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Quantitative High-Throughput Screening Using a Live-Cell cAMP Assay Identifies Small-Molecule Agonists of the TSH Receptor

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The thyroid-stimulating hormone (TSH; thyrotropin) receptor belongs to the glycoprotein hormone receptor subfamily of 7-transmembrane spanning receptors. TSH receptor (TSHR) is expressed mainly in thyroid follicular cells and is activated by TSH, which regulates the growth and function of thyroid follicular cells. Recombinant TSH is used in diagnostic screens for thyroid cancer, especially in patients after thyroid cancer surgery. Currently, no selective small-molecule agonists of the TSHR are available. To screen for novel TSHR agonists, the authors miniaturized a commercially available cell-based cyclic adenosine 3′,5′ monophosphate (cAMP) assay into a 1536-well plate format. This assay uses an HEK293 cell line stably transfected with the TSHR coupled to a cyclic nucleotide gated ion channel as a biosensor. From a quantitative high-throughput screen of 73,180 compounds in parallel with a parental cell line (without the TSHR), 276 primary active compounds were identified. The activities of the selected active compounds were further confirmed in an orthogonal homogeneous time-resolved fluorescence cAMP-based assay. Forty-nine compounds in several structural classes have been confirmed as the small-molecule TSHR agonists that will serve as a starting point for chemical optimization and studies of thyroid physiology in health and disease. (*Journal of Biomolecular Screening* 2008:120-127)

Key words: thyroid-stimulating hormone, TSH, TSHR, TSHR agonist, quantitative high-throughput screening, qHTS, HTS, probe identification, CNG, PubChem

INTRODUCTION

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, luteinizing hormone, and follicle-stimulating hormone.[1] The actions of TSH are mediated by a 7-transmembrane receptor, which, upon TSH binding, couples preferentially to the G-α (s) protein (Gs), resulting in activation of adenylate cyclase and an increase in cyclic adenosine 3′,5′ monophosphate (cAMP).[1] The TSH receptor (THSR) is mainly expressed in thyroid follicular cells[2] and regulates their growth and function.[3] Recombinant TSH is used to activate TSHR in patients with thyroid cancer receiving thyroid hormone suppression therapy and to screen for residual tumor after surgery,[4] but it is expensive and must be administered intramuscularly. An orally active small-molecule TSHR agonist would serve as an invaluable research tool for studying TSHR pharmacology and physiology and would have multiple advantages in therapeutic settings. However, no selective small-molecule agonist of the TSHR exists, and no small-molecule screen for TSHR agonists has ever been reported.

Several assays have been used to measure the interaction between TSH and TSHR, but none have been ideal for identifying TSHR agonists via high-throughput screening (HTS). The traditional radiolabeled TSHR binding assay[5,6] is sensitive but does not measure direct activation of the receptor. Measurement of the amount of intracellular cAMP generated upon the activation of the TSHR more directly reflects activity at the receptor; however, cAMP radioimmunoassays[7] and enzyme-linked immunosorbent assays[8] require multiple assay steps and washes, making them nontransferable to HTS. Indirect TSHR activation readouts, such as by the use of cAMP response element (CRE) reporter-gene

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assays or downstream gene expression measurements,[9] are more amenable to HTS, but because they report activity at any point along the extended TSHR signaling pathway, including gene transcription and protein synthesis, they may produce large numbers of actives, which require time-consuming deconvolution to identify TSHR agonists.

Recently, several more HTS-amenable cAMP assays have been developed and used to screen Gs–coupled G protein–coupled receptors (GPCRs), including fluorescence polarization,[10] homogeneous time-resolved fluorescence (HTRF),[11] enzyme fragmentation complementation,[12] and cyclic nucleotide gated ion channel (CNG)–coupled[13] approaches. In CNG-coupled cAMP assays, the cAMP signal is amplified and measured in live cells by a change in membrane potential monitored by a membrane potential dye. Here, we describe the use of a CNG-coupled cAMP assay in a quantitative HTS (qHTS) of a 73,180-member compound library and the resulting identification of several structural classes of small-molecule TSHR agonists.

MATERIALS AND METHODS

Materials

TSH, RO 20-1724, and forskolin were purchased from Sigma-Aldrich (St. Louis, MO). The membrane potential dye kit was purchased from BD Biosciences (Rockville, MD). The TSHR transfected HEK293 cells coexpressed with modified CNG channels and their parental cells (without the receptor) were obtained from BD Biosciences (named as ACT:One cells). The cell culture medium (DMEM) was purchased from Invitrogen (Carlsbad, CA), and fetal calf serum (FCS) was from HyClone (Logan, UT).

Cell culture and frozen cell preparation

Both TSHR transfected and parental HEK293 cells (expressing a modified CNG channel) were maintained in DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 µg/mL geneticin (Invitrogen) at 37 °C in 5% CO_2. For the TSHR expression cell line, an additional 1 µg/ml puromycin (Invitrogen) was added during the cell culture. The cells were seeded at a density of 3 to 4 million cells in a T175 flask (Corning, Corning, NY) containing 35 ml of media and were allowed to grow for 3 to 4 d to reach 80% to 90% confluence. A flask of HEK293 cells at this density generally yielded 30 million total cells.

CNG channel–coupled cAMP assay

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 d 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 µl of resuspended cells was dispensed into each well of black, clear-bottom, 1536-well tissue culture–treated plates (Greiner Bio-One, Kremsmuenster, Austria) using a Multidrop Combi dispenser (Thermo Fisher Scientific, Waltham, MA). After overnight culture at 37 °C with 5% CO_2, the cells were generally 60% to 70% confluent in the 1536-well plates.

Table 1. Thyroid-Stimulating Hormone (TSHR) Quantitative High-Throughput Screening Protocol

Step	*Parameter*	*Value*	*Description*
1	Reagent	4 µl	1000 TSHR or parental cells/well
2	Incubation time	18-30 h	37 °C, 5% CO_2
3	Reagent	4 µl	Membrane potential dye
4	Incubation time	30 min	At room temperature
5	Compounds	23 nL	Library compounds in 0.128 µM to 10 mM titration series or control
6	Incubation time	30 min	At room temperature
7	Assay readout	Excitation = 535 and emission = 590 nm	Envision plate reader

After overnight incubation, 4 µl of membrane potential dye containing 50 µM 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 23 nl of compound or positive control (30 nM TSH or 10 µM forskolin for TSHR or parental, respectively) in DMSO was added to each well using a pintool (Kalypsys, San Diego, CA), and the plates were further incubated for 30 min at room temperature. Plates were measured in the Envision plate reader with an excitation of 535/20 nm, an emission of 590/20 nm, a gain of 150, and 5 flashes per well on a PerkinElmer plate reader (PerkinElmer, Boston, MA) (**Table 1**).

HTRF cAMP assay

All follow-up compounds were assayed using an HTRF cAMP detection kit (Cisbio, Bedford, MA) on both the TSHR cell line and parental cell line. Briefly, 750 cells were plated in 2.5 µl/well of complete media (DMEM containing 10% FCS) in 1536-well, solid-bottom, white plates, and 23 nl/well compound in DMSO solution or controls was added. Following a 30-min incubation at room temperature, 2.5 µl/well of labeled d2 cAMP and 2.5 µl/well of anti-cAMP antibody (both diluted 1:20 in lysis buffer) were added to each well using a flying reagent dispenser (Aurora Discovery, San Diego, CA). Plates were measured using the Envision plate reader (PerkinElmer), with excitation at 330 nm and emissions of 615 nm and 660 nm.

Compound library preparation and qHTS

A library of 73,180 structurally diverse compounds was serially diluted 1:5 or 1:2.236 in DMSO in 384-well plates to yield 7 (from 0.64 μM to 10 mM) or 15 concentrations (from 0.128 μM to 10 mM) and formatted into 1536-well plates at 7 μl/well. A qHTS was performed as described previously[14] using a fully automated robotic screening system (Kalypsys). Final compound concentrations during cell incubation ranged from 0.36 nM to 28.8 μM.

Data analysis

Primary screen data were analyzed with customized software developed internally. The maximal response (100% activity) was determined by the response of 30 nM TSH, and the basal response (0% activity) was measured by the DMSO control in the TSH screen. In the counterscreen with the parental cell line, 10 μM forskolin was added for the maximal response. The concentration responses of all the compounds were analyzed using methods described by Inglese and others,[14] and structural clustering of active compounds was performed using Leadscope Hosted Client (Leadscope Inc., Columbus, OH). The EC_{50} values of compounds in the confirmation and follow-up experiments were calculated from the concentration-response curves by nonlinear regression analysis using Prism software (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Assay optimization and miniaturization

The assay used here allowed us to measure elevation in intracellular cAMP produced by TSHR activation in live cells. This HEK293 cell line stably expresses both the TSHR and an CNG cation channel[13,15] (**Fig. 1**). Activation of the TSHR by an agonist results in Gs-adenylate cyclase coupling and an increase in intracellular cAMP, which binds to and activates the CNG channel. CNG channel activation produces an influx of Ca^{2+} and Na^{+} cations and subsequent membrane depolarization, which can be measured using a calcium-sensitive dye in a kinetic fluorescence plate reader or even more conveniently using a membrane potential dye[16] in a standard fluorescence plate reader.

The original assay was developed in a 96-well plate format. To adapt it to a 1536-well plate format, the number of cells per well was first examined in the presence of TSH stimulation. The signal-to-basal ratio was similar, with cell densities from 500 to 3000 cells/well; 1000 cells/well was selected because it had the highest signal-to-basal ratio after a 60-min incubation period (**Fig. 2a**). Time-course experiments showed that the signal-to-basal ratio was 2.67- and 2.92-fold at 30 and 60 min after addition of the agonist, respectively (**Fig. 2b**). Thus, an incubation time of 30 min after agonist addition was chosen for

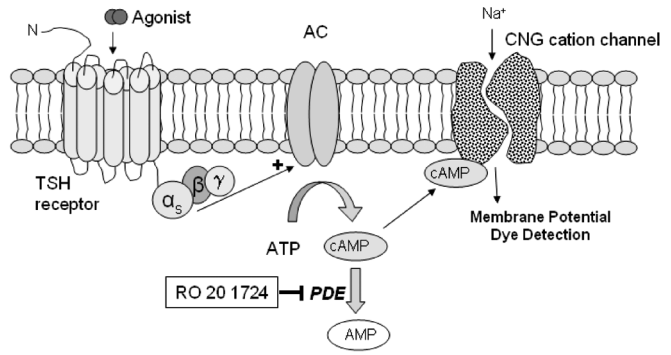

FIG. 1. Schematic diagram of the detection mechanism of the ACT:One cyclic adenosine 3′,5′ monophosphate (cAMP) assay (BD Biosciences, Rockville, MD). The thyroid-stimulating hormone (TSH) receptor was cotransfected in the HEK293 cells with a modified cyclic nucleotide gated ion (CNG) channel, which serves as a biosensor to detect changes in the intracellular cAMP level. TSH stimulates the TSH receptor, which increases the intracellular cAMP level, resulting in the activation of CNG cation channels. The membrane depolarization occurs after the influx of cations such as sodium and calcium, which can be detected by the membrane potential dye in the live cells.

the assay considering the convenience for HTS and sensitivity of the compound screen. DMSO was also tested, and it was found to have no effect on the assay at concentrations less than 1% (v/v); the final DMSO concentration in the assay wells in the screen was 0.25%.

With the assay conditions defined as 1000 cells/well and a 30-min incubation after agonist addition, concentration response to the native agonist TSH was tested in a 1536-well plate format. The EC_{50} value of TSH was 1.40 nM (95% confidence interval [CI = 2.48-4.62 nM]) in a 1536-well format compared with the EC_{50} value of 3.89 nM measured in a 96-well format; **Fig. 3**), showing that the potency of TSH was similar in both assay formats.

Assay parameters were then determined in the miniaturized 1536-well assay format using a DMSO plate. The signal-to-basal ratio was 2.5-fold, and the coefficient of variation (CV) and Z′ factor were 6.3% and 0.83, respectively (**Fig. 4**), measured in a fluorescence plate reader. These results indicated that the assay's performance was adequate for qHTS and that a kinetic reader was not needed for this cAMP assay.

Quantitative high-throughput screen

A total of 73,180 compounds were screened using a fully automated screening system in the qHTS format.[14] All the compounds were assayed at 7 or 15 concentrations, ranging from 0.36 nM to 28.8 μM. The response to the control agonists (TSH or forskolin) was measured in each plate and was stable across the entire assay (data not shown).

Freshly prepared cells were dispensed into 1536-well assay plates at 4 μl/well at a rate of 100 plates per hour and incubated

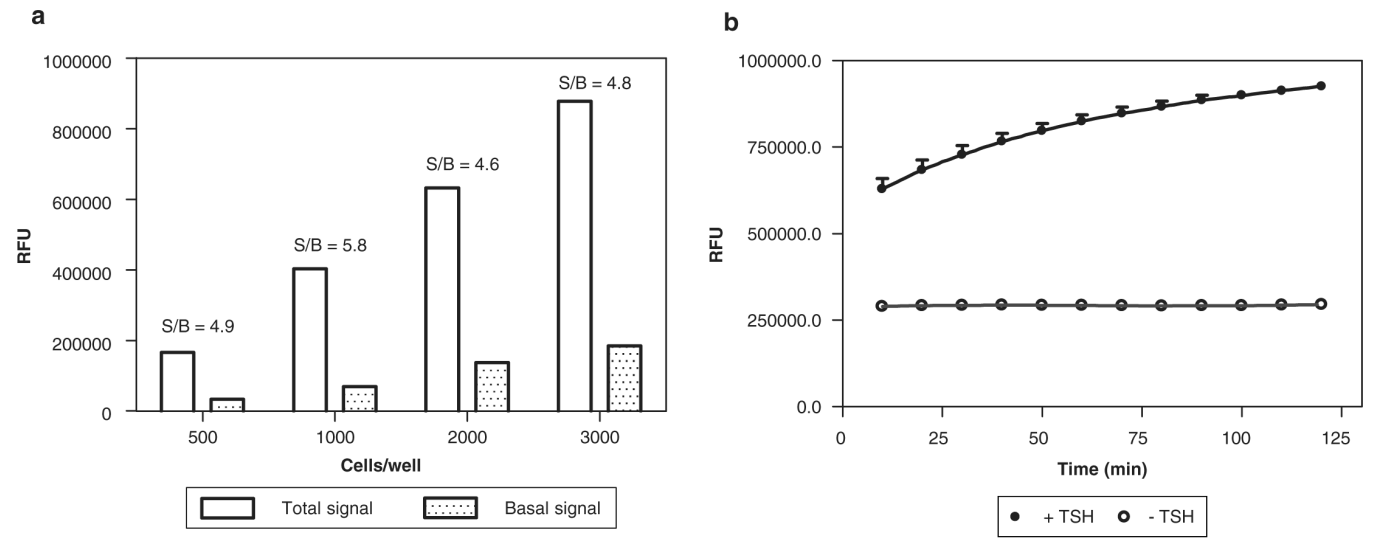

FIG. 2. The effects of cell density and agonist incubation time on the cyclic adenosine 3′,5′ monophosphate (cAMP) assay. (**a**) Cell number titration in the presence and absence of 30 nM thyroid-stimulating hormone (TSH). Cells were plated at 500, 1000, 2000, and 3000 cells/well in a 1536-well plate 24 h before stimulation with 30 nM TSH for 60 min. (**b**) Time course of the agonist incubation time. Cells were seeded at 1000 cells/well in 1536-well plates and stimulated 24 h later with 30 nM TSH and compared with cells without TSH stimulation. The fluorescence signals of the membrane potential dye were measured every 10 min from 10 to 120 min after the agonist addition.

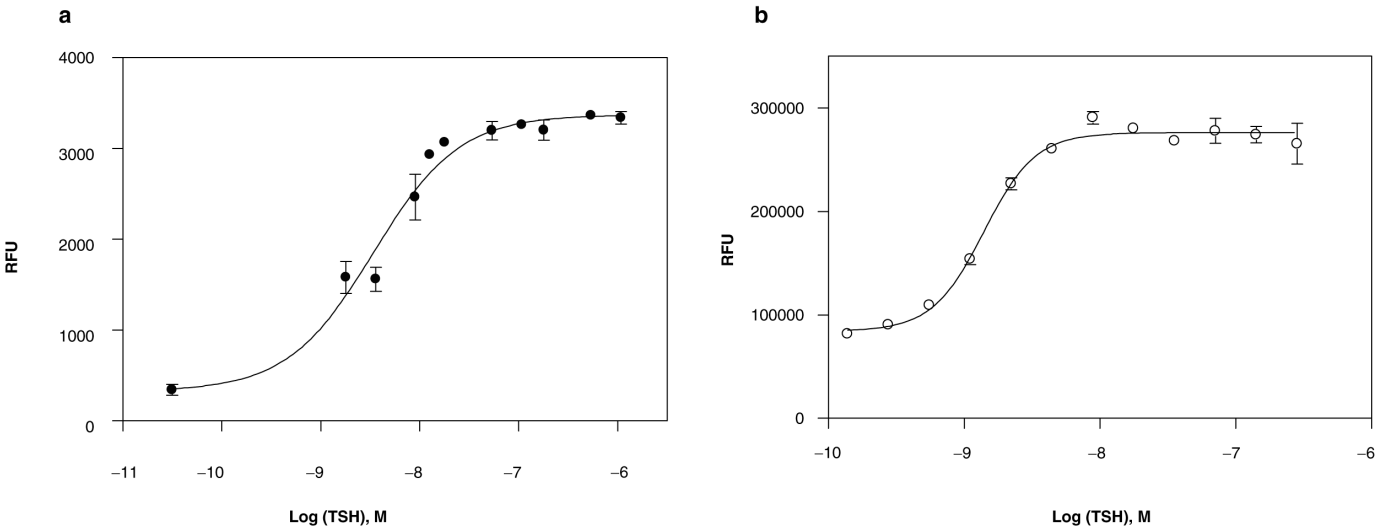

FIG. 3. Concentration response of thyroid-stimulating hormone (TSH) determined from the cyclic nucleotide gated ion (CNG)–coupled cyclic adenosine 3′,5′ monophosphate (cAMP) assay. (**a**) TSH response determined in a 96-well format. In the 96-well plate assay, 60,000 cells/well were plated and incubated for 24 h before the measurement. TSH EC_{50} was 3.38 nM, and the 95% confidence interval (CI) was 2.48 to 4.62 nM. (**b**) TSH response determined in a 1536-well format. In the 1536-well plate assay, 1000 cells/well were plated in a 1536-well plate and incubated for 24 h before the cAMP assay. TSH EC_{50} was 1.90 nM, and the 95% CI was 1.19 to 1.62 nM. Different plate readers and gain settings were used in the 96- and 1536-well plate assays, accounting for the observed differences in relative fluorescence units (RFUs) in the 2 formats.

at 37 °C with 5% CO_2 for 20 to 36 h before the robotic screening. The screen was initiated by the addition of 4 µl/well membrane potential dye, followed by a 60-min incubation at room temperature to allow for dye equilibration across the cell membrane. Then, 23 nl/well compound or control was added to the assay plates, which were incubated for an additional 30 min before reading a fluorescence plate reader in bottom-read mode for fluorescence intensity (emission at 590 nm). Both TSHR and parental (lacking TSHR) lines were screened across 463 compound plates (for a total of 926 plates). The average

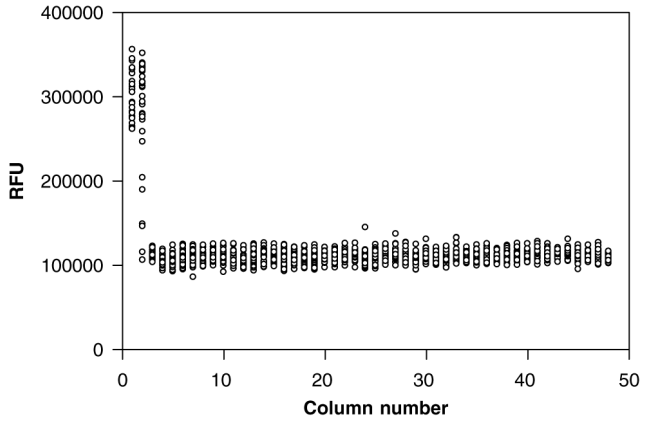

FIG. 4. Scatter plot of the results from a 1536-well DMSO plate to establish assay performance parameters. Cells were seeded at a density of 1000 cells/well. Columns 1 to 4 were controls: 30 nM thyroid-stimulating hormone (TSH) was added to the wells in column 1 to establish a maximum signal, wells in column 2 received a TSH titration ranging from 0.1 nM to 1 μM in duplicate, columns 3 and 4 received DMSO as the negative control, and DMSO was added to columns 5 to 48. The resulting signal-to-basal ratio was 2.5, the coefficient of variation was 6.3%, and the Z′ factor was 0.83.

signal-to-basal ratio for the entire screen was 2.01, the CV was 12.1%, and Z′ value was 0.4 (**Fig. 5**). Plates with a Z′ factor less than 0.2 (approximately 5% of the total plates) were rescreened. The relatively low Z′ was likely due to the use of HEK293 cells, which typically do not adhere well after treatment with membrane potential dyes.[17] In addition, although only 1 pipette tip was used to dispense cells and reagents into assay plates for the DMSO-only test, 8 pipette tips were used simultaneously to rapidly dispense cells and reagents in the robotic screen; dispensing variation among the 8 tips likely contributed to the difference in the Z′ factor between the DMSO plate test and the qHTS. However, the qHTS Z′ factors of between 0.2 and 0.5 were acceptable for HTS,[18] particularly when performed in titration-response qHTS mode, which can produce reliable data in the presence of relatively low Z′ values.[14]

qHTS data analysis

Because the screen was run in qHTS mode, concentration-response curves, potencies, efficacies, and structure-activity relationship data on all compounds were available immediately after the primary screen. Active compound series and singletons were initially denoted on the basis of high-confidence curve class (1.1, 1.2, and 2.1; details discussed by Inglese and others[14]) and compound potency (<10 μM). A total of 1012 compounds (1.4% of screened compounds) were designated as provisional actives from the primary screen.

Elimination of assay false positives

Large numbers of false positives are commonly found in cell-based phenotypic and cell-signaling assays, and considerable time and effort are frequently required to identify the compounds acting via the desired mechanism (in this case, the TSHR agonism). Although qHTS data are relatively refractory to many sources of false positives, we wished to eliminate other assay-related false positives before moving to the next stages of follow-up. These would include compounds that directly activated the CNG channel or cAMP cascade (e.g., adenylate cyclase, G proteins, or other endogenous GPCRs expressed by HEK293 cells), fluorescent compounds, or noncompound assay fluorescence due to dust or lint. We were able to rapidly eliminate compounds with undesired mechanisms using a progressive series of experimental and computational tests (**Fig. 6**).

We first examined the activity of the 1012 compounds identified in the TSHR qHTS in the parental cell line, which expresses the CNG channel without the TSH receptor; 736 compounds were found to have activity that was not dependent

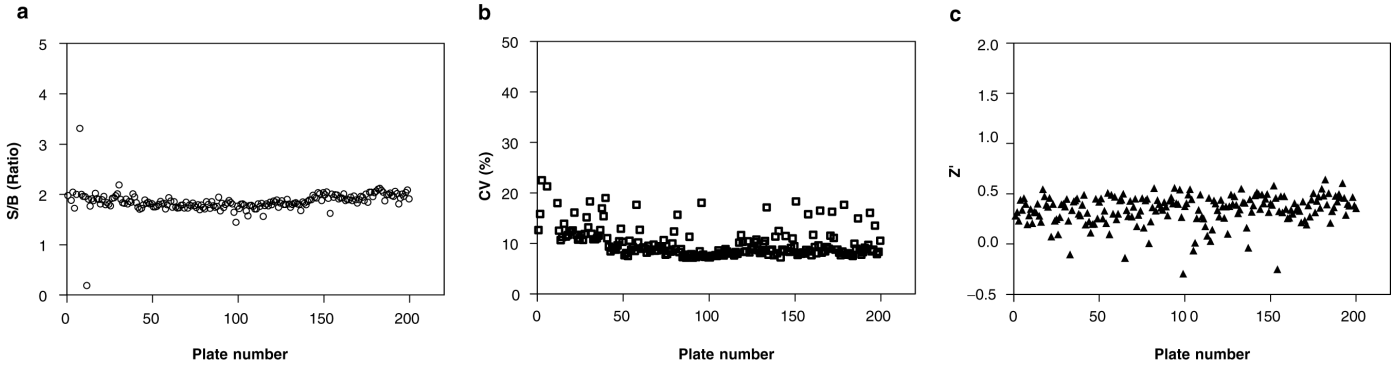

FIG. 5. High-throughput screening performance of 200 plates. (**a**) Signal-to-basal ratio. The total signals were obtained from the wells in columns 1 and 2 (with 30 nM thyroid-stimulating hormone [TSH]), and the basal signals were collected from the wells in column 4, which received DMSO. (**b**) Coefficient of variation (CV), calculated as the standard deviation from the wells in column 4 divided by the standard deviation from the wells in columns 1 and 2. (**c**) Z′ factor, calculated by $Z' = 1 - (3 \times \text{SD of total signal} + 3 \times \text{SD of basal signal})/(\text{total signal} - \text{basal signal})$.[18]

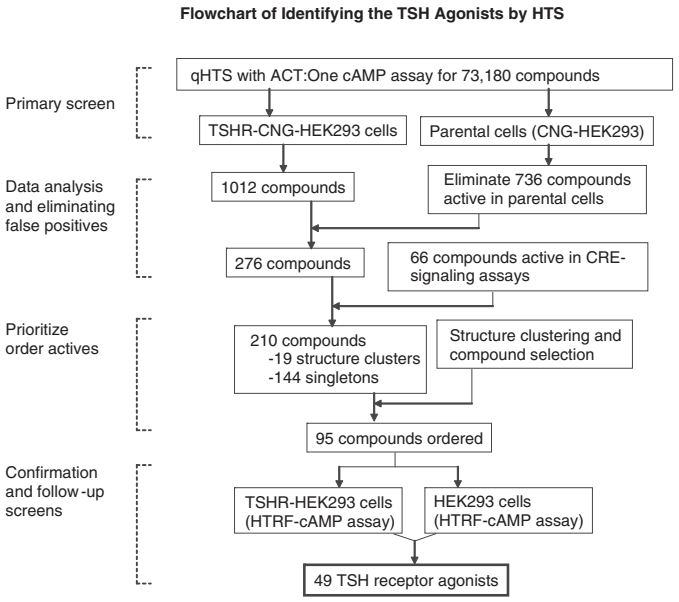

FIG. 6. Flowchart for rapid triaging of primary hits to identify the thyroid-stimulating hormone (TSH) agonists. The process of TSH receptor (TSHR) agonist discovery included the quantitative high-throughput screening (qHTS), false-positive elimination, active compound selection, and confirmation. For details, see the text. cAMP = cyclic adenosine 3′,5′ monophosphate; CNG = cyclic nucleotide gated ion channel; CRE = cAMP response element; HTRF = homogeneous time-resolved fluorescence.

on TSHR (i.e., similar activity was seen in both cell lines) and were eliminated. A further 66 compounds showed activity in a CRE-signaling pathway assay previously screened in our laboratory (Xia et al., unpublished data; PubChem Assay ID 662), indicating direct activity on the cAMP cascade, and were also eliminated. This left 210 active compounds (0.29% of the screened compounds), which comprised 19 structural classes and 144 singletons. Of these, 95 compounds representing each of the scaffolds and the most potent, efficacious singletons were selected for confirmation and further studies. The primary screening results have been deposited into PubChem (AID 926, http://pubchem.ncbi.nlm.nih.gov/).

TSH agonist confirmation

The 95 selected compounds were tested for TSHR agonism in an alternate-readout HTRF cAMP assay, which measures cAMP concentrations in cell lysates using a cAMP antibody and the time-resolved fluorescence resonance energy transfer (TR-FRET) detection technology. 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 HTRF cAMP assay. These 49 compounds were then tested in the parental cell line (without TSHR) in the same HTRF cAMP assay and showed no activity (data not shown). Taken together, these results indicate that these 49 compounds are true small-molecule TSHR agonists. **Figure 7** shows 4 representative compounds of these confirmed small-molecule TSH agonists. Studies further characterizing these compounds in physiological and disease-relevant systems are currently in progress.

The obvious advantages of the cAMP assay with a CNG cation channel as biosensor are 1) unlike the other cAMP determination methods, the changes of cAMP level in response to the activation of Gs-/Gi-coupled GPCRs are measured in living cells, which allows screening of agonists, allosteric modulators, and antagonists in the same assay plate; 2) high sensitivity due to the amplified signal by the CNG channels enables the cAMP measurement with endogenous GPCRs; and 3) potential coupling to high-content analysis and multiplexing assays. The disadvantages include additional false positives due to the activation/inhibition of the CNG channels, need of cotransfection with the CNG channels, and moderate well-to-well variation due to the unattachment of HEK293 cells after the treatment with membrane potential dye. The TR-FRET–based cAMP assays including HTRF and LANCE produce fewer false positives and have better CV and Z′ factor numbers because of the ratio metric measurement. But both assays may be less sensitive in some cell lines in which the GPRCs are expressed in low levels (such as the endogenous receptors). In addition, the agonist and antagonist screenings cannot be done in the same plate because the cells are lysed when the cAMP is measured.

In conclusion, we have optimized a cAMP assay using a CNG cation channel as a biosensor in HEK293 cells expressing the TSHR and miniaturized it to a 1536-well plate format. This assay was used to screen a library of 73,180 compounds in qHTS format. A total of 210 compounds in 19 structural classes, representing an active rate of 0.29%, were identified as true actives after excluding a large number of primary screen actives by experimental and computational counterscreening. TSHR agonist activity was confirmed in 49 of 95 tested compounds comprising several medicinally attractive structural classes in an orthogonal TSHR cAMP assay using a different detection technology. These compounds, the 1st small-molecule TSHR agonists identified by HTS, will serve as chemical probes for further studies of TSHR pharmacology and as an important starting point in medicinal chemistry optimization to produce TSHR agonists for in vivo use.

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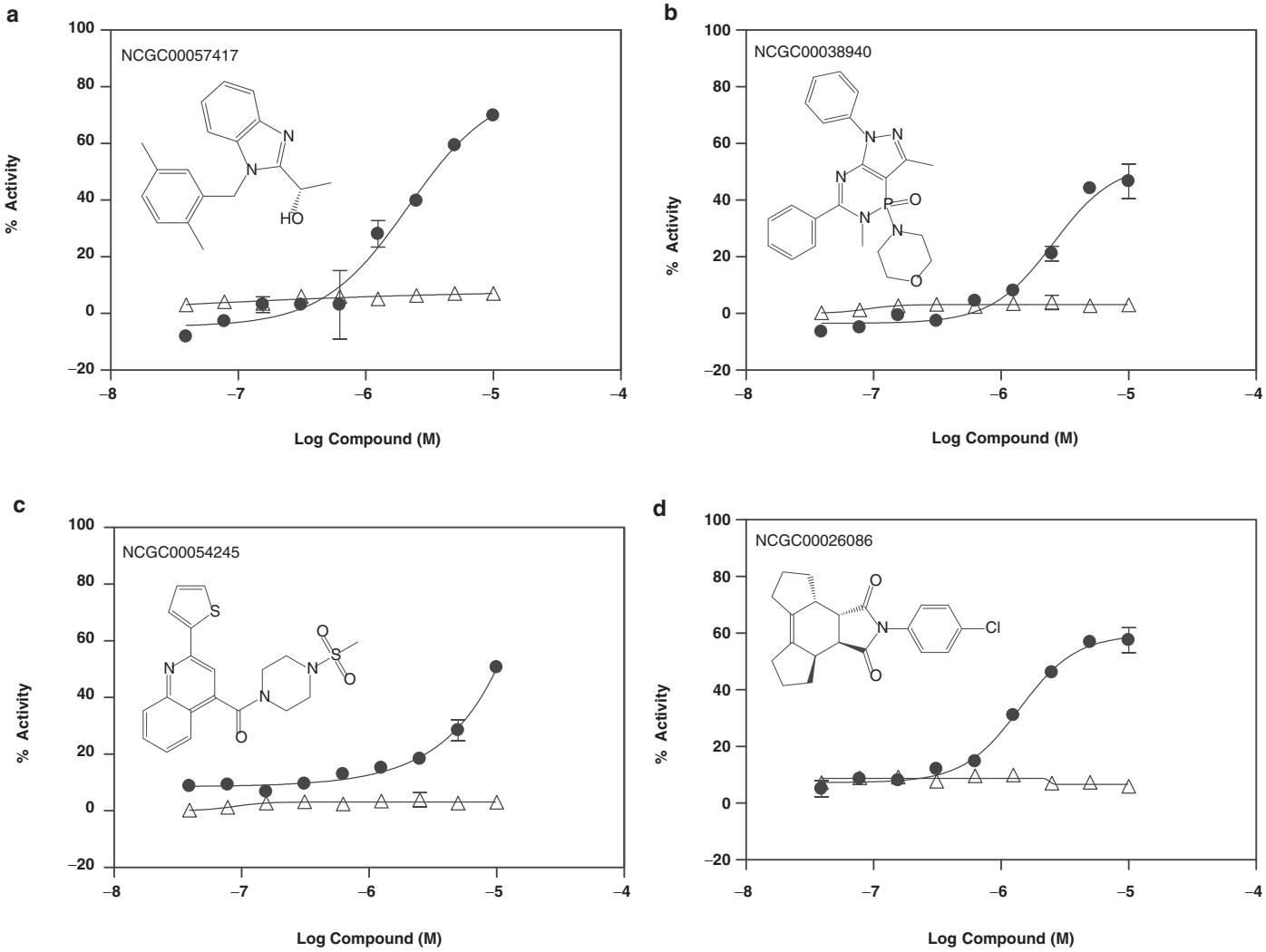

FIG. 7. Dose-response curves and structures of 4 representative thyroid-stimulating hormone (TSH) receptor agonists. Activities of compounds were normalized to the maximal response of TSH (30 nM) and the DMSO control in the TSH receptor cell line (closed circles) and the parental cell line (open triangles). All 4 compounds were inactive in the parental cell line (without the TSH receptor). (**a**) NCGC00057417 with an EC_{50} of 2.15 μM and 69.8% maximal response at a concentration of 10 μM. (**b**) NCGC00038940 with an EC_{50} of 2.57 μM and 46.6% maximal response at a concentration of 10 μM. (**c**) NCGC00054245 with an EC_{50} of 10.1 μM and 50.6% maximal response at a concentration of 10 μM. (**d**) NCGC00026086 with an EC_{50} of 1.70 μM and 54.5% maximal response at a concentration of 10 μM.

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