Original Article

High-throughput screening of antagonists for the orphan G-protein coupled receptor GPR139

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Aim: To discover antagonists of the orphan G-protein coupled receptor GPR139 through high-throughput screening of a collection of diverse small molecules.

Methods: Calcium mobilization assays were used to identify initial hits and for subsequent confirmation studies.

Results: Five small molecule antagonists, representing 4 different scaffolds, were identified following high-throughput screening of 16 000 synthetic compounds.

Conclusion: The findings provide important tools for further study of this orphan G-protein coupled receptor.

Keywords: orphan G-protein coupled receptor; GPR139; high-throughput screening; calcium mobilization assay; antagonist

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Introduction

G-protein coupled receptors (GPCRs) are the largest family of cell surface communicating molecules, and they play crucial roles in numerous physiological processes and disease states. GPCRs share high levels of homology and contain seven transmembrane helices separated by intra- and extracellular loops^[1]. They signal via heterotrimeric G proteins composed of G α , β , and γ subunits, and there are four major G α protein subfamilies: Gq, Gs, Gi, and G_{12/13}. The Gs and Gi subunits regulate adenylyl cyclase activity, whereas the Gq subunit regulates phospholipase C and the subsequent production of diacylglycerol and inositol phosphate, which initiates calcium release from intracellular stores. The G_{12/13} subunit regulates the activities of the small G protein RhoA^[2].

The human *GPR139* gene was identified by searching the GenBank genomic database^[2–5]. The human GPR139 (also known as hGPRg1 or hGPCR12) protein is a 345-amino acid orphan receptor located on chromosome 16p12.3. It shares 96% amino acid homology with murine orthologs^[2]. Expression studies in mice have revealed that the transcription of *GPR139* is more evident in the brain, both in the adult stage and during development^[3]. Human *GPR139* mRNA is pre-

dominantly expressed in the fetal and adult central nervous system (CNS), especially in the basal ganglia and the hypothalamus^[2, 3], which are involved in movement control, regulation of food intake and metabolism^[2, 3, 6]. The consistent expression of *GPR139* mRNA in the CNS of different species suggests that it plays specific roles in the modulation of brain functions. Thus, it is implicated as a potential drug target for diabetes, obesity and Parkinson's disease.

Understanding the signaling pathway of a receptor is critical for the development of *in vitro* bioassays to support drug discovery. The signal transduction pathway of GPR139, however, is still not fully defined due to a lack of natural or synthetic ligands.

To date, three groups have reported small molecule ligands for GPR139 (Figure 1). Shi *et al* identified compound **1** as a GPR139 receptor agonist with an EC₅₀ of 39 nmol/L in a calcium mobilization assay for a CHO-K1 cell line stably expressing the human GPR139 for high-throughput screening (HTS)^[7]. Isberg *et al* discovered dipeptides and L- α -amino acids as GPR139 agonists by building a pharmacophore model based on the characterization of 13 compounds reported by Shi *et al*^[8]. Four dipeptides [TyrTrp (2), TyrPhe, TrpTyr, and TyrHis] consisting of aromatic amino acids were obtained. Among them, TyrTrp (2) and TyrPhe satisfied all five elements of the pharmacophore model^[9] and therefore displayed the highest potencies (EC₅₀=160 μ mol/L for TyrTrp and 180 μ mol/L for

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Figure 1. Reported small molecule ligands for GPR139.

TyrPhe), whereas TrpTyr lacks a hydrogen bond acceptor element, and TyrHis lacks an aromatic element. In addition, two amino acids, Trp and Phe, and their *L*- and *D*-isomers were found to activate GPR139 with an $E_{\max}$ similar to compound 1.

