

Probe Report

Title: Identification of Potent and Selective Thyroid Stimulating Hormone Receptor Agonists

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Version #4

Assigned Assay Grant #: X01 MH080680

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Chemistry Center Name & PI: NIH Chemical Genomics Center, Christopher Austin

Assay Submitter & Institution: Marvin Gershengorn, NIDDK

PubChem Summary Bioassay Identifier (AID): 1401

Abstract:

Thyroid Stimulating Hormone (TSH) is a heterodimeric glycoprotein hormone that regulates thyroid homeostasis upon interaction with the TSH receptor (TSHR). TSH binds to the TSH receptor, 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). In this report, we describe the discovery and SAR studies of a series of dihydroquinazolin-4-ones as TSHR agonists. ML109 (CID 25246343) is the first selective and orally available small-molecule TSHR agonist, and the probe will be a useful pharmacological tool to study TSHR biology in thyroidal and extrathyroidal tissues.

Probe Structure & Characteristics:

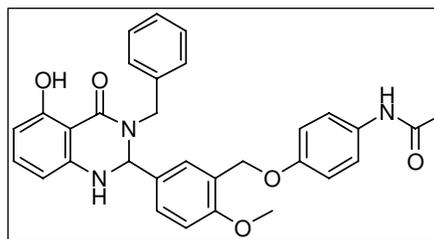
PubChem CID: 25246343

Probe MLSMR ID: MLS002576689

Internal ID: NCGC00161870

Chemical Formula: C₃₁H₂₉N₃O₅

Exact Mass: 523.21

**ML109**

CID/ML#	Target Name	IC ₅₀ /EC ₅₀ (nM) [SID, AID]	Anti-target Name(s)	IC ₅₀ /EC ₅₀ (μM) [SID, AID]	Fold Selective	Secondary Assay(s) Name: IC ₅₀ /EC ₅₀ (nM) [SID, AID]
25246343/ ML109	TSHR	7900nM [26755506, 1401]	FSHR	>100μM [26755506, 1403]	>10 fold	TSHR cAMP ELISA 90nM [26755506, 2104]
			LHR	>100μM [26755506, 1402]	>10 fold	

Recommendations for scientific use of the probe:

This probe is a member of a series of TSHR agonists. The current state of the art is lacking any small molecule TSHR agonists. This probe can be used to study TSHR functions *in vitro* and *in vivo*, and could be used as a lead for development of drugs to replace recombinant human TSH in patients with thyroid cancer. It is highly selective for human TSHR over other glycoprotein hormone receptors, such as LHCGR and FSHR, and interacts with the receptor's serpentine domain. In primary cultures of human thyrocytes, this compound increases mRNA levels for thyroglobulin, thyroperoxidase, sodium iodide symporter, and deiodinase type 2, as well as deiodinase type2 enzyme activity. Moreover, oral administration of the agonist stimulated thyroid function in mice, resulting in increased serum thyroxine and thyroidal radioiodide uptake¹. This report supplants the previous report of activity with the [2-(furan-2-ylmethylamino)-2-oxoethyl] adamantane-1-carboxylate series.



Thyroid Stimulating Hormone (TSH) is an α/β heterodimeric glycoprotein hormone secreted from the anterior pituitary gland. It belongs to the glycoprotein hormone family, including Chorionic Gonadotropin (CG), Luteinizing Hormone (LH), and Follicle Stimulating Hormone (FSH)^{2,3}. 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). TSHR is mainly expressed in thyroid follicular cells⁴ and regulates their growth and function⁵. Recombinant TSH is currently used for follow-up in patients with thyroid cancer who are receiving thyroid hormone suppression therapy, specifically for screening of residual tumor after surgery⁶, but it is difficult to produce 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, especially the extrathyroidal role of TSHR, and would have multiple advantages for therapeutic applications.

There are few literature reports of TSHR agonists (all from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) group), and most are confined to a class of thienopyrimidines with little structural diversity and no selectivity for TSHR over LHR or FSHR. The thienopyrimidine **Org 41841 (Fig. 1)** was first described in 2002 as an orally active LH agonist with *in vivo* efficacy in mice following oral administration⁷. It was later found to have modest TSHR activity, with an EC₅₀ of 7700nM and 220nM for TSHR and LHCGR, respectively⁸. This finding was followed with a series of synthesized analogs for SAR studies. Some of the analogs achieved LHR selectivity, but none were TSHR selective⁹. Another TSHR agonist is from a high throughput screen at NCGC. **MLS000098157** was identified as a 2 μ M agonist of hTSHR from the 2-(furan-2-ylmethylamino)-2-oxoethyl] adamantane-1-carboxylate series (**Fig. 1**). That series is supplanted by this report.

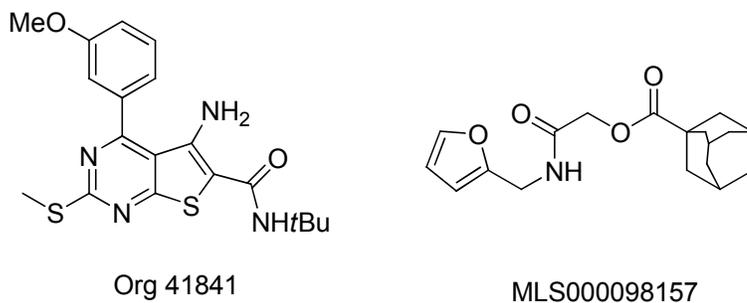


Figure 1. TSHR agonists

2 Materials and Methods

All commercially available reagents and solvents were purchased and used without further purification. All microwave reactions were carried out in a sealed microwave vial equipped with a magnetic stir bar and heated in a Biotage Initiator Microwave Synthesizer. All compounds for biological testing were purified using a Waters semi-preparative HPLC equipped with a Phenomenex Luna[®] C18 reverse phase (5 micron, 30 x 75 mm) column having a flow rate of 45 ml/min. The mobile phase was a mixture of acetonitrile and H₂O, each containing 0.1% trifluoroacetic acid. During purification, a gradient of 30% to 80% acetonitrile over 8 minutes was used with fraction collection triggered by UV detection (220 nM). Pure fractions passed through PL-HCO₃ MP SPE (Varian) to remove trifluoroacetic acid and concentrated under vacuum on a lyophilizer. ¹H spectra were recorded using an Inova 400 (100) MHz spectrometer (Varian).

2.1 Assays

i. qHTS Assay for agonists of the TSH receptor [AID 926; Primary DR]

Assay Description:

TSHR is a seven-transmembrane receptor that 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^{9,10,11}. The membrane potential dye was used to detect the 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 the TSHR as well as a modified CNG was purchased from BD biosciences (TSHR ACTOne cell line, Rockville, MD). When the 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. 2**). 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 µg/ml Puromycin (Invitrogen) at 37°C 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.

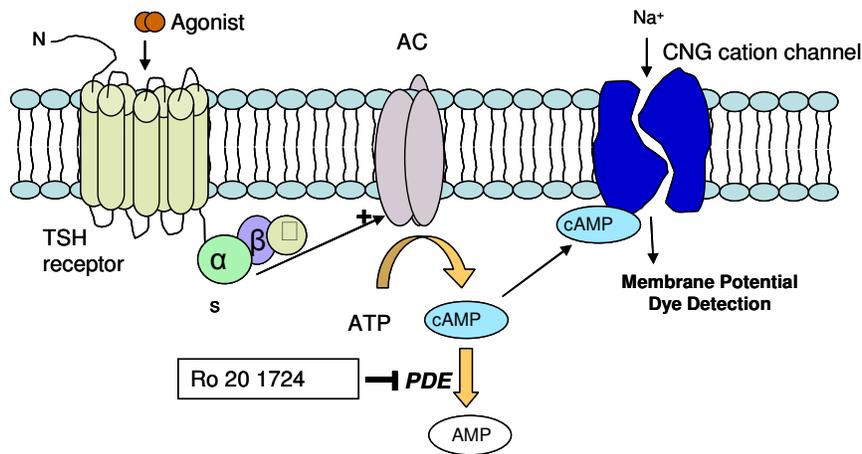


Figure 2. Schematic illustration of the assay principle of the ACT:One cAMP assay for TSHR. A TSHR was co-expressed in a HEK293 cell line with a modified CNG channel. When TSH binds to TSHR, it increases the intracellular cAMP level. The CNG channels are activated by the increased cAMP, which results in membrane depolarization after the influx of cations, such as sodium and calcium.

