

Development of a High-Throughput Screening-Compatible Assay for Discovery of GPR3 Inverse Agonists Using a cAMP Biosensor

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Abstract

While G-protein-coupled receptors (GPCRs) represent the largest class of cell surface proteins, there are ≥ 100 orphan GPCRs whose endogenous ligands are unknown. Accordingly, these could prove to be potential therapeutic targets for the pharmaceutical intervention of various diseases. Constitutively active orphan GPCRs are activated without ligands; thus, inverse agonists may be very useful pharmacological tools for inhibiting constitutive activity. However, in general, inverse agonist screening is considered more difficult to perform with high quality than antagonist screening, particularly due to the narrow assay window. We developed a high-throughput screening (HTS)-compatible assay to identify inverse agonists of GPR3. GPR3 is expressed in the central nervous system (CNS) and is known to be related to Alzheimer's disease and other CNS diseases. The GPR3 inducible cell line was established using T-REx 293 cells that stably expressed the tetracycline repressor protein, and the cAMP biosensor, GloSensor, was stably co-expressed. After optimization of the induction level of GPR3 and assay conditions, the GloSensor assay showed an approximately 20-fold signal-to-background ratio and high sensitivity. Using the HTS method, we successfully screened a library of hundreds of thousands of compounds for the inhibition of constitutive activity with good quality and excellent reproducibility. Finally, 35 compounds were identified as GPR3 selective inverse agonists. This inverse agonist screening approach using GloSensor in combination with the inducible expression of orphan GPCR indicates universal applicability to the search for inverse agonists of constitutively active orphan GPCRs.

Keywords

HTS, inverse agonist, constitutively active orphan GPCR, GloSensor, GPR3

Introduction

G-protein-coupled receptors (GPCRs) represent a large membrane protein family, encoding as many as 800 genes in the human genome.^{1,2} GPCRs regulate a wide variety of physiological functions, including those related to vision, heart rate, neurotransmission, and the immune system, through the binding of each ligand.³ Indeed, the targets of approximately 30% of all Food and Drug Administration (FDA)-approved drugs involve the GPCR family, highlighting the fact that GPCRs are attractive targets for drug discovery.⁴ Therefore, it is important to facilitate the functional analysis of those GPCRs that remain uncharacterized.

Traditionally, both academia and the pharmaceutical industry have attempted to screen natural or synthetic ligands for GPCRs, followed by the characterization of the receptors pharmacologically using these ligands. Nevertheless, approximately 150 GPCRs are still considered orphan receptors

whose endogenous ligands have not yet been identified.⁵ On the other hand, Costa and Herz have proposed the concept of constitutively active GPCRs by demonstrating that delta

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opioid receptors have an intrinsic activity in the absence of an agonist.⁶ Some orphan GPCRs may exert their physiological function through constitutive activity without any endogenous ligand present. Recently, it has been demonstrated that GPR21, a constitutively active orphan GPCR, contributes to the development of insulin resistance and energy homeostasis.⁷ This may be one example of a (patho)physiological function of an orphan GPCR without the presence of an endogenous ligand. Furthermore, it has been reported that increased constitutive activity caused by the overexpression or mutation of constitutively active GPCRs is associated with various pathologies. In fact, mutations linked to increased constitutive activity in the rhodopsin gene and in the thyrotropin receptor gene are known to cause retinitis pigmentosa⁸ and hyperthyroidism,⁹ respectively.

Inverse agonists, but not neutral antagonists, can inhibit the agonist-independent activities of these constitutively active GPCRs. For example, histamine H₂ receptor ligands, cimetidine, ranitidine, and famotidine have been used as therapeutic drugs for duodenal or gastric ulcers and are categorized as neutral antagonists.¹⁰ However, several studies have reported that the histamine H₂ receptor possesses constitutive activity, and these compounds have currently been recognized as inverse agonists.^{11,12} These inverse agonists can convert constitutively active receptors into an inactive state.¹³ Thus, inverse agonists may be beneficial tools for the detailed (patho)physiological analyses of constitutively active orphan GPCRs where there is very little known regarding their function. Ultimately, inverse agonists may have the potential to be therapeutic drugs.

Screening for inverse agonists that inhibit the function of constitutively active GPCRs is typically performed without agonists, whereas agonists are necessary for the screening of antagonists that inhibit GPCR responses elicited by agonists.¹⁴ In general, inverse agonist screening is more difficult to carry out with high quality than antagonist screening since constitutive activity in the absence of an agonist is often lower than the agonist response. Therefore, the utilization of artificially mutated receptors is one strategy to augment the constitutive activity for screening.¹⁴ However, the compounds obtained from the screening using artificially mutated GPCRs may not necessarily exhibit inverse agonist activity in native GPCRs. Thus, it is important to evaluate constitutive activity with high sensitivity in native GPCRs. GloSensor is a novel technology based on a luciferase-based biosensor that can detect real-time cAMP levels in live cells with high sensitivity.¹⁵ Therefore, this methodology may be suitable for the screening of inverse agonists that inhibit constitutive activity. However, earlier studies on screening using GloSensor were limited to small-scale screening.^{16–18} To our knowledge, no comprehensive study has been described regarding a large-scale screening of inverse agonists.

Transfection with small amounts of GPR3 expression plasmid DNA elicited a significant increase in intracellular cAMP levels.¹⁹ Furthermore, knockdown of endogenous GPR3 inhibited its functions, including neurite outgrowth in neurons.²⁰ Thus, GPR3 has been recognized as a constitutively active Gs-coupled orphan GPCR that results in elevated intracellular cAMP levels in a wide variety of cells;^{19–21} however, we cannot completely exclude the possibility that unidentified endogenous agonists activate GPR3. GPR3 is highly expressed in the central nervous system (CNS), particularly in the hippocampus, hypothalamus, cerebral cortex, and cerebellum.^{20,22} Recent studies have suggested the involvement of GPR3 in neurological disorders, such as Alzheimer's disease,²³ anxiety, and depression, based on studies using GPR3 knockout mice.²⁴ In particular, GPR3 inverse agonists may prove to be therapeutic drugs for Alzheimer's disease since GPR3 facilitates amyloid-beta production. However, very few GPR3 selective inverse agonists have been reported.²⁵

In this report, we demonstrated that the GloSensor assay is applicable for a large-scale HTS campaign to identify inverse agonists of GPR3. The receptor activity of constitutively active GPCRs is correlated with its gene expression. Thus, in this study, an inducible expression system was utilized to develop and verify the assay systems for the GPR3 GloSensor assay and the counterassay. To our knowledge, this is the first comprehensive report of a large-scale HTS campaign for inverse agonists toward constitutively active orphan GPCRs using GloSensor in combination with an inducible expression system. Moreover, multiple GPR3 inverse agonists were identified through this HTS campaign.

Materials and Methods

Cells

T-REx 293 cells stably expressing the tetracycline repressor protein (Thermo Fisher Scientific, Waltham, MA) were used to develop a stable cell line expressing human GPR3 (T-REx-293/hGPR3). The pT-REx-DEST30/hGPR3 plasmid was constructed by recombining hGPR3 complementary DNA with an amino-terminal HA tag cloned in the pENTR/D-TOPO vector into the pT-REx-DEST30 (mammalian expression vector with the cytomegalovirus (CMV) promoter/enhancer and a tetracycline repressor operator sequence) using LR clonase II (Thermo Fisher Scientific). T-REx 293 cells were then transfected with the pT-REx-DEST30/hGPR3 plasmid using Lipofectamine 2000 reagent (Thermo Fisher Scientific) and further selected in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, 5 µg/mL blasticidin S, and

250 $\mu\text{g/mL}$ G-418 at 37 °C with 5% CO_2 in a humidified atmosphere. Cell clones resistant to G-418 were isolated and selected by their ability to induce hGPR3 expression following the addition of tetracycline. The selected cell line, T-REx-293/hGPR3, was then transfected with the pGloSensor-22F cAMP plasmid (Promega Corporation, Fitchburg, WI) and cultured in the above-described medium supplemented with 300 $\mu\text{g/mL}$ hygromycin B. Resistant clones were isolated and selected based on luminescence increased by 3-isobutyl-1-methylxanthine (IBMX) in the GloSensor assay following tetracycline addition. The selected clone was referred to as T-REx-293/hGPR3-GS22F.

HEK 293 cells (American Type Culture Collection, Manassas, VA) were used to develop a stable cell line expressing the human melanocortin 4 receptor (hMC4R) and GloSensor 20F (HEK293/hMC4R-GS20F). The pcDNA3.1(+)-hMC4R plasmid was constructed by ligating hMC4R complementary DNA into the multiple cloning sites of the mammalian expression vector pcDNA3.1(+) (Thermo Fisher Scientific). HEK 293 cells were transfected with the pcDNA3.1(+)-hMC4R and pGloSensor-20F cAMP plasmids (Promega Corporation), transfected using Lipofectamine 2000 reagent, and selected in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, 500 $\mu\text{g/mL}$ G-418, and 300 $\mu\text{g/mL}$ hygromycin B at 37 °C with 5% CO_2 in a humidified atmosphere. Resistant clones were isolated and selected by constitutive activity and responsiveness to an hMC4R inverse agonist, agouti-related protein (AGRP; Peptide Institute Inc., Osaka, Japan), in the GloSensor assay. The selected clone was referred to as HEK293/hMC4R-GS20F.

In the present study, cryopreserved cell stocks were prepared for the HTS campaign. T-REx293/hGPR3-GS22F cells were pretreated overnight with 0.3 $\mu\text{g/mL}$ tetracycline and collected using phosphate-buffered saline (PBS)-based Cell Dissociation Buffer (Thermo Fisher Scientific). Collected cells were suspended in a serum-free cell cryopreservation medium, CELLBANKER 2 (Nippon Zenyaku Kogyo Co. Ltd., Koriyama, Japan) and stored at -80°C for the hGPR3 GloSensor assay. T-REx293/hGPR3-GS22F cells without pretreatment of tetracycline and HEK293/hMC4R-GS20F cells were cryopreserved in the same manner for the counterassay and the hMC4R GloSensor assay, respectively.

GloSensor cAMP Assay Using Cultured Cells

T-REx-293/hGPR3-GS22F cells were seeded at 5000 cells/well in a 384-well white-well/clear-bottom plate (Corning Inc., Corning, NY) and cultured overnight in the presence of 0.1 $\mu\text{g/mL}$ tetracycline unless stated otherwise, at 37 °C with 5% CO_2 in a humidified atmosphere, in order to induce hGPR3 expression. The culture media were then removed

and replaced with 20 μL of CO_2 -independent medium (Thermo Fisher Scientific) containing 10% fetal bovine serum and 2% GloSensor cAMP Stock Reagent (Promega Corporation). The cells were then incubated for 2–3 h at room temperature under light-shielded conditions. The luminescence of each well was determined at 30 min after the addition of the test compound solution (5 μL) with a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA) unless stated otherwise.

GloSensor cAMP Assay Using Cryopreserved Cells

Cryopreserved cells were thawed at 37 °C and suspended in CO_2 -independent medium containing 10% fetal bovine serum and 2% GloSensor cAMP Stock Reagent. The cells were dispensed at 5000 cells/20 μL /well in white, tissue-cultured, 384-well plates with MultiDrop Combi (Thermo Fisher Scientific). Cells were incubated for more than 3 h at room temperature under light-shielded conditions. The luminescence of each well was determined at 30 min after the addition of the test compound solution (5 μL) using an EnVision 2104 Multimode Plate Reader (PerkinElmer, Waltham, MA) unless stated otherwise.

HTS Campaign

Primary screening for human GPR3 inverse agonists was conducted using cryopreserved stocks of T-REx-293/hGPR3-GS22F pretreated with 0.3 $\mu\text{g/mL}$ tetracycline, as described above. Briefly, 5 μL of the compound solution was added to 20 μL of cell suspension using EDR-384UX (Biotech Co. Ltd., Tokyo, Japan). We evaluated the inhibitory effect of a test compound at 10 μM on the constitutive activity of hGPR3 in the presence of 0.5 mM IBMX. Luminescence was measured at 60 min after the addition of the compound solution using an EnVision 2104 Multimode Plate Reader. The inverse agonist activity of each test compound was expressed as inhibitory activity toward the cAMP response (percent inhibition). Luminescent values in the presence and absence of 0.5 mM IBMX were defined as 0% and 100% inhibition, respectively, in the GloSensor assay for hGPR3. The cryopreserved stocks of T-REx-293/hGPR3-GS22F without pretreatment of tetracycline were used for the counterassay. We evaluated the inhibitory effect of a test compound at 10 μM on the cAMP response stimulated by 0.5 μM forskolin in T-REx-293/hGPR3-GS22F without induction of hGPR3 expression in order to exclude nonspecific compounds. Luminescent values in the presence of 0.5 mM IBMX with 0.5 μM forskolin and 0.5 mM IBMX without forskolin were defined as 0% and 100% inhibition, respectively, in the counterassay.

Concentration–response curves for both hGPR3 and hMC4R were generated using the cryopreserved stocks of

T-REx-293/hGPR3-GS22F and HEK293/hMC4R-GS20F with different concentrations of test compounds in the same manner as the primary screen. Luminescent values in the presence of 0.5 mM IBMX and 0.5 mM IBMX with 1 μ M AGRP, which has been reported as a full inverse agonist for hMC4R,^{26,27} were defined as 0% and 100% inhibition, respectively, in the GloSensor assay for hMC4R.

Homogeneous Time-Resolved Fluorescence cAMP Assay Using Cultured Cells

T-REx-293/hGPR3-GS22F cells were seeded and cultured overnight in the presence of 0.1 μ g/mL tetracycline in the same manner as those for the GloSensor assay using cultured cells. cAMP levels were determined with a cAMP HiRange Kit (Cisbio Bioassays, Codolet, France) according to the manufacturer's protocol. Briefly, the culture media were replaced with Hanks' Balanced Salt Solution containing 20 mM HEPES (pH 7.4), cAMP-d2, and test compound, and incubated at room temperature for 30 min. Lysis buffer containing anti-cAMP cryptate was added to each well and incubated for 1 h. The homogeneous time-resolved fluorescence (HTRF) ratio was measured for fluorescence emission at 616 nm for the donor and at 665 nm for the acceptor using a SpectraMax Paradigm Multi-Mode Microplate Reader.

Data Analyses and Statistics

Data analyses were performed using GraphPad Prism (version 8.1.1; GraphPad Software, San Diego, CA) and TIBCO Spotfire Analyst (version 7.11.1; TIBCO, Palo Alto, CA). Concentration–response curves and standard curves for cAMP were fitted using nonlinear regression analysis using the following equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\log EC_{50} - X) * \text{Hill Slope})}) \quad (1)$$

The Z' factor,²⁸ the index for assay quality assessment in assay development and optimization, was determined using the following equation:

$$Z' = 1 - (3\sigma_{C+} + 3\sigma_{C-}) / |\mu_{C+} - \mu_{C-}| \quad (2)$$

where μ_{C+} and μ_{C-} are the means of positive control signal (0.5 mM IBMX) and negative control signal (DMSO), respectively, and σ_{C+} and σ_{C-} are the standard deviations of the corresponding signals. For the counterassay, positive and negative controls are defined as 0.5 mM IBMX with 0.5 μ M forskolin and 0.5 mM IBMX without forskolin, respectively.

Results

GPR3 is known as a constitutively active Gs-coupled orphan GPCR. Thus, the expression of GPR3 without an agonist could stimulate adenylate cyclase via Gs, resulting in increased intracellular cAMP levels.^{19–21} T-REx-293/hGPR3-GS22F is a cell line stably expressing GloSensor 22F and it inducibly expresses hGPR3 under the control of the CMV promoter/enhancer with a tetracycline repressor operator sequence. This cell line was used to evaluate the usefulness of the GloSensor assay in the HTS for GPR3 inverse agonists. Induction of hGPR3 expression in T-REx-293/hGPR3-GS22F cells with pretreatment of tetracycline increased the luminescence in the presence of 1 mM IBMX in the GloSensor assay, suggesting that hGPR3 expression elicits a cAMP response without the presence of an agonist, which is consistent with previous studies (Fig. 1A).^{19–21}

We evaluated the relationship between intracellular cAMP concentrations and luminescence of the GloSensor assay using the conventional HTRF assay. To this end, the GloSensor assay and the HTRF assay were conducted at the same time, and intracellular cAMP levels in the cells were determined in the presence of different concentrations of IBMX. IBMX increased luminescence in the GloSensor assay using T-REx-293/hGPR3-GS22F cells pretreated with 0.1 μ g/mL tetracycline (Fig. 1B). The HTRF assay is based on the competitive immunoassay between endogenous cAMP produced in cells and exogenous cAMP labeled with fluorescent dye. Therefore, an increase in intracellular cAMP levels results in a decreased HTRF ratio. IBMX decreased the HTRF ratio in the HTRF assay, indicating that increased intracellular cAMP levels occurred in the cells, which is consistent with our results obtained from the GloSensor assay (Fig. 1C). The absolute amount of cAMP in each sample was determined by the HTRF assay using a cAMP standard curve. Each luminescence value in the GloSensor assay was plotted against the cAMP amount in cells treated at the corresponding concentrations of IBMX (Fig. 1D). Luminescence in the GloSensor assay showed a linear correlation with the cAMP amount up to 0.698 pmol of cAMP, which was produced in the cells treated with 0.5 mM IBMX. However, luminescence in the GloSensor assay exhibited a saturation at 1.18 pmol of cAMP produced in the cells treated with 1 mM IBMX. The signal-to-background (S/B) ratio in the GloSensor assay was 18.8.

T-REx-293/hGPR3-GS22F cells were cryopreserved after pretreatment with tetracycline. Cryopreserved cells were thawed and used for the GloSensor assay immediately after thawing without cell culture. In the GloSensor assay using cryopreserved T-REx-293/hGPR3-GS22F cells, IBMX increased intracellular cAMP levels in a concentration-dependent manner (Fig. 2A). The EC_{50} value of IBMX was 166 ± 15.1 μ M (mean $\pm$ SEM, $n = 4$), which is

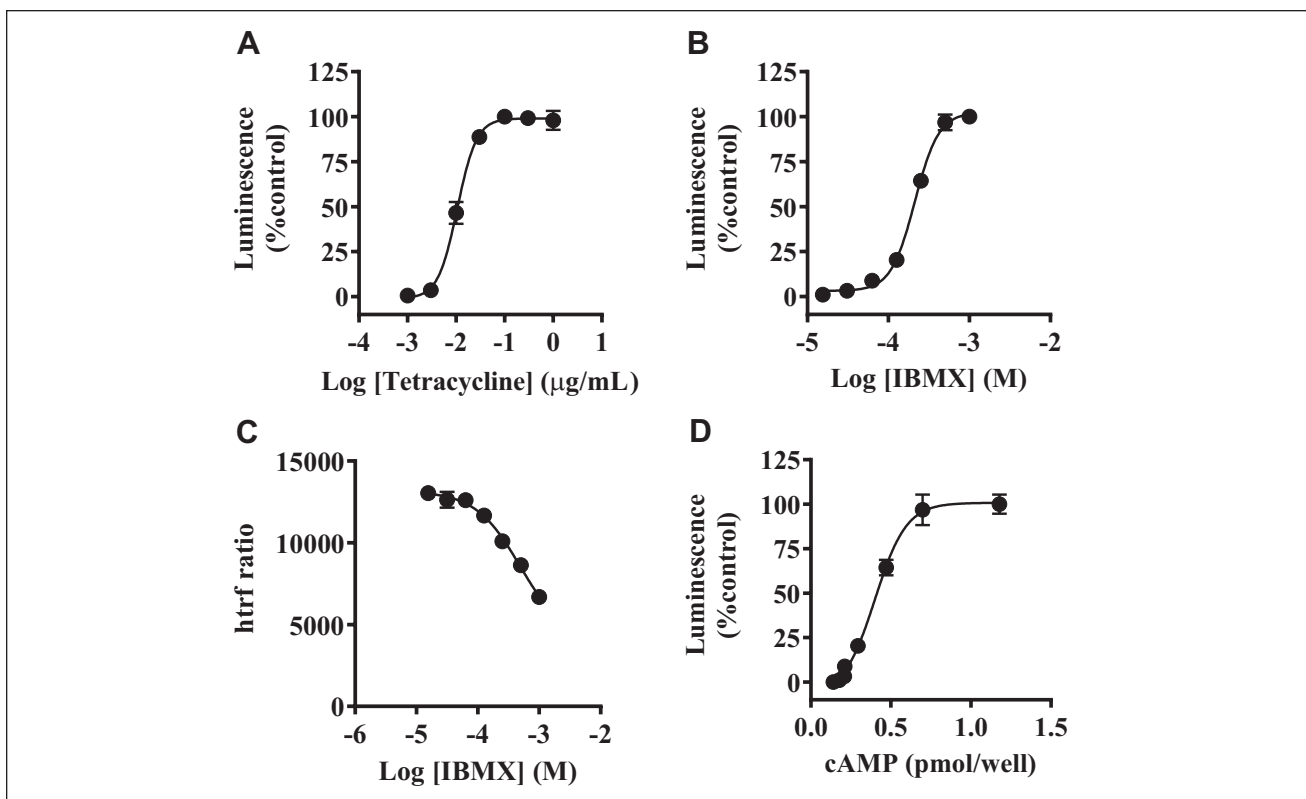


Figure 1. GloSensor cAMP assay using T-REx-293/hGPR3-GS22F. **(A)** T-REx-293/hGPR3-GS22F cells were pretreated overnight with different concentrations of tetracycline to induce hGPR3 expression. Intracellular cAMP levels in the cells were determined in the GloSensor assay. Tetracycline increased intracellular cAMP levels in a concentration-dependent manner in the presence of 1 mM IBMX. The EC_{50} value of tetracycline was $0.011 \pm 0.0025 \mu\text{g/mL}$ ($n = 4$). Results are expressed as a percentage of the response in the cells pretreated with 0.1 $\mu\text{g/mL}$ tetracycline and are means $\pm$ SEM from four individual experiments performed in more than duplicate. Relative luminescence units (RFUs) in the presence and absence of 0.1 $\mu\text{g/mL}$ tetracycline were $63,200 \pm 12,600$ and 2590 ± 1830 , respectively. T-REx-293/hGPR3-GS22F cells were pretreated overnight with 0.1 $\mu\text{g/mL}$ tetracycline. Intracellular cAMP levels in the cells were determined in the presence of different concentrations of IBMX in the GloSensor assay **(B)** and the HTRF assay **(C)** at the same time. IBMX increased luminescence in the GloSensor assay and decreased the HTRF ratio in the HTRF assay, indicating increased intracellular cAMP levels. **(D)** Each luminescence value obtained from the GloSensor assay was plotted against the cAMP amount in the cells treated at the corresponding concentrations of IBMX. Results are expressed as a percentage of the response in the cells treated with 1 mM IBMX. Data are the means $\pm$ SD of a representative experiment performed in quadruplicate out of two independent experiments **(B–D)**. RFUs in the GloSensor assay were $67,600 \pm 3470$ and 3600 ± 2160 in the presence and absence of 1 mM IBMX, respectively **(B,D)**.

consistent with those obtained from cultured cells without cryopreservation (mean 187 μM , $n = 2$). There was no significant effect on the hGPR3 GloSensor assay in the presence of 0.5%–2.5% DMSO (**Suppl. Fig. S1**).

In the present study, we defined GPR3 inverse agonist activity as inhibitory activity of test compounds on the increase in intracellular cAMP levels in the presence of 0.5 mM IBMX in T-REx-293/hGPR3-GS22F cells with the induction of hGPR3 expression. The counterassay was designed to discriminate between compounds with inverse agonist activity toward hGPR3 and nonspecific compounds reducing intracellular cAMP levels regardless of the constitutive activity of hGPR3. For the counterassay, the forskolin-stimulated cAMP response was evaluated in

T-REx-293/hGPR3-GS22F cells without induction of hGPR3 expression in the same assay format. Forskolin in the presence of 0.5 mM IBMX increased intracellular cAMP levels in the GloSensor assay using cryopreserved T-REx-293/hGPR3-GS22F cells without induction of hGPR3 expression (**Fig. 2B**). The EC_{50} value for forskolin was $1.05 \pm 0.174 \mu\text{M}$ (mean $\pm$ SEM, $n = 3$). Luminescent values in the hGPR3 GloSensor assay with 0.5 mM IBMX were equivalent to those in the counterassay with 0.5 μM forskolin (**Fig. 2C**). Therefore, 0.5 μM forskolin was determined as the concentration of forskolin in the counterassay using T-REx-293/hGPR3-GS22F cells without induction of hGPR3 expression. Furthermore, the hMC4R GloSensor assay was also developed in order to evaluate the selectivity

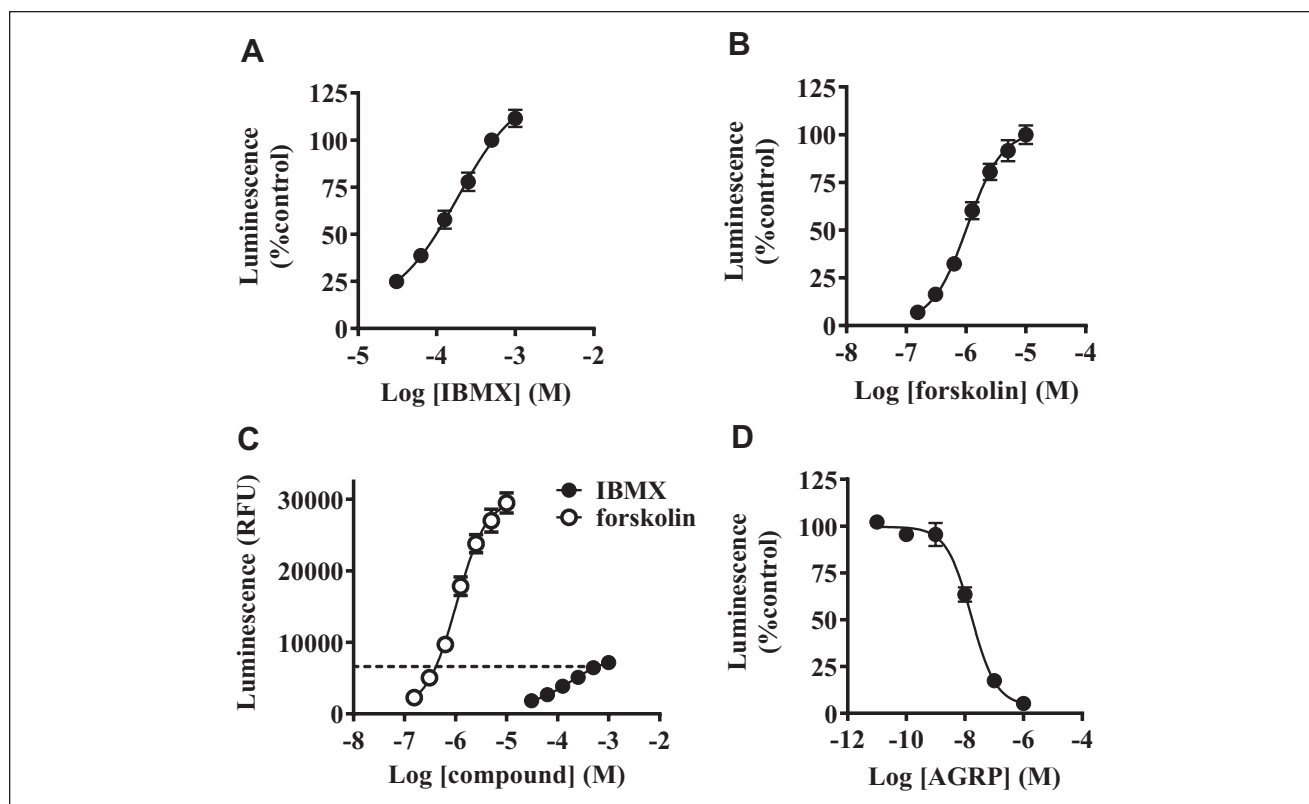


Figure 2. GloSensor cAMP assay using cryopreserved cells. **(A)** T-REx-293/hGPR3-GS22F cells were cultured overnight in the presence of tetracycline and then cryopreserved. The hGPR3 GloSensor assay was conducted using the cryopreserved cells immediately after thawing without cell culture for recovery. The EC_{50} value of IBMX was $166 \pm 15.1 \mu\text{M}$ (mean $\pm$ SEM, $n = 4$). **(B)** T-REx-293/hGPR3-GS22F cells were cultured without tetracycline, and the cryopreserved cells without induction of hGPR3 expression were used for the counterassay. Forskolin with 0.5 mM IBMX was used in the counterassay to increase intracellular cAMP levels instead of hGPR3 expression. The EC_{50} value of forskolin was $1.05 \pm 0.174 \mu\text{M}$ (mean $\pm$ SEM, $n = 3$). Results are expressed as a percentage of the response in the presence of 0.5 mM IBMX **(A)** and $10 \mu\text{M}$ forskolin with 0.5 mM IBMX **(B)**, respectively. **(C)** Luminescent values were compared in the hGPR3 GloSensor assay and the counterassay. The luminescent value in the hGPR3 GloSensor assay with 0.5 mM IBMX was equivalent to that in the counterassay with $0.5 \mu\text{M}$ forskolin (horizontal dotted line). **(D)** Cryopreserved HEK293/hMC4R-GS20F cells were used for the hMC4R GloSensor assay. The EC_{50} value of the MC4R inverse agonist, AGRP, was $10.7 \pm 2.98 \text{ nM}$ (mean $\pm$ SEM, $n = 3$). Data are means $\pm$ SD of a representative experiment performed in quadruplicate. RFUs in the hGPR3 GloSensor assay were 7170 ± 283 and 300 ± 37 (mean $\pm$ SD) in the presence and absence of 1 mM IBMX, respectively. RFUs in the counterassay were $29,500 \pm 1420$ and 265 ± 34 (mean $\pm$ SD) in the presence of 0.5 mM IBMX with $10 \mu\text{M}$ forskolin and without forskolin, respectively. RFUs in the hMC4R GloSensor assay were $17,910 \pm 619$ and 1810 ± 68 (mean $\pm$ SD) in the presence and absence of 0.5 mM IBMX, respectively.

of hit compounds toward hMC4R (**Fig. 2D**). The GloSensor assay using cryopreserved HEK293/hMC4R-GS20F cells exhibited constitutive activity in the presence of 0.5 mM IBMX. The hMC4R inverse agonist, AGRP, fully inhibited constitutive activity with an EC_{50} value of $10.7 \pm 2.98 \text{ nM}$ (mean $\pm$ SEM, $n = 3$).

Luminescence in the hGPR3 GloSensor assay was sequentially monitored until 300 min after the addition of IBMX (**Fig. 3A**). The time course experiment indicated that higher luminescence was observed at 30 min, and it gradually decreased at 60 min, whereas a stable luminescence was obtained even at 300 min after addition of IBMX. Z' factor calculations estimated the statistical significance and

the viability of the assays. Z' factors at 60, 90, and 120 min were higher than those at earlier and later time points, whereas the Z' factor at 300 min was still more than 0.7 (**Fig. 3B**). The percent coefficient of variation (%CV) values at 15 and 30 min were higher than those at later time points, and the values at 60 min and later were also lower (**Fig. 3C**). Thus, we measured luminescence in the GloSensor assay at 60 min after the addition of IBMX in the HTS campaign.

Before the initiation of a whole HTS campaign, we carried out a pilot screen for the 4218 test compounds at final concentrations of $10 \mu\text{M}$ with 0.5% DMSO. In the pilot screen, the hGPR3 GloSensor assay and the counterassay

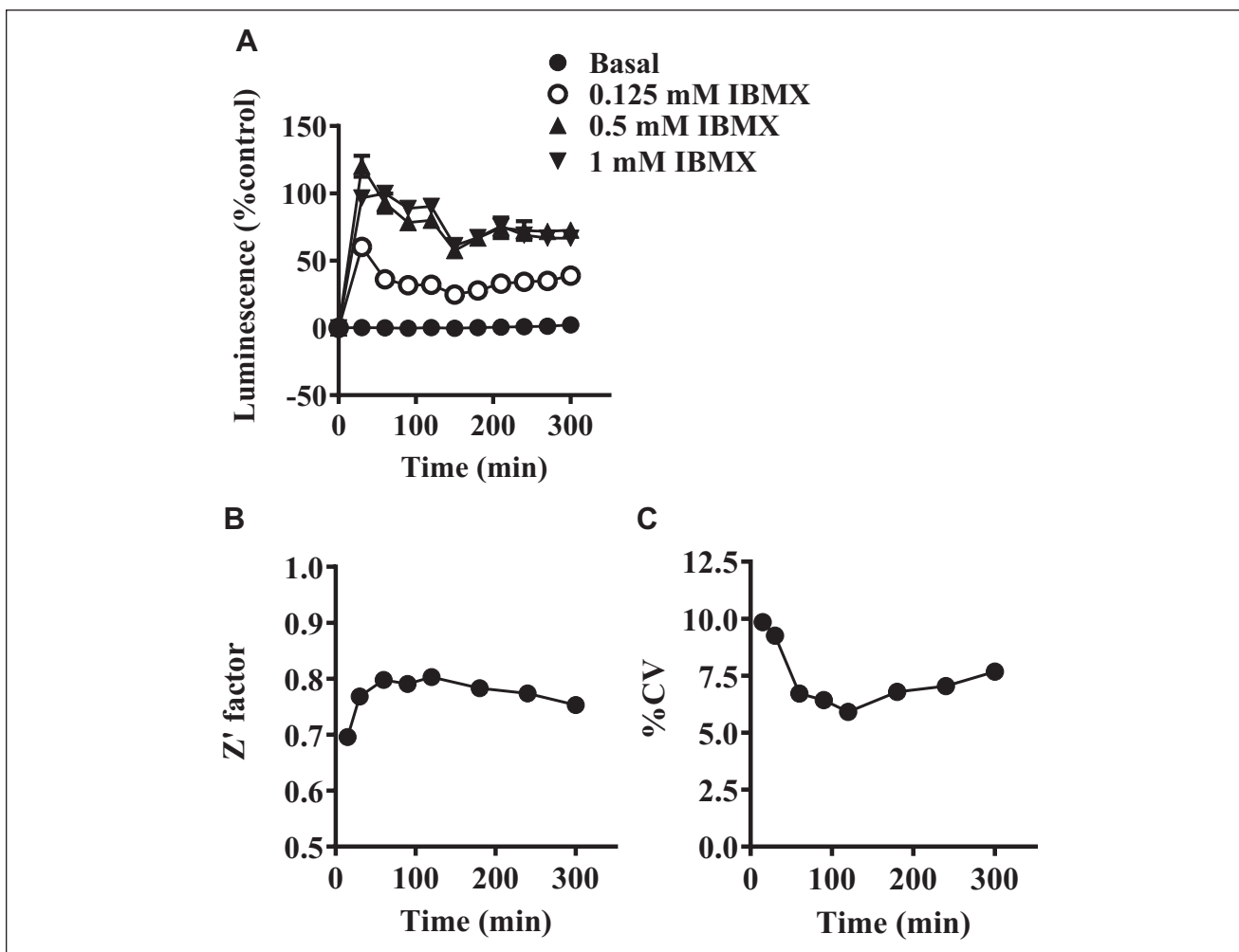


Figure 3. Time course of luminescence in the hGPR3 GloSensor cAMP assay. **(A)** Luminescence was sequentially monitored from 30 to 300 min after addition of IBMX. Results are expressed as a percentage of the response in the presence of 1 mM IBMX at 60 min. Data are means $\pm$ SD of a representative experiment performed in quadruplicate. RFUs were 16022 ± 270 and 307 ± 50 (mean $\pm$ SD) in the presence and absence of 1 mM IBMX, respectively. Z' factors **(B)** and %CV values **(C)** were determined from 15 to 300 min after addition of IBMX. A representative experiment was shown from two independent experiments.

were conducted in triplicate. Z' factors in the hGPR3 GloSensor assay and the counterassay were 0.84 ± 0.04 and 0.85 ± 0.03 (mean $\pm$ SD), respectively. The S/B ratios in the hGPR3 GloSensor assay and the counterassay were 25.0 ± 2.2 and 37.6 ± 2.4 (mean $\pm$ SD), respectively. hGPR3 inverse agonist activity of the tested compounds was expressed as the percent inhibition in the hGPR3 GloSensor assay. The activity of the compounds was calculated as the mean percent inhibition of triplicates in the hGPR3 GloSensor assay and in the counterassay, respectively. The calculated mean + 3 SD of the percent inhibition in the hGPR3 GloSensor assay among all tested compounds was 27.8% (Suppl. Fig. S2A). If a compound with 30% or more inhibition in the hGPR3 GloSensor assay was defined as a hit compound, 76 out of 4218 compounds

were selected through the pilot screen, resulting in a hit rate of 1.8%. Then, the counterassay revealed that only 1 out of the 76 compounds had hGPR3 selective activity (Suppl. Fig. S2B).

In the whole HTS campaign, more than 300,000 compounds were tested at a final concentration of 10 μ M with 0.5% DMSO for the hGPR3 GloSensor assay. About 900 plates were evaluated in 10 days. The Z' factor was 0.84 ± 0.04 (mean $\pm$ SD) with an S/B ratio of 19.7 ± 2.7 (mean $\pm$ SD) (Fig. 4A) through the whole HTS campaign. In the definition for a hit compound the same as the pilot screen, 5124 compounds were selected as hit compounds (Fig. 4B). To confirm the inhibitory activity and exclude any nonspecific compounds, 5124 compounds were cherry-picked and retested in triplicate at 10 μ M. We conducted

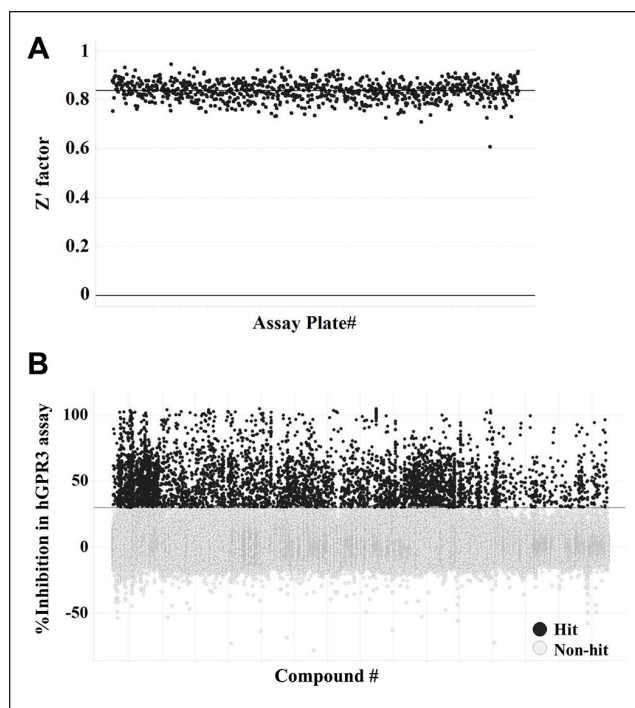


Figure 4. Results of the primary screen. More than 300,000 compounds were screened at the final concentration of 10 μ M using the hGPR3 GloSensor assay. **(A)** The Z' factor was calculated during the primary screen. The mean $\pm$ SD was calculated to be 0.84 ± 0.04 . **(B)** Scatterplot of all compounds tested in the primary screen. The x axis represents compound ID versus the y axis, which corresponds to percent inhibition in the hGPR3 GloSensor assay. Each closed circle represents a hit compound showing more than 30% inhibition in hGPR3 GloSensor assay. Light gray circles represent compounds with less than 30% inhibition in hGPR3 GloSensor assay, suggesting less active compounds. We selected 5124 compounds as hit compounds (closed circle).

the hGPR3 GloSensor assay that was run under the same conditions as the primary screen and the counterassay. The Z' factor and the S/B ratio in the retest of the hGPR3 GloSensor assay were 0.85 ± 0.03 and 17.0 ± 1.1 (mean $\pm$ SD), respectively. The Z' factor and the S/B ratio in the counterassay were 0.81 ± 0.04 and 27.0 ± 1.3 (mean $\pm$ SD), respectively. High reproducibility was observed from comparison of the percent inhibition in the primary screen with that in the retest. There were 4981 compounds (97.2% of all compounds tested in the confirmation assay) with 20% or less variance in the percent inhibition between the primary screen and the retest (**Fig. 5A**). On the other hand, the results of the counterassay indicated that many hit compounds were nonspecific compounds, similar to the results of the pilot screen. Finally, 50 compounds were confirmed as hit compounds. Among them, 48 out of 50 compounds showed more than 40% inhibition in the hGPR3 GloSensor assay and less than 20% inhibition in the counterassay

(**Fig. 5B**). The remaining two compounds exhibited more than 90% inhibition in the hGPR3 GloSensor assay, with 20%–30% inhibition in the counterassay. To evaluate the potency and selectivity of these 50 compounds, we performed concentration–response assays for hGPR3 and hMC4R using the GloSensor assay in the presence of varying concentrations ranging from 0.1 to 30 μ M (**Fig. 6**). Thirty-five compounds out of 50 were identified as hGPR3 selective inverse agonists with EC_{50} values of less than 20 μ M and more than 10-fold selectivity toward hMC4R.

