Of these, 95 compounds representing each of the scaffolds and the most potent, efficacious singletons were selected for confirmation and further studies (Fig. 2).

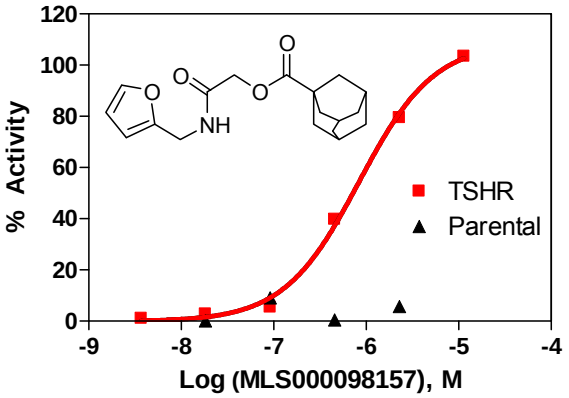


Fig 3. A representative positive compound, MLS000098157, in the TSHR cell line (red squares) and parental cells (black triangles). The EC_{50} value in the TSHR cell line was 0.79 uM. It was not active in the parental cell line. The sample was reordered and confirmed EC_{50} value of 2.0 uM.

Follow up assay to confirm selected compounds:

Recently, a time resolved fluorescence resonance energy transfer (TR-FRET) based probe of cAMP was introduced by Cisbio (10,11). It uses an anti-cAMP antibody labeled with Eu^{3+}, which binds to a d2-dye labeled cAMP tracer, resulting in TR-FRET. The cAMP from the cell lysate can displace the d2 labeled cAMP tracer that interrupts the TR-FRET (Fig. 4). Because this cAMP assay uses a different detection system, it can help to further eliminate the false positive compounds.

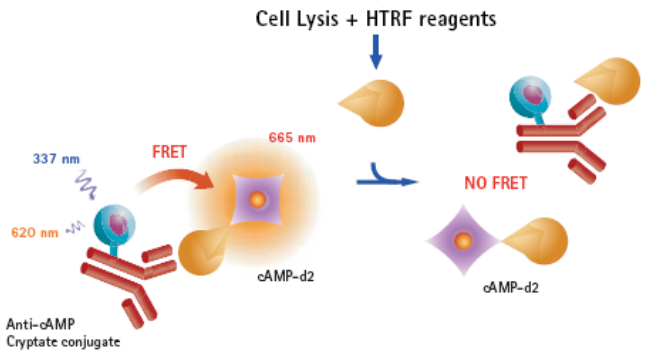


Fig. 2. Schematic of cAMP competitive immunoassay.

The 95 selected compounds were tested for TSHR agonism in the TR-FRET based cAMP assay, which measures cAMP concentrations in cell lysates. The readout in the HTRF cAMP assay is distinct from that in the membrane potential dye measurement assay used in the qHTS, allowing us to further eliminate assay-related false positive compounds. Of the 95 compounds, 49 showed TSH agonist activity in the TSHR cell line without the activities in the parental cell line. Taken together, these results indicate that these 49 compounds are true small molecule TSHR agonists.

Counter-screen assays:

HEK 293 cell lines stably expressing the TSH, FSH, or LH receptors were used as the counter-screens for these compounds in the TR-FRET cAMP assay format. These lines did

not contain the CNG and were therefore incapable of being used in the primary assay format. An assay protocol was summarized in Table 2.

Table 2. A protocol for TR-FRET based cAMP assay.

TSHR, FSHR, and LHR			
Step	**Parameter**	**Value**	**Description**
1	Cells	2.5 uL	750 cells/well
2	Compound	23 nL	In DMSO solution
3	Incubation time	30 min	Room temperature
4	Reagents	2.5 ul + 2.5 ul	d2-cAMP and antibody (separately) both diluted 1:20 in lysis buffer
5	Incubation time	30 min	Room temperature
6	Detection	EX=330/Em1=615 and Em2=660 nm	Envision plate reader

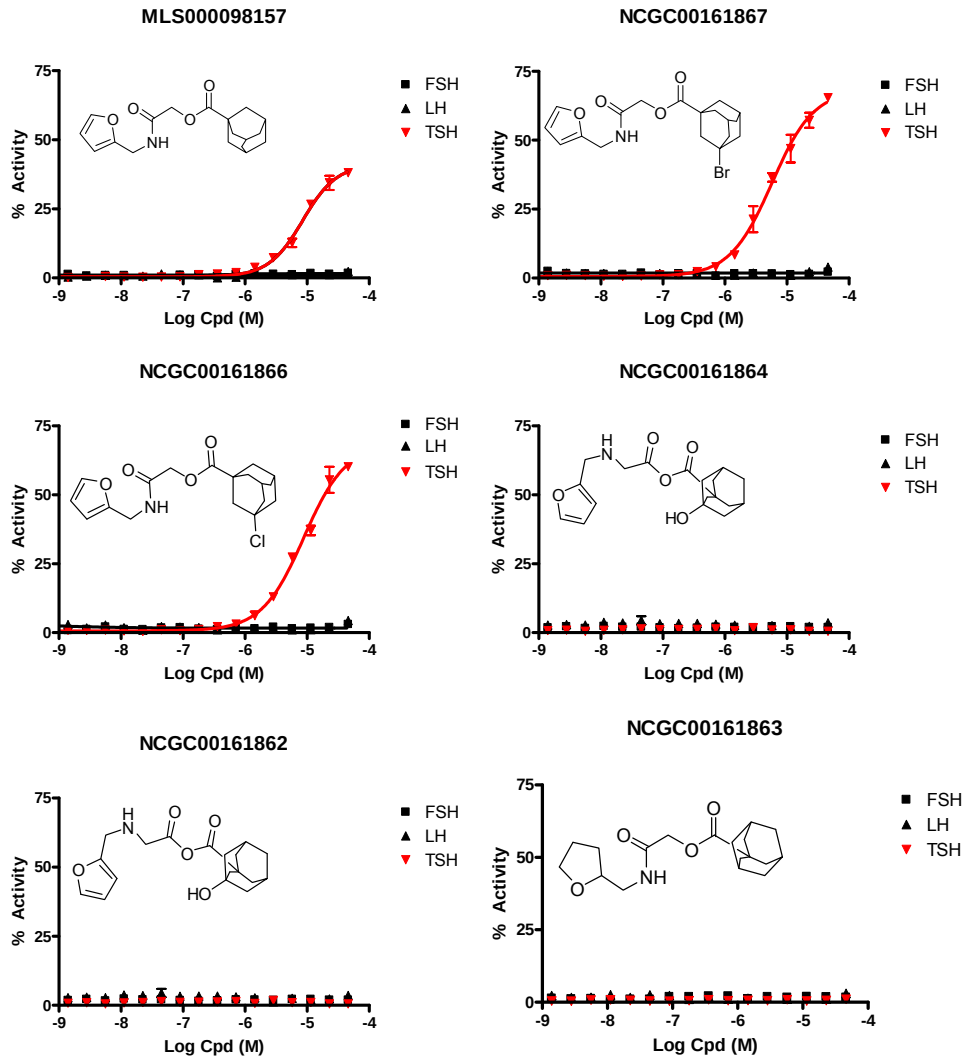


Fig. 4. Concentration-response curves of representative compounds in TR-FRET based cAMP assays in the cell lines with TSHR (red triangles), FSHR (black squares), and LHR (open circles). MLS000098157 showed activity specific to the TSH cell line and had an EC50 value of 12.6 uM. NCGC00161867-01 had an EC 50 value of 10.0 uM in the TSH cell line and was inactive in FSH and LH. NCGC00161866-01 had an EC50 value of 14.1 uM in the TSH cell line and was inactive in FSH and LH. NCGC00161862-01 and NCGC00161863-01 were inactive in all 3 cell lines.

Probe Characterization

Prior Art for the TSH receptor: There have been no reported TSHR agonists that are specific to the TSH receptor. Compounds such as Organon exhibit agonistic activity in the TSHR, as well as the two closely related GPCR family members FSH and LH.

***Initial Probes specific to TSHR*:** We purchased four analogs for the lead structure series. The chemical purities of these compounds were all greater than 95%, as verified by LC-MS. The activity of TSHR agonism was confirmed using a secondary cAMP assay based on TR-FRET. In order to determine specificity of the compounds to the TSH receptor, the compounds were also examined in the cells expressing FSHR and LHR (Fig. 4). Two compounds, NCGC00161862-01 and NCGC00161863-01 were inactive in all three cell lines. Fresh powder of MLS000098157 was obtained (NCGC00039523-03). An approximately 10 fold difference in potency between the primary screen (Fig. 3) and the follow-up TR-FRET based cAMP assay (Fig. 4). Two other analog compounds, NCGC00161866-01 and NCGC00161867-01, had higher efficacies than NCGC00039523-03 (61%, 65%, versus 40% respectively) but similar potencies (15.8, 10.0, and 8.5 uM respectively). All three confirmed compounds, NCGC00039523, NCGC00161866 and NCGC00161867, were not active in the TR-FRET based cAMP assay with FSHR and LHR, indicating high selectivity of these compounds.

Known probe properties:

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:

MLS000098157

Molecular Weight	317.37952 [g/mol]
Molecular Formula	$C_{18}H_{23}NO_4$
XLogP	2.8
H-Bond Donor	1
H-Bond Acceptor	4
Rotatable Bond Count	6
Tautomer Count	2
Exact Mass	317.162708
MonoIsotopic Mass	317.162708
Topological Polar Surface Area	68.5
Heavy Atom Count	23

Has this compound been provided to the MLSMR:

MLS000098157 (NCGC00039523) is from the MLSMR library.

Canonical SMILES:
C1C2CC3CC1CC(C2)(C3)C(=O)OCC(=O)NCC4=CC=CO4

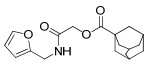
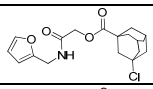
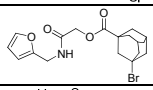
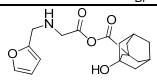
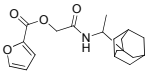
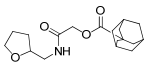
InChI: InChI=1/C18H23NO4/c20-16(19-10-15-2-1-3-22-15)11-23-17(21)18-7-12-4-13(8-18)6-14(5-12)9-18/h1-3,12-14H,4-11H2,(H,19,20)/f/h19H

Compound preparation: Compound is prepared in DMSO at 10 mM stock concentration.

Probe availability: Aliquots of MLS000098157 are available from the NCGC upon request.

Appendices-A

Table 4. Results of confirmation and counter-screen for TSHR probe and its analogues.

PubChem SID Internal ID	STRUCTURE	TSHR MP IC_{50} (uM)	TSHR TR-FRET IC_{50} (uM)	FSHR IC_{50} (uM)	LHR IC_{50} (uM)	Supplier - ID
3714076 MLS000098157		2.00	12.6	>100	>100	Enamine 002759
26755502 NCGC00161866		ND	14.1	>100	>100	Ambinter T5939111
26755503 NCGC00161867		ND	10.0	>100	>100	Ambinter T5939130
26755500 NCGC00161864		ND	>100	>100	>100	Ambinter T5645274
26755498 NCGC00161862		ND	>100	>100	>100	Ambinter T5547929
26755499 NCGC00161863		ND	>100	>100	>100	Ambinter ZT-3482300

MP = membrane potential, CNG assay of TSHR activity; TR-FRET = resolved fluorescence resonance energy transfer (TR-FRET) based probe of intracellular cAMP; ND = not determined.

Bibliography

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