Table 1. Reagents and resources for the TSH receptor screen

Reagents	Resources	Catalog Number
Membrane potential Dye	BD Biosciences	80500-210
Puromycin	Sigma	P9620
DMEM Media	Invitrogen	11765
Geneticin	Invitrogen	10131027
Fetal Bovine Serum	Hyclone	SH30071.03
Penicillin-Streptomycin	Invitrogen	15140
RO 20-1724	Sigma	B8279
TSH	Sigma	T8931
Forskolin	Sigma	F6686

Assay Protocol:

Reagents used are listed in **Table 1**. Cells from 4 confluent T175 flasks were seeded into 25 to 30 T175 flasks at a density of 3 to 4 million cells per flask. After 3 days of growth, the flasks were harvested, and the fresh cells were used for dispensing into 1536-well plates. This process was repeated on a daily basis for screening purposes. The cells were resuspended in 2% FCS DMEM medium at 500,000 cells/ml, and 4 μ l of resuspended cells were dispensed into each well of black, clear bottom, 1536-well tissue culture-treated plates using a Multidrop Combi dispenser. After overnight culture at 37°C with 5% CO₂, the cells were generally 60% to 70% confluent in the 1536-well plates. After overnight incubation, 4 μ l of membrane potential dye containing 50 μ M of the phosphodiesterase inhibitor RO 20-1724 was added to each well, and the plates were incubated for 60 min at room temperature. A total of 23nl of compound or positive control (30nM TSH or 10 μ M forskolin for TSHR-expressing or parental cells, respectively) in DMSO was added to each well using a pintoole (Kalypsys, San Diego, CA), and the plates were further incubated for 30 min at room temperature. Plates were measured with an excitation of 535/20 nm, an emission of 590/20 nm, a gain of 150, and 5 flashes per well on an Envision plate reader (PerkinElmer, Boston, MA) (**Table 2**).

Table 2. Assay protocol for the TSHR and parental cell lines in 1536 well format

Step	Parameter	Value	Description
1	<i>Cells</i>	<i>4μl</i>	<i>1000 TSHR or parental cells/well</i>
2	<i>Incubation</i>	<i>18 - 30 hours</i>	<i>37°C; 5% CO₂</i>
3	<i>Reagent</i>	<i>4μl</i>	<i>Membrane potential dye</i>
4	<i>Incubation</i>	<i>30 min</i>	<i>At room temperature</i>
5	<i>Compound</i>	<i>20nl</i>	<i>Compound Libraries or control</i>
6	<i>Incubation</i>	<i>30 min</i>	<i>At room temperature</i>
7	<i>Detector</i>	<i>Ex = 535 and Em = 590 nm</i>	<i>Envision plate reader</i>

Center Summary of Results:

Both TSHR and parental (lacking TSHR) lines were screened across 463 compound plates (for a total of 926 plates). The average signal-to-basal ratio for the entire screen was 2.01, the CV was 12.1%, and the Z' value was 0.4. Plates with a Z' factor less than 0.2 (approximately 5% of the total plates) were rescreened. The relatively low Z' is likely due to the use of HEK293 cells, which typically do not adhere well after treatment with membrane potential dyes.

ii. Counterscreen in ACTOne parental cell line for TSHR agonists. [AID:398; Primary DR]

Assay Description:

All 72,030 compounds were tested in qHTS format in the parental cell line lacking the TSH receptor.

Assay Protocol:

The assay protocol and reagents are identical to: qHTS Assay for TSHR agonists Primary screen [AID: 926; Primary] but screened in the parental HEK 293 cell line, which lacks the TSH receptor.

Center Summary of Results

Prioritized compounds had no activity in the parental cell line.

iii. Confirmation Concentration-Response Assay for TSHR agonists in ACTOne TSHR cells [AID:939; Confirmatory DR]

Assay Description:

To confirm activity in the original assay, select samples active in the primary screen were obtained in DMSO solution from the MLSMR and/or as powders from compound vendors.

Assay Protocol:

The assay protocol and reagents are identical to: qHTS Assay for TSHR agonists Primary screen [AID: 926; Primary]

Center Summary of Results:

Prioritized compounds had a high confirmation rate.

iv. **Counterscreen in ACTOne parental cell line for TSHR agonists. [AID:953; Confirmatory DR]**

Assay Description:

To confirm activity in the original assay, select samples active in the primary screen were obtained in DMSO solution from the MLSMR and/or as powders from compound vendors.

Assay Protocol:

The assay protocol and reagents are identical to: qHTS Assay for TSHR agonists Primary screen [AID: 926; Primary], but screened in the parental HEK 293 cell line, which lacks the TSH receptor.

Center Summary of Results:

Prioritized compounds were inactive in the parental cell line.

v. **Counterscreen in TSHR cell line using Homogeneous Time Resolved Fluorescence (HTRF) cAMP assay in TSHR cells [AID:933; Confirmatory DR]**

Assay Description:

Recently, a time resolved fluorescence resonance energy transfer (TR-FRET) based cAMP assay has been available for screens from Cisbio (12,13). 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. 3**). Because this cAMP assay uses a different detection system, it can help to further eliminate the false positive compounds.

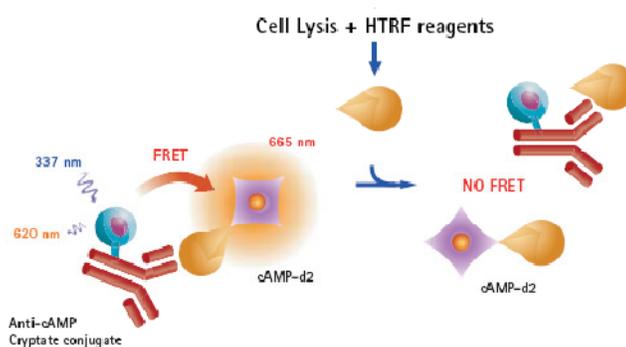


Figure 3. Schematic of cAMP competitive immunoassay

Assay Protocol:

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.

Table 3. HTRF assay in TSH, LH, and FSH cell lines

TSHR, FSHR, and LHR			
Step	Parameter	Value	Description
1	Cells	2.5 μ l	750 cells/well
2	Compound	23nl	In DMSO solution
3	Incubation time	30 min	Room temperature
4	Reagents	2.5 μ l + 2.5 μ l	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

Assay Protocol

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**. Cells were plated in white, solid bottom 1536 well tissue culture treated plates and assayed as described in **Table 3**.

Table 4. Reagents for HTRF counterscreen.

Reagents	Resources	Catalog Number
HTRF cAMP <i>dynamic</i> 2 kit	Cisbio	62AM4PEJ
DMEM Media	Invitrogen	11765
Geneticin	Invitrogen	10131027
Fetal Bovine Serum	Hyclone	SH30071.03
Penicillin-Streptomycin	Invitrogen	15140
RO 20-1724	Sigma	B8279

vi. Counterscreen in TSHR cell line using Homogeneous Time Resolved Fluorescence (HTRF) cAMP assay in LH cells [AID:1402; Confirmatory DR]

Assay Description

To confirm activity in the original assay, select samples active in the primary screen were obtained in DMSO solution from the MLSMR and/or as powders from compound vendors.

Assay Protocol

The assay protocol and reagents are identical to the HTRF assay run in TSH cells qHTS Assay [AID: 933; Primary].

Center Summary of Results

Prioritized compounds had a high confirmation rate.

vii. Counterscreen in TSHR cell line using Homogeneous Time Resolved Fluorescence (HTRF) cAMP assay in FSH cells [AID:1403; Confirmatory DR]

Assay Description

To confirm activity in the original assay, select samples active in the primary screen were obtained in DMSO solution from the MLSMR and/or as powders from compound vendors.

Assay Protocol

The assay protocol and reagents are identical to the HTRF assay run in TSH cells qHTS Assay [AID: 933; Primary].

Center Summary of Results

Prioritized compounds had a high confirmation rate.

viii. Secondary Concentration-Response Assay for Agonists of the Thyroid Stimulating Hormone Receptor: ELISA Activity Detection [AID:2104; Confirmatory DR]

Assay Description:

To confirm activity in the original assay, select samples active in the confirmatory assays were resynthesized and assayed by the low-throughput ELISA assay format.

Assay Protocol:

The assay protocol and reagents are described in detail in reference 11 (1) [AID: 2104; Secondary]. Transiently transfected HEK-EM293 cells or cells stably expressing TSHR, LHCGR, or FSHR were seeded into 96-well plates at a density of 50,000 cells/well in DMEM containing 10% FBS. Cells were cultured for 24 h before incubation for 1 h in serum-free DMEM containing 1mM 3-isobutyl-1-methylxanthine (Sigma) and TSH, LH, FSH, or small-molecule ligands in a humidified 5% CO₂ incubator at 37°C. Following aspiration of the media, cells were lysed using lysis buffer of the cAMP-Screen Direct system (Applied Biosystems). The cAMP content of the cell lysate was determined using the method described in the manufacturer's protocol. Reagents are listed in **Table 5**.

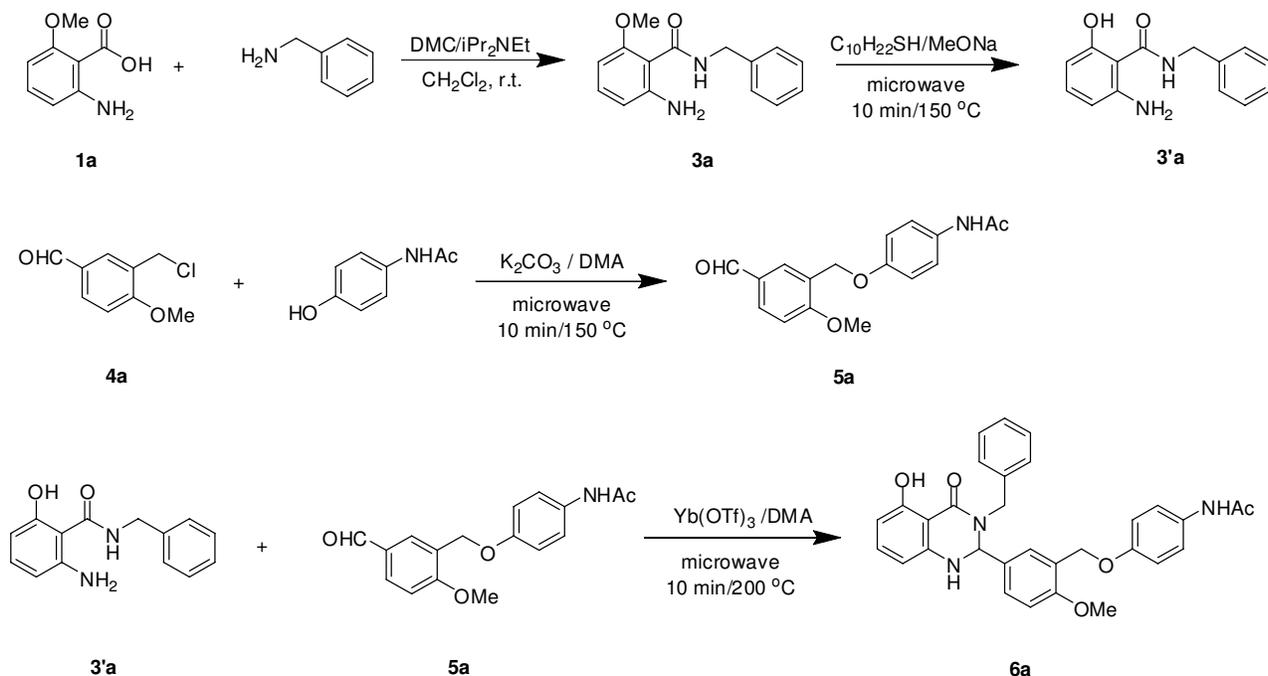
Table 5. Reagents for ELISA secondary screen.

<i>Reagents</i>	<i>Resources</i>	<i>Catalog Number</i>
<i>cAMP-Screen Direct</i>	<i>Applied Biosystems</i>	<i>T1500</i>
<i>DMEM Media</i>	<i>Invitrogen</i>	<i>11765</i>
<i>Geneticin</i>	<i>Invitrogen</i>	<i>10131027</i>
<i>Fetal Bovine Serum</i>	<i>Hyclone</i>	<i>SH30071.03</i>
<i>Penicillin-Streptomycin</i>	<i>Invitrogen</i>	<i>15140</i>
<i>RO 20-1724</i>	<i>Sigma</i>	<i>B8279</i>

Center Summary of Results:

Compounds were much more potent using ELISA assay than HTRF format assay.

2.2 Probe Chemical Characterization



Scheme 1. Synthesis of probe

Synthesis of probe molecule:

The synthesis of the probe molecule commenced with amide formation between commercially available acid **1a** and benzyl amine to afford **3a** and subsequent demethylation to **3'a** (Scheme 1). The second component of the aminal was synthesized via ether formation between benzyl chloride **4a** and p-acetamide phenol to afford **5a**. The ytterbium catalyzed condensation between **3'a** and **5a** afforded the probe molecule **6a**.

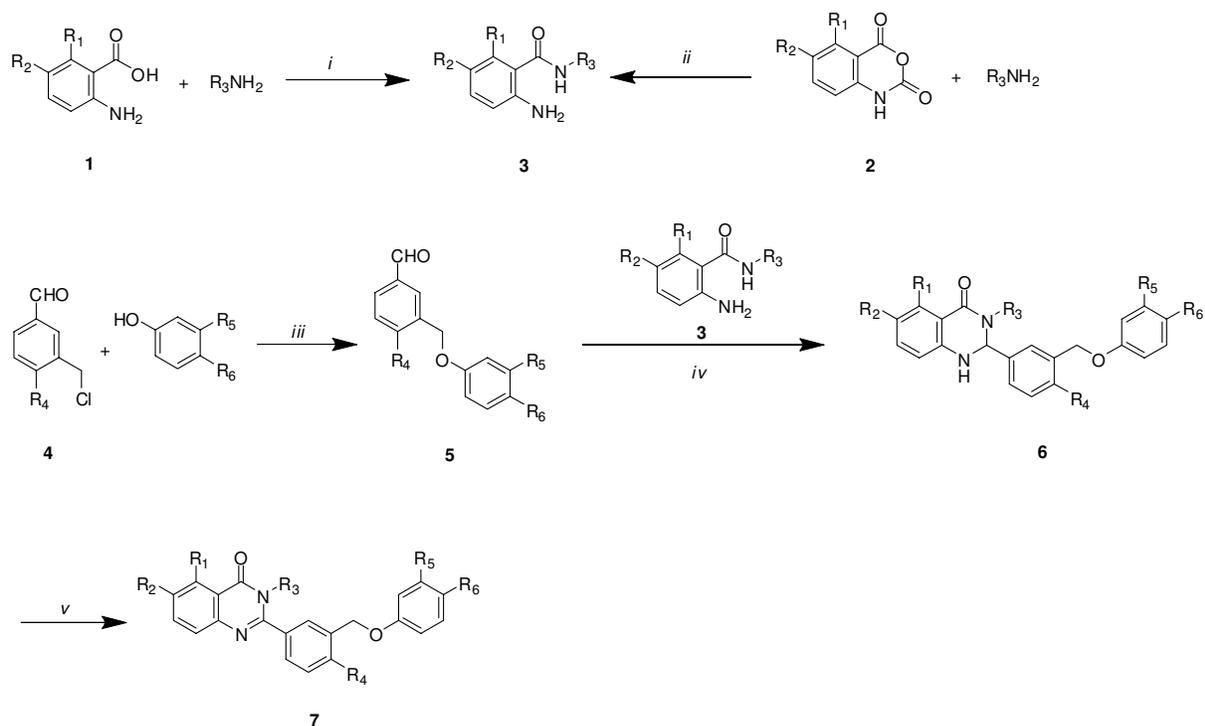
MLS002576689 (CID: 25246343/ML109):

¹H NMR (400 MHz, DMSO-*d*₆) δppm 2.00 (s, 3 H), 3.78 (d, *J*=15.5Hz, 1H), 3.80 (s, 3H), 4.96 (s, 2 H), 5.15 (d, *J*=15.3 Hz, 1 H), 5.73 (d, *J*=1.96 Hz, 1 H), 6.05-6.08 (m, 2 H), 6.86-6.90 (m, 2 H), 7.01 - 7.11 (m, 2 H), 7.19 - 7.36 (m, 6 H), 7.41-7.48 (m, 4 H) 9.77 (s, 1 H), 12.26 (s, 1 H); HPLC: *t*_R = 6.19 min, UV₂₅₄ = 96%; HRMS (ESI): *m/z* calcd for C₃₁H₂₉N₃O₅ [M+1]⁺ 524.2185, found 524.2184. Solubility (PBS, pH = 7.4, 23 °C): 2μM.

Table 6. Compounds submitted to the MLSMR:

MLS002576689	Probe
MLS003221407	Analogue
MLS003221408	Analogue
MLS003221409	Analogue
MLS003221410	Analogue
MLS003221411	Analogue

2.3 Probe Preparation



Reagents and conditions: (i) DMC, DIPEA, r.t. 12 h; (ii) ACN, r.t.-50 °C. (iii) K₂CO₃, DMA, microwave heating, 150 °C, 10 min; (iv) Yb(OTf)₃, DMA, microwave heating, 200 °C, 10 min; (v) DDQ, r.t. 1 h

Scheme 2. Analog synthesis

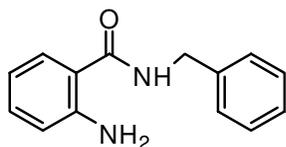
Synthesis of 2,3-dihydroquinazolin-4(1H)-one (6) and quinazolin-4(1H)one (7) analogs:

As depicted in scheme 2, 2-aminobenzamides 3 were prepared by either amide couplings of 2-aminobenzoic acids 1 with different amines or reactions of isoic anhydrides 2 with amines. Reactions of benzyl chlorides 4 with different phenols under microwave irradiation generated aldehydes 5. Condensations of aldehydes 5 with 2-aminobenzamides 3 yielded 2,3-dihydroquinazolin-4-ones 6. The 2,3-dihydroquinazolin-4-ones 6 were rapidly oxidized by DDQ at room temperature to produce quinazolin-4-ones 7.

General procedure for the synthesis of 2-aminobenzamides from isoic anhydride:

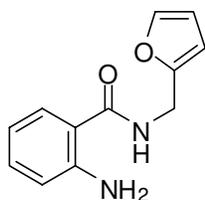
Amines (1.05 mmol, 1.05 equiv) at room temperature were added to a solution of isoic anhydride (0.163 g, 1.0 mmol, 1.0 equiv) in 10 mL of anhydrous acetonitrile. The resulting mixture was stirred at room temperature for 2 hours and heated at 50°C for 4 hours. Then, it was concentrated *in vacuo* to yield the products as solids in 90-99% yields.

2-Amino-N-benzylbenzamide:



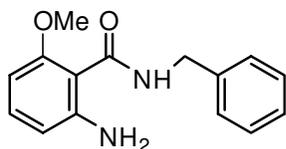
^1H NMR (400 MHz, CHLOROFORM-*d*) δ 4.61 (s, 1 H), 4.63 (s, 1 H), 5.58 (br. s., 2 H), 6.33 (br. s., 1 H), 6.62-6.66 (m, 1 H), 6.69-6.71 (m, 1 H), 7.19-7.25 (m, 1 H), 7.28 - 7.43 (m, 6 H); LCMS: (electrospray +ve), m/z 227.1 (MH) $^+$; HPLC: t_R = 4.38 min, UV₂₅₄ = 96%.

2-Amino-N-(furan-2-ylmethyl)benzamide:



^1H NMR (400 MHz, CHLOROFORM-*d*) δ 4.60 (s, 1 H), 4.61 (s, 1 H), 5.57 (br. s., 2 H), 6.24 - 6.42 (m, 3 H), 6.59 - 6.74 (m, 2 H), 7.16 - 7.25 (m, 1 H), 7.33-7.39 (m, 2 H); LCMS: (electrospray +ve), m/z 217.1 (MH) $^+$; HPLC: t_R = 3.77 min, UV₂₅₄ = 98%.

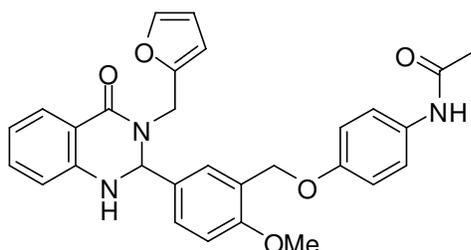
2-Amino-N-benzyl-6-methoxybenzamide:



2-chloro-1,3-dimethylimidazolium chloride (1.099g, 6.5mmol, 1.3equiv) at room temperature was added to a solution of 2-amino-6-methoxybenzoic acid (0.841g, 5.0mmol, 1.0equiv), benzylamine (0.643g, 6.0mmol, 1.2equiv), and diisopropylethylamine (1.935g, 15.0mmol, 3.0equiv) in 50ml of dichloromethane. The mixture was stirred at room temperature for 6 hours, poured into water, and extracted with dichloromethane. The organic solution was successively washed with aqueous saturated NaHCO₃ and water. The organic layer was dried over MgSO₄ and the solvent was removed by rotary evaporator. The residue was purified by column chromatography (silica gel, 2% 2.0 M ammonia MeOH solution in CH₂Cl₂) to give 2-Amino-N-benzyl-6-methoxybenzamide (0.593g, 46%) as a solid. ^1H NMR (400 MHz, CHLOROFORM-*d*) δ 3.81 (s, 3 H), 4.62 (s, 1 H), 4.63 (s, 1 H), 6.07 (vb.s, 2 H), 6.19 (d, $J=8.2$ Hz, 1 H), 6.32 (d, $J=8.2$ Hz, 1 H), 7.07 (t, $J=8.2$ Hz, 1 H), 7.14 - 7.54 (m, 5 H), 8.05 (br. s., 1 H);

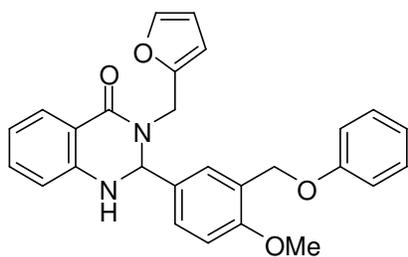
HPLC: $t_R = 4.72$ min, $UV_{254} = 99\%$; HRMS (ESI): m/z calcd for $C_{15}H_{16}N_2O_2$ $[M+1]^+$ 257.1296, found 257.1294.