Discussion

cAMP is an important second messenger molecule produced by adenylyl cyclase that is mainly regulated via activation of Gs and Gi proteins.²⁹ Several methods for quantifying intracellular cAMP levels have been developed, including the radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), scintillation proximity assay (SPA), cAMP response element-luciferase reporter assay (CRE-Luc assay), and time-resolved fluorescence resonance energy transfer (TR-FRET) assay, such as HTRF.^{30–35} Among these, RIA has historically been used but needs a radioisotope, and ELISA is a well-established method as an alternative of RIA; however, both are considered low-throughput assays because of the laborious steps involved, such as multiple washes. SPA technology is based on a homogeneous assay without wash steps prior to detection, but this method is relatively expensive for screening a large number of compounds and still requires a radioisotope. The CRE-Luc assay is relatively easy to set up and allows for the conduction of cell-based HTS. However, this assay is characterized by high false-positive hit rates since it can detect compounds interfering with the downstream components of the cAMP pathway and gene expression. Furthermore, longer incubations of the compounds in the assay tend to detect compounds with cellular toxicity as false positives. On the other hand, the features of HTRF include a simple mix-and-measure protocol, and it is a safer nonradioisotope method in comparison with RIA, ELISA, and SPA. In addition, it provides more direct detection of cAMP and shorter compound incubation in comparison with the CRE-Luc assay. For these reasons, a TR-FRET assay, such as HTRF, is currently widely used in HTS.³⁶

GloSensor is a relatively new luciferase-based biosensor that detects intracellular cAMP levels and can easily measure the kinetics of live cells with a wide dynamic range.¹⁵ These characteristics provide many advantages over other methods for measuring intracellular cAMP levels. In fact, we monitored luminescence sequentially until 300 min and were able to easily find an optimum condition for evaluating compounds (**Fig. 3**). For stop time assays, such as HTRF, many wells need to be prepared for each time to

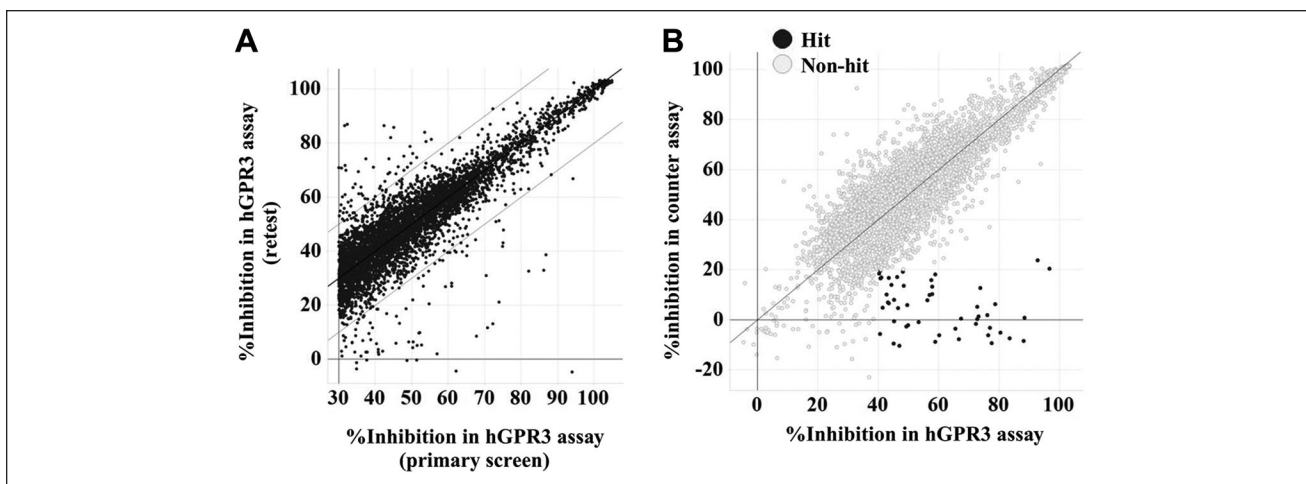


Figure 5. Results of the confirmation assay. Compounds (5124) were tested at a final concentration of 10 μ M using the hGPR3 GloSensor assay and the counterassay. **(A)** Comparison of compound activities between the primary screen and the retest in the hGPR3 GloSensor assay. Almost all compounds show similar percent inhibition in the primary screen and the retest, suggesting good reproducibility. **(B)** Comparison of compound activities between the retest of the hGPR3 GloSensor assay and the counterassay. The x axis and the y axis represent percent inhibition in the hGPR3 GloSensor assay and percent inhibition in the counterassay, respectively. Each closed circle represents a hit compound showing more than 40% inhibition in the hGPR3 GloSensor assay without significant activity in the counterassay. Light gray circles represent compounds with less than 40% inhibition in the hGPR3 GloSensor assay or showing inhibition similar to the counterassay. We selected 50 compounds as hit compounds (closed circle). Data are the means of triplicate experiments.

monitor time-dependent changes. In contrast, continuous assays such as the GloSensor assay enable the sequential monitoring of the same wells; thus, fewer wells are enough to optimize the experimental conditions. Furthermore, the GloSensor assay exhibited a high S/B ratio and a high DMSO tolerance (Suppl. Fig. S1). Considering that $>1\%$ DMSO often causes a significant effect in cellular assays,³⁷ the DMSO tolerance in the GloSensor assay is very high. Collectively, GloSensor appears to be a more optimum method to identify the hGPR3 inverse agonists. As expected, the primary screen revealed a high S/B ratio and stable Z' factor, shown in Figure 4A. An IC_{50} value of approximately 10 μ M was set as one of the final hit criteria in the HTS campaigns. Thus, taking experimental errors into consideration, we set more than 30% and 40% inhibitions as the cutoff values in the primary screening and the retest of the cherry-picked compounds, respectively. Furthermore, the mean + 3 SD of inhibitory activities of all compounds was 27.8% in the primary screening (Suppl. Fig. S2), indicating that these cutoff values were statistically significant. In addition, the mean + 3 SD of inhibitory activities of DMSO was approximately 20% in the counterassay of the pilot screening, suggesting that $<20\%$ inhibition is not significant. Thus, 20% was set as the cutoff value in the counterassay. When a hit confirmation rate was defined as the percentage of confirmed positive hits out of the total hits collected from the primary screen, it was approximately 90% (4595 of 5140). The hit confirmation rate is ultimately

high. Accordingly, the GloSensor is characterized by a simple protocol, larger assay window, and excellent reproducibility, suggesting that it is a useful tool for large-scale HTS.