Hu *et al* identified compound 3 as a GPR139 agonist after screening a large library of small molecules^[6]. Compound 3 specifically increased cAMP levels by 8- to 10-fold in cells expressing GPR139 but not in cells expressing GPR142, a GPR139-related receptor with 50% amino acid identity. Furthermore, this compound was unable to induce Ca^{2+} mobilization in a fluorescent imaging plate reader (FLIPR) based assay, indicating the absence of a $\text{G}\alpha_q$ -mediated response. Antagonist screening with an identified agonist yielded two classes of compounds, including the triazolo-pyrimidine series, represented by compound 4, and the sulfonamide series, represented by compound 5, which specifically inhibited compound 3 induced cAMP accumulation in GPR139-expressing cells with IC_{50} values of 0.67 and 0.64 $\mu\text{mol/L}$, respectively.

In this study, we report the identification of four new scaffolds of GPR139 antagonists following high-throughput screening of 16 000 synthetic compounds using a calcium mobilization assay.

Materials and methods

Materials

Fluo-4 NW calcium assay kit, F-12 Kaighn's Nutrient Mixture (+*L*-Glutamine), fetal bovine serum, penicillin-streptomycin, sodium pyruvate, geneticin and 0.5% trypsin-EDTA were bought from Life Technologies (Carlsbad, CA, USA). DMSO was purchased from Sigma (St Louis, MO, USA) and the assay plates were procured from Corning (Tewksbury, MA, USA). Compound 1 was supplied by H Lundbeck A/S, Denmark.

Cell culture

CHO-K1 cells stably transfected with human GPR139 was provided by H Lundbeck A/S, Denmark. Cells were maintained in F-12 Kaighn's medium containing 10% fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin, 1 mmol/L sodium pyruvate and 0.5 mg/mL geneticin in a 5% CO_2 incubator.

Compound library

The compound library used in this study consists of both synthetic and natural compounds. The structural diversity covers heterocycles, lactams, sulfonates, sulfonamides, amines, secondary amides and natural product-derived compounds. The compounds were highly purified, and the stock was pre-solubilized in 100% DMSO prior to application in the HTS campaign, performed with an average concentration of 10 $\mu\text{mol/L}$ for each compound.

HTS campaign

The cells mentioned above were seeded in 96-well microtiter plates (Corning; 14 000 cells per 100 μL growth medium per well) and rested for 45–60 min at room temperature before incubating at 37°C, 5% CO_2 and 95% humidity chamber for two days. The dye mix solution was prepared from the Fluo-4 NW calcium assay kit according to the manufacturer's protocol by adding 100 μL assay buffer (HBSS/HEPS) and 1 mL probenecid stock solution (250 mmol/L) to the dye and shaking the resultant solution vigorously for 2 min (stored in dark).

Upon removal of growth medium 100 μL of the dye mix solution was added to each well and incubated for 30 min at 37°C, then at room temperature for an additional 45 min with plate top covered with tinfoil. This was followed by measurement of fluorescence signals in the first column (8 wells) for 10 s to obtain a baseline, using a FlexStation^{II} microplate reader (Molecular Devices, Sunnyvale, CA, USA). The library compounds in DMSO (1 mg/mL) were diluted in the assay buffer and 25 μL of which was dispensed to each well (final concentration was approximately 10 $\mu\text{mol/L}$ in 0.5% DMSO). After the first measurement for 1 min, the plates were incubated for 9 min at room temperature prior to introduction of the agonist compound 1 (25 μL at a final concentration of 100 nmol/L) followed by the second measurement for 1 min. Intracellular calcium change was recorded at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Dose-response study

Dose-response study was performed essentially as the same as described above except that test compounds were hand-picked (10 mmol/L concentration in DMSO) and serially diluted (1:5) seven times to give a total of eight different concentrations. This reflects a final compound dilution factor ranging from 1:96 to 1:136. For a starting concentration of 10 mmol/L this corresponded to a concentration range between 103.6 and 80.2 $\mu\text{mol/L}$. Each compound was tested in duplicate and the dose-response curves were fitted by Prism 5 software (GraphPad, San Diego, CA, USA) using nonlinear least square regression in a sigmoidal model with variable slope.