N-(4-(5-(3-(Furan-2-ylmethyl)-4-oxo-1,2,3,4-tetrahydroquinazolin-2-yl)-2-methoxybenzyloxy)phenyl)acetamide:



K_2CO_3 (207mg, 1.5mmol, 5equiv) was added to a solution of 3-(chloromethyl)-4-methoxybenzaldehyde (55.2mg, 300 μ mol, 1.0equiv) and 4-acetamidophenol (54.4mg, 360 μ mol, 1.2equiv) in 1.5ml of anhydrous DMA. The mixture was heated in a microwave for 10 min at 150°C. After filtering off the solid, 2-amino-*N*-(furan-2-ylmethyl)benzamide (77.8mg, 360 μ mol, 1.2equiv) and Ytterbium trifluoromethanesulfonate (93mg, 150 μ mol, 0.5equiv) were added to the clear solution. The resulting mixture was heated in a microwave for 10 min at 200 °C. The product was isolated via preparative HPLC purification and solvent was removed by a GeneVac to give *N*-(4-(5-(3-(furan-2-ylmethyl)-4-oxo-1,2,3,4-tetrahydroquinazolin-2-yl)-2-methoxybenzyloxy)phenyl)acetamide (47.8mg, 32%) as a solid. 1H NMR (400 MHz, $CDCl_3$) δ 2.11 (s, 3 H), 3.72 (d, $J=15.6$ Hz, 1 H), 3.83 (s, 3 H), 4.25 (v.b.s, 1H), 5.01 (s, 2 H), 5.26 (d, $J=15.6$ Hz, 1 H), 5.71 (s, 1 H), 6.03 - 6.29 (m, 2 H), 6.48 (d, $J=7.8$ Hz, 1 H), 6.83 (dd, $J=18.2, 8.8$ Hz, 3 H), 7.10 - 7.61 (m, 7 H), 7.92 (d, $J=7.0$ Hz, 1 H); HPLC: $t_R = 5.40$ min, $UV_{254} = 91\%$; HRMS (ESI): m/z calcd for $C_{29}H_{27}N_3O_5$ $[M+1]^+$ 498.2029, found 498.2025.

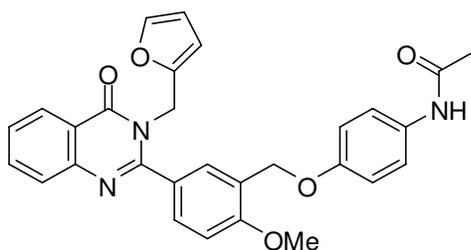
3-(Furan-2-ylmethyl)-2-(4-methoxy-3-(phenoxy)methyl)phenyl)-2,3-dihydroquinazolin-4(1H)-one:



K_2CO_3 (319mg, 2.3mmol, 5equiv) was added to a solution of 3-(chloromethyl)-4-methoxybenzaldehyde (85mg, 462 μ mol, 1.0equiv) and phenol (52mg, 554 μ mol, 1.2equiv) in 2.0ml of anhydrous DMA. The mixture was heated in a microwave for 10 min at 150°C. After filtering off the solid, 2-amino-*N*-(furan-

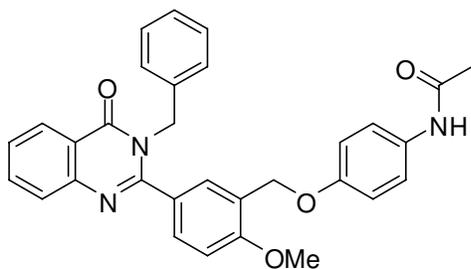
2-ylmethyl)benzamide (100mg, 462 μ mol, 1.0equiv) and Ytterbium trifluoromethanesulfonate (286 mg, 231 μ mol, 0.5equiv) were added to the clear solution. The resulting mixture was heated in a microwave for 10 min at 200°C. The product was isolated via preparative HPLC purification and solvent was removed via reduced pressure lyophilization to give 3-(furan-2-ylmethyl)-2-(4-methoxy-3-(phenoxy)methyl)phenyl)-2,3-dihydroquinazolin-4(1H)-one (41.5mg, 20%) as a white solid after triturating with diethyl ether. ¹H NMR (400 MHz, CDCl₃) δ 3.74 (d, *J*=15.6 Hz, 1 H), 3.86 (s, 3 H), 4.40 (v.b.s, 1H), 5.08 (s, 2 H), 5.31 (d, *J*=16.0 Hz, 1 H), 5.74 (s, 1 H), 6.13 (dd, *J*=3.3, 0.6 Hz, 1 H), 6.25 (dd, *J*=3.1, 1.9 Hz, 1 H), 6.48 (dd, *J*=8.0, 0.4 Hz, 1 H), 6.81-6.85 (m, 2H), 6.94-6.98 (m, 3H), 7.21 - 7.32 (m, 5 H), 7.48 (d, *J*=2.4 Hz, 1 H), 7.98 (dd, *J*=7.8, 1.4Hz, 1H); HPLC: *t*_R = 6.42 min, UV₂₅₄ = 92%; HRMS (ESI): *m/z* calcd for C₂₇H₂₄N₂O₄ [M+1]⁺ 441.1820, found 441.1821.

N-(4-(5-(3-(Furan-2-ylmethyl)-4-oxo-3,4-dihydroquinazolin-2-yl)-2-methoxybenzyloxy)phenyl)acetamide:



¹H NMR (400 MHz, CHLOROFORM-*d*) δ 2.15 (s, 3 H), 3.95 (s, 3 H), 5.12 (s, 2 H), 5.19 (s, 2 H), 6.12 (d, *J*=2.7 Hz, 1 H), 6.22 - 6.28 (m, 1 H), 6.93 (d, *J*=9.0 Hz, 2 H), 7.00 (d, *J*=8.6 Hz, 1 H), 7.12 (br. s., 1 H), 7.24 (s, 1 H), 7.39 (d, *J*=9.0 Hz, 2 H), 7.46 - 7.56 (m, 2 H), 7.64 - 7.82 (m, 3 H), 8.33 (d, *J*=8.2 Hz, 1 H); HPLC: *t*_R = 5.49 min, UV₂₅₄ = 95%; HRMS (ESI): *m/z* calcd for C₂₉H₂₅N₃O₅ [M+1]⁺ 496.1872, found 496.1873.

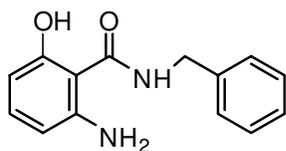
N-(4-(5-(3-Benzyl-4-oxo-3,4-dihydroquinazolin-2-yl)-2-methoxybenzyloxy)phenyl)acetamide:



^1H NMR (400 MHz, CHLOROFORM-*d*) δ 2.13 (s, 3 H), 3.91 (s, 3 H), 5.04 (s, 2 H), 5.26 (s, 2 H), 6.81 - 7.04 (m, 5 H), 7.11 - 7.26 (m, 4 H), 7.38 (d, $J=9.0$ Hz, 2 H), 7.46 - 7.58 (m, 2 H), 7.71 - 7.83 (m, 2 H), 8.36 (d, $J=7.8$ Hz, 1 H); HPLC: $t_{\text{R}} = 5.73$ min, $\text{UV}_{254} = 98\%$; HRMS (ESI): m/z calcd for $\text{C}_{31}\text{H}_{27}\text{N}_3\text{O}_4$ $[\text{M}+1]^+$ 506.2086, found 506.2082.

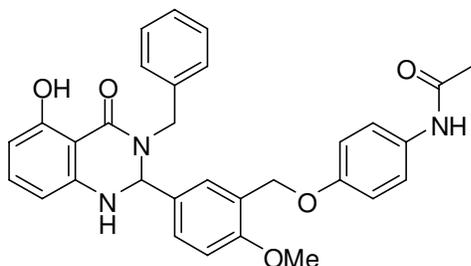
Synthesis of the probe:

2-Amino-*N*-benzyl-6-hydroxybenzamide (**3a**):



1-dodecanethiol (0.360g, 1.78 mmol, 2.0equiv) was added to a solution of 2-amino-*N*-benzyl-6-methoxybenzamide (0.228g, 0.89mmol, 1.0equiv) in 3 mL of anhydrous DMF, followed by adding NaOMe (0.385g of 25% solution in MeOH, 1.78, 2.0equiv). Five copies of this reaction were prepared, and the reaction mixtures were heated in a microwave at 150 °C for 10 min. The reaction mixtures were combined and passed through a silica gel plug, which was washed with an ethyl acetate-methanol mixture (1:1). The solvents were removed and the residue was purified by column chromatography (silica gel, eluent gradient 2:98 ethyl acetate- CH_2Cl_2 to 1:1 ethyl acetate- CH_2Cl_2). The desired 2-Amino-*N*-benzyl-6-hydroxybenzamide (0.780 g, 72%) was isolated as an off-white solid after triturating with diethyl ether/Hexanes. ^1H NMR (400 MHz, CHLOROFORM-*d*) δ 3.55 - 4.20 (vb.s., 2 H), 4.60 (s, 1 H), 4.61 (s, 1 H), 6.25 (d, $J=7.8$ Hz, 1 H), 6.50 (d, $J=8.2$ Hz, 1 H), 7.09 (t, $J=8.0$ Hz, 1 H), 7.16 - 7.52 (m, 5 H), 8.58 (br. s., 1 H), 12.05 (br. s., 1 H); HPLC: $t_{\text{R}} = 3.97$ min, $\text{UV}_{254} = 98\%$; HRMS (ESI): m/z calcd for $\text{C}_{14}\text{H}_{14}\text{N}_2\text{O}_2$ $[\text{M}+1]^+$ 243.1140, found 243.1134.

N-(4-(5-(3-Benzyl-5-hydroxy-4-oxo-1,2,3,4-tetrahydroquinazolin-2-yl)-2-methoxybenzyloxy)phenyl)acetamide (**6a**):



K_2CO_3 (690 mg, 5.0mmol, 5equiv) was added to a solution of 3-(chloromethyl)-4-methoxybenzaldehyde (184.6 mg, 1.00 mmol, 1.0equiv) and *N*-(4-hydroxyphenyl)acetamide (181mg, 1.20mmol, 1.2equiv) in 5.0ml of anhydrous DMA. The mixture was heated in a microwave at 150°C for 10 min. The solid was filtered and the clear solution was added to a microwave tube containing 2-amino-*N*-benzyl-6-hydroxybenzamide (264mg, 1.09mmol, 1.1equiv). After adding Ytterbium trifluoromethanesulfonate (310mg, 0.5mmol, 0.5equiv), the mixture was heated in a microwave at 200°C for 10 min. The reaction mixture was purified by HPLC. Pure fractions were dried down under vacuum on a lyophilizer. The desired product (132.3mg, 28%) was obtained as a solid after triturating with diethyl ether. 1H NMR (400 MHz, $DMSO-d_6$) δ ppm 2.00 (s, 3 H), 3.78 (d, $J=15.5$ Hz, 1H), 3.80 (s, 3H), 4.96 (s, 2 H), 5.15 (d, $J=15.3$ Hz, 1 H), 5.73 (d, $J=1.96$ Hz, 1 H), 6.05-6.08 (m, 2 H), 6.86-6.90 (m, 2 H), 7.01 - 7.11 (m, 2 H), 7.19 - 7.36 (m, 6 H), 7.41-7.48 (m, 4 H) 9.77 (s, 1 H), 12.26 (s, 1 H); HPLC: $t_R = 6.19$ min, $UV_{254} = 96\%$; HRMS (ESI): m/z calcd for $C_{31}H_{29}N_3O_5$ $[M+1]^+$ 524.2185, found 524.2184.

3 Results

3.1 Summary of Screening Results

72,030 compounds were screened in the primary assay¹⁴. Some active compounds were chosen for confirmation according to the scheme shown in **Fig. 4**.

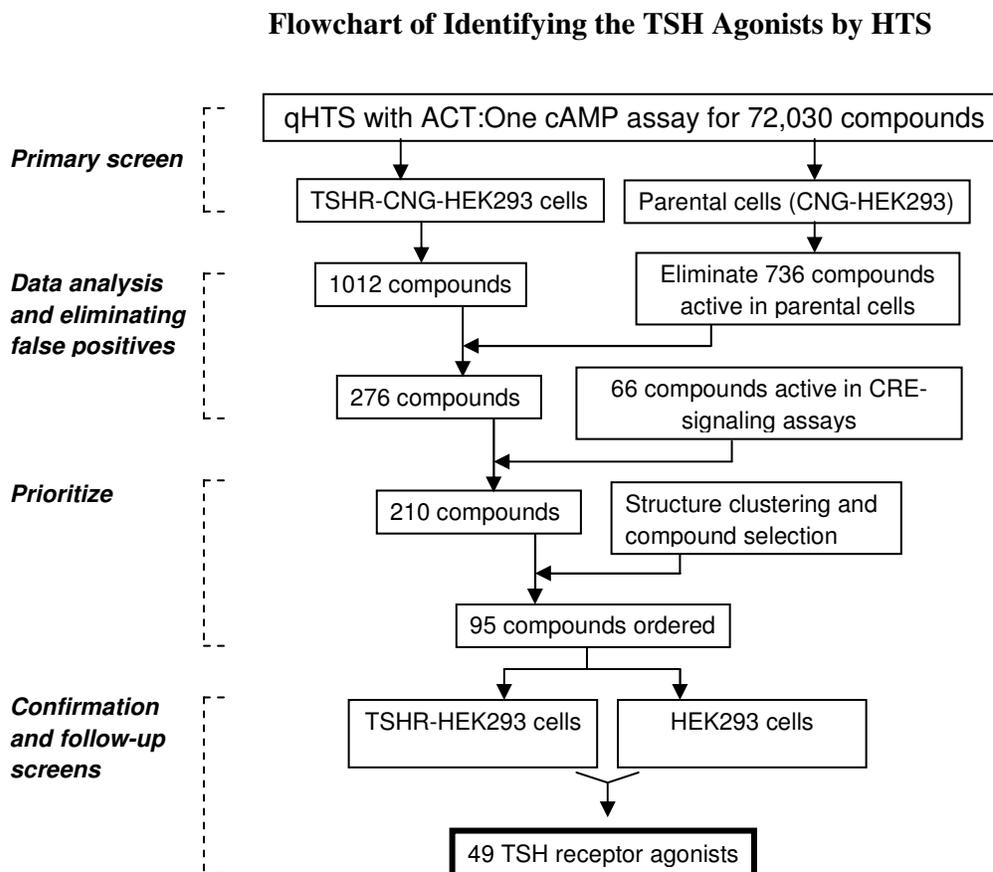


Figure 4. High-throughput screen

3.2 Dose Response Curves for Probe

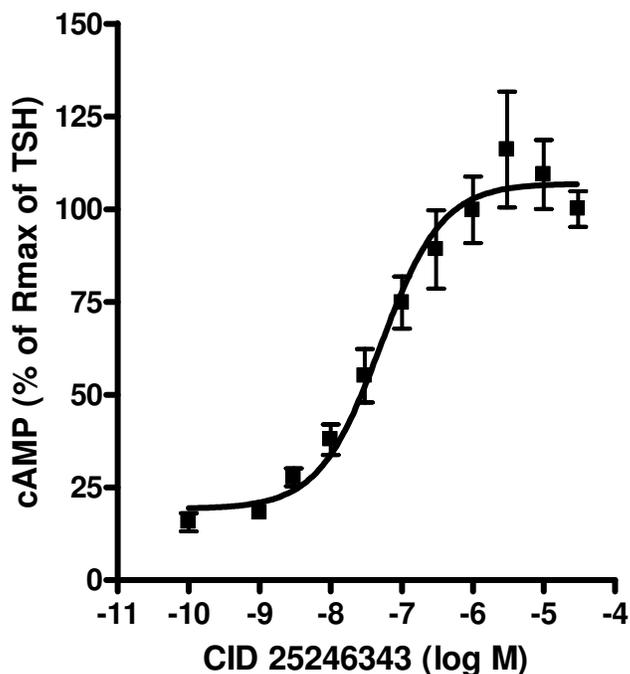


Figure 5. Activity of MLS002576689 in the ELISA cAMP assay

3.3 Scaffold/Moiety Chemical Liabilities

A potential site for undesired chemical reactivity in the probe would be the aminal, which could undergo oxidation or hydrolysis to the amine and aldehyde. We found experimentally that the rate of oxidation is dependent on the aryl ring substitution. The 5-hydroxy substituted probe molecule is particularly resistant to oxidation. It was dissolved in acetonitrile and oxygen was bubbled through the solution, but no oxidation was witnessed, even after several hours. In fact, the probe compound only underwent oxidation when treated with MnO_2 . The other chemical concern for this scaffold would be hydrolysis. The hydrolytic stability of this scaffold was monitored at neutral and basic pH and the $t_{1/2}$ was 16 h. The stability, not surprisingly, suffered at low pH and the $t_{1/2}$ was 3 h¹.

3.4 SAR Tables

*Table 7. Structure-Activity relationship study**

No	SID	CID	R1	R2	R3	R4	R5	R6	X	Y	EC50	Emax
1	26755506	661788	H	H		OMe	H	NHAc	NH	O	1.16	101
2	29216393	16759579	H	H	Bn	OMe	H	NHAc	NH	O	0.29	99.8
3	29216998	17757110	H	H	n-Bu	OMe	H	NHAc	NH	O	0.83	112
4	29218134	17757294	H	H		OMe	H	NHAc	NH	O	3.98	70.3
5	29216997	17757109	H	H	H	OMe	H	NHAc	NH	O	n.d.	39.5
6	29218154	17757314	H	H	Bn	OMe	H	NHAc	N	O	0.96	97.4
7	57655046	25246367	H	OMe	Bn	OMe	H	NHAc	NH	O	4.48	49.1
8	26755506	25246343	H	OH	Bn	OMe	H	NHAc	NH	O	0.09	100
9	29218135	17757295	H	H		OMe	H	NHAc	NH	O	n.d.	10.1
10	29216999	17757111	H	H		OMe	H	NHAc	NH	O	n.d.	19.7
11	29218136	17757296	H	H		OMe	H	NHAc	NH	O	0.43	102.5
12	103073331	49789101	OH	H	Bn	OMe	H	NHAc	NH	O	0.33	93.3
13	103073332	49789102	H	H	Bn	H	H	NHAc	NH	O	1.86	36.8
14	103073333	49789103	H	F	Bn	OMe	H	NHAc	NH	O	0.28	94.7
15	29216969	2887852	H	H		OMe	H	NHAc	NH	O	5.09	75.1
16	29218141	17757301	H	H	Bn	OMe	H	OMe	NH	O	2.16	46.3
17	103073337	49789106	H	H	Bn	OMe	H	H	NH	O	2.57	25.6
18	103073338	49789107	H	H	Bn	OMe	NHAc	H	NH	O	4.34	24.8
19	103073339	46190126	H	H	Bn	OMe	H	NHAc	NH	S	0.08	95.7
20	29216989	17757102	H	H		OMe	H	NHAc	N	O	0.93	59
21	103073340	49789108	H	OH	Bn	OMe	-	-	NH	-	n.d.	17.7
22	103073341	49789109	H	NH2	Bn	OMe	H	NHAc	NH	O	3.78	72.6
23	103073342	49789110	H	NHMs	Bn	OMe	H	NHAc	NH	O	0.98	90.8
24	29218140	17757300	H	H	Bn	OMe	H	CN	NH	O	n.d.	11.7
25	103073343	49789111	H	H	Bn	OMe	F	H	NH	O	n.d.	7.4
26	103073344	49789112	H	OH	Bn	OMe	H	NHAc	NH	S	0.018	69.6
27	103073345	49789113	H	OH	Bn	OMe	H	NHAc	N	O	0.55	98.6

* All analogs were synthesized. Activities (EC50) in μM were obtained from the Elisa assay (AID 2104). E_{max} is expressed as % of the maximal response of CID 25246343/ML109, set at 100%.

3.5 Cellular Activity

Both primary and secondary assays are cell-based assays.

3.6 Profiling Assays

Table 8. Probe (CID25246343/ML109) ADME properties

Study	Activity/Result	Activity/Result 2
Turbidometric aqueous solubility	EPR = 2.0 μM	
Microsomal stability	Clint = 195	$t_{1/2}$ = 7.1 min
Bi-directional Caco-2 permeability	P_{app} = 38.6 (A-B)	P_{app} = 11.8 (B-A)
Pgp substrate identification	Efflux Ratio = 0.25	
5-isoform/substrate CYP inhibition	>25 μM	
Plasma protein binding	fu = 0.14	

In vitro ADME studies were performed on the probe molecule (Table 8). This data indicates that the probe precipitates out of solution at 2 μM , and exhibits a high rate of clearance and good absorption. Additionally, the probe does not inhibit CYP, is modestly affected by the Pgp inhibitor.

Table 9. Summary of data in PubChem:

PubChem AID	Type	Target	Conc. Range	Samples Tested
926	Primary DR	TSHR cell line	0.5nM-46µM	72030
938	Primary DR counterscreen	Parental cell line	0.5nM-46µM	72030
939	Counterscreen	TSHR cell line	0.5nM-46µM	151
933	Counterscreen	TSHR cell line HTRF	0.5nM-46µM	346
953	Counterscreen	Parental cell line	0.5nM-46µM	151
1403	Counterscreen	FSHR cell line HTRF	0.5nM-46µM	29
1402	Counterscreen	LHR cell line HTRF	0.5nM-46µM	31
2104	Secondary	TSHR cell line	0.5nM-46µM	35
1401	Summary	N/A	N/A	N/A

4 Discussion

Among the confirmed hits, NCGC00168126 (CID 661788) was the most selective for TSH receptor, with no detectable agonist activity at the closely related LHCGR or FSHR, and was therefore selected for chemistry optimization. We explored substitution patterns throughout the scaffold. The analogs were first evaluated using the HTRF cAMP assay (AID 933). However, due to increased satisfaction with the consistency of results from the ELISA assay (AID 2104), the ELISA assay was used to guide our SAR study. As shown in **Table 7**, when R1 was either an OH or H (entries 12 and 2), there was no substantial change in activity. However, the R2 substitution influences activity. OH improved activity (entry 8), but NH₂, NHMs and OMe (entries, 22, 23, and 7) all resulted in a loss of activity. Furyl, benzyl, butyl and phenethyl groups were tolerated at R3 (entries 1, 2, 3 and 11), but pyridyl, tertiary amine, or an alcohol (entries 4, 9 and 10) resulted in a loss of activity. This indicates that lipophilic groups are well tolerated at R3, but polar groups that could behave as either hydrogen bond donors or acceptors are detrimental to activity. When R3 was an H (entry 5), activity was completely lost. The removal of the OMe moiety at R4 resulted in an inactive compound (entry 13). Activity was lost when R5 was a functionality other than H (entries 18 and 25), or when R6 was a functionality other than NHAc (entries 16, 17, 18, 24 and 25). The R5 and R6 containing aryl ring is crucial for activity and very sensitive to substitution patterns. This was further demonstrated in the inactive analog where this entire ring was removed (entry 21). Y was oxygen in most analogs tested, but the activity improved when Y was sulfur (entries 19 and 26). Most analogs tested were with X as NH, but several oxidized compounds (X = N) were tested (entries 6, 20, and 27). The oxidized compounds were less active than the un-oxidized counterparts, but remain interesting analogs due to the improved air and acid stability. *In vivo* studies (**Figure 6**) showed that CID 25246343/ML109 could increase secretion of T4 and thyroidal iodide uptake in mice after administration by esophageal gavage, suggesting CID 25246343/ML109 is an orally available small molecule that can stimulate thyroid gland function¹.

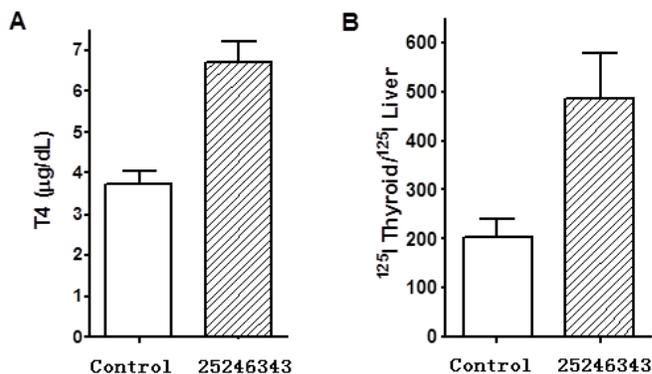


Figure 6. Stimulation by CID 25246343/ML109 of thyroxine (T4) secretion and thyroidal radioiodide uptake in mice. **A:** Vehicle (PEG 300, control) or CID 25246343/ML109 (2.5 mg) was given by esophageal gavage and serum T4 was measured 2 hr later. **B:** Vehicle (control) or CID 25246343/ML109 (2.5 mg) was given by esophageal gavage on days 1 and 2. On the morning of the 3rd day, Na¹²⁵I (20 µCi) was administered by esophageal gavage and the mice were sacrificed 2 hr later. The thyroid glands and small pieces of liver were excised and counted for ¹²⁵I radioactivity. The data are presented as the ratio of radioactivity in the thyroid/liver.

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

The probe is the first orally active TSHR agonist. The probe series represents the most potent and selective small molecule TSHR agonists reported to date.

4.2 Mechanism of Action Studies

There have been several studies to validate the probe's exact binding site on TSHR. It was predicted to bind to the serpentine domain of TSHR, which is different from the amino terminal ectodomain binding site of endogenous TSH. This was validated by testing its binding to a TSHR mutant with the amino-terminal ectodomain deleted (KFLR). KFLR is not activated by TSH, but the probe molecule did activate KFLR, albeit with 12% lower efficacy and lower potency (EC50 = 1.7 µM). The binding was then modeled and N5.47 was predicted to be critical for activity, and a site specific mutant validated this prediction¹ (Figure 7).

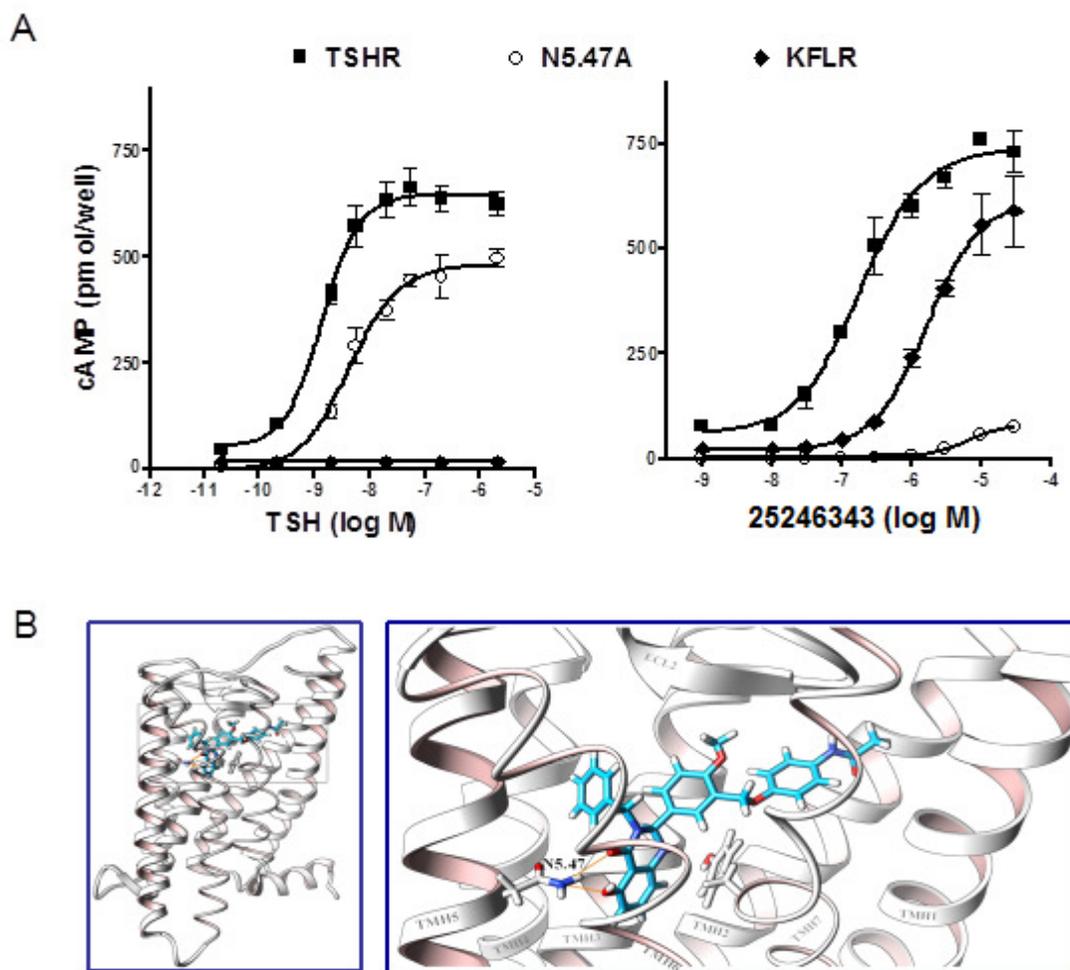


Figure 7. CID 25246343/ML109 activates TSHR by binding to the transmembrane helical bundle. **A** The effects of CID 25246343/ML109 or TSH on cAMP accumulation in cells expressing TSHR, TSHR-KFLR in which the large amino-terminal ectodomain to which TSH binds, is deleted, or N5.47A in which Asn at position-5.47 was mutated to Ala. **B** Docking of CID 25246343/ML109 into the homology model of TSHR predicts CID 25246343/ML109 binds within the transmembrane helical bundle (left panel). Enlargement of the boxed region in the left panel (right panel) shows an interaction of CID 25246343/ML109 with an Asn in TMH5 (N5.47).

4.3 Planned Future Studies

The oxidized compounds present an attractive scaffold for further optimization due to the improved air and acid stability over the probe molecule.

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