The activity of the constitutively active orphan GPCR such as GPR3 correlates with the expression level on the cell surface.³⁸ The T-REx system was utilized as an inducible expression system in the present study. The inducible expression system was useful for the development and validation of the assay systems for GPR3 since activity can be easily modulated by the simple addition of tetracycline without GPCR ligands (Fig. 1A). In the pilot screening, as shown in Supplemental Figure S2B, only 1 compound satisfied the hit criteria, whereas 76 compounds showed more than 30% inhibition in the hGPR3 GloSensor assay. In comparison with the percent inhibition in the hGPR3 GloSensor assay with the percent inhibition in the counterassay, most compounds showed nearly similar activity (percent inhibition) between the two assays (Suppl. Fig. S2B). These results indicate that the GloSensor assay may detect false positives at least in our compound library. As expected from the pilot screening, many false positives were detected in the primary screen (Fig. 5B). Since these compounds inhibited GloSensor activity regardless of hGPR3 expression, these false positives may inhibit the luciferase activity of the GloSensor or adenylate cyclase producing cAMP. Alternatively, compounds may have cytotoxicity resulting in decreased GloSensor activity. Luciferase is necessary for the GloSensor, and it is inevitable to eliminate such false positives as long

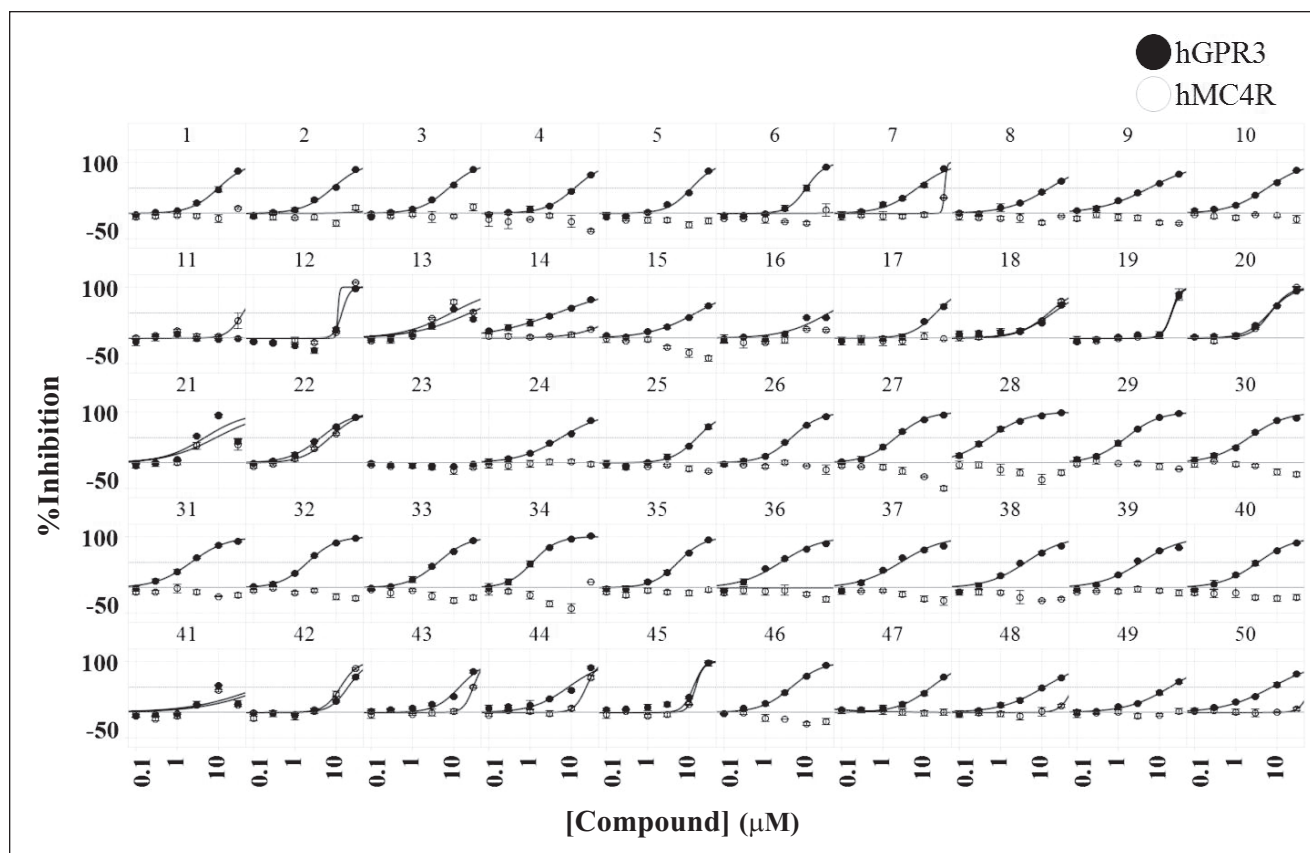


Figure 6. Concentration–response curves of 50 selected compounds. The hGPR3 GloSensor assay and the hMC4R GloSensor assay were conducted in the presence of different concentrations ranging from 0.1 to 30 μM . Closed circles and open circles represent percent inhibition in the hGPR3 GloSensor assay and percent inhibition in the hMC4R GloSensor assay, respectively. Data are means $\pm$ SD of quadruplicate experiments.

as GloSensor is used. Thus, it is important to set up an appropriate counterassay. In this report, we used the same cell line except that no induction of hGPR3 was used for the counterassay. This means that only the triggering of signal transduction was different between the hGPR3 GloSensor assay and the counterassay; thus, the influence on the difference between cell lines or cell clones was eliminated. Consequently, the counterassay using cells without the induction of hGPR3 could work well as an effective filter for excluding nonspecific compounds. Although almost all false positives were eliminated using the counterassay, some compounds lacked selectivity toward hMC4R (Fig. 6). hMC4R is known as the Gs coupling constitutively active GPCR, but it is not related to hGPR3, sharing only 35% amino acid homology. Taking this low homology between hGPR3 and hMC4R into consideration, it is unlikely that the same compound directly binds to both receptors. In addition, forskolin used in the counterassay is a direct activator of adenylyl cyclase in a Gs-protein-independent manner. Collectively, the nonselective compounds identified in the

hMC4R GloSensor assay may inhibit the upstream components of adenylyl cyclase, such as the Gs protein.

Among the 35 compounds with selectivity against hMC4R, 18 compounds were found to be derivatives of triazolopyrimidine that have already been reported as a GPR3 inverse agonist (Suppl. Fig. S3).²⁵ Furthermore, our HTS campaign revealed multiple novel GPR3 inverse agonists with the potency in the single-digit micromolar range (Fig. 6). By virtue of a sufficient S/B ratio and the stability observed in large-scale screening, we were able to successfully identify these compounds with somewhat less potent activity than the triazolopyrimidine derivatives. These results indicate that the GloSensor assay reported in the present article is an effective method for identifying diverse GPR3 inverse agonists. The feature of not only a real-time biosensor but also a wide dynamic range made the best use of the GloSensor in this report. This approach for inverse agonist screening with GloSensor may thus be applicable to other constitutively active orphan GPCRs in which attempts to establish assays compatible for HTS have failed due to

various limitations such as insufficient S/B ratios, narrow dynamic ranges, a large variance, and low reproducibility.

In conclusion, we demonstrated the feasibility of the GloSensor in large-scale screening for inverse agonists of constitutively active orphan GPCR in combination with an inducible expression system. The GloSensor assay can be set up easily and fast with high sensitivity, large assay windows, and remarkable reproducibility. Thus, it could facilitate the screening for constitutively active GPCR screening and provide an opportunity for further understanding of lesser known GPCRs.

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