Statistical analysis

The results were analyzed using Prism 5 (GraphPad) and representatives of three independent experiments in duplicate.

$$Z' = 1 - 3 \times \frac{(SD_{PC} + SD_{NC})}{|M_{PC} - M_{NC}|}$$

$$\text{Inhibition \%} = \left(1 - \frac{\text{Signal}_{\text{test}} - \text{Signal}_{\text{NC}}}{\text{Signal}_{\text{PC}} - \text{Signal}_{\text{NC}}} \right) \times 100\%$$

PC indicates the average of cells in positive control wells, and NC indicates the average of cells in negative control wells.

Results

The GPR139 agonist compound **1** was employed to screen antagonists of GPR139. A total of 16 000 compounds with diverse structures were screened at 10 $\mu\text{mol/L}$ in the presence of 100 nmol/L of compound **1** using a calcium mobilization assay. The Z' of the screening assay was 0.74 with an S/B ratio of 22 and a coefficient of variation (CV) value of 5.0% (Figure 2). These parameters suggest that the assay system is of high quality and is suitable for HTS^[10]. The scatter plots of HTS are shown in Figure 3.

In the primary screening, 162 hits were identified ($\geq 50\%$ inhibition of compound **1** induced activation). Five hits were subsequently confirmed (Figure 4) in dose-response assays.

Figure 2. Z' value and S/B ratio determination. The assay was performed under optimized conditions with GPR139-expressing CHO-K1 cells. The background (open circles) indicates the relative fluorescence units in 0.5% DMSO. The signal (closed circles) indicates the relative fluorescence units in 100 nmol/L of compound **1**. Forty replicates of the S/B readouts were examined. RFU=relative fluorescence units.

Figure 3. HTS of 16 000 small molecule compounds using a calcium mobilization assay. The results are expressed as the percentage of inhibition of each sample on calcium mobilization responses induced by the GPR139 agonist compound **1**.

Figure 4. Concentration-dependent characteristics of the five confirmed hits. (A) The calcium mobilization responses of NCRW0001-C02 (●), NCRW0005-F05 (■), NCRW0008-C04 (▲), NCRW0095-F03 (▼), NCRW0105-E06 (◆) and compound **1** (○) in CHO-K1 cells stably transfected with human GPR139. (B) Antagonism of NCRW0001-C02 (●), NCRW0005-F05 (■), NCRW0008-C04 (▲), NCRW0095-F03 (▼) and NCRW0105-E06 (◆) to compound **1** induced calcium mobilization. The IC_{50} values were calculated by nonlinear regression analysis of the dose-response curves generated using the Prism 5 program. All data points are representative of three independent experiments performed in duplicate.

They represent 4 different structural scaffolds; NCRW0001-C02 and NCRW0005-F05 share the same core and their IC_{50} values are similar (0.2 and 0.4 $\mu\text{mol/L}$, respectively). The other 3 confirmed hits, namely NCRW0008-C04, NCRW0095-F03, and NCRW0105-E06, showed variable antagonist activities with IC_{50} values ranging from 0.4 to 2.1 $\mu\text{mol/L}$ (Table 1).

Table 1. Confirmed hits showing consistent antagonistic effects on GPR139 agonist compound **1** and their corresponding structures. IC₅₀ values are mean±SEM. *n*=3.

No	Structure	Chemical formula	Molecular weight	IC ₅₀ (μmol/L)
NCRW0001-C02		C ₁₅ H ₁₀ NOF ₃	277.24	0.42±0.10
NCRW0005-F05		C ₁₆ H ₁₃ NO ₂ F ₂	289.28	0.21±0.01
NCRW0008-C04		C ₁₄ H ₉ N ₃ F ₃ Cl	311.69	2.1±0.34
NCRW0095-F03		C ₁₃ H ₇ NS ₂	241.33	0.83±0.15
NCRW0105-E06		C ₁₆ H ₈ O ₃ F ₂ Cl	340.68	0.43±0.17

Discussion

GPR139 was identified by searching the genomic database and has characteristics of the rhodopsin family of GPCRs^[3]. It is abundantly expressed in distinct regions of the brain, both in humans and in mice. In the caudate putamen, habenular nucleus, zona incerta and medial mammillary nucleus, the expression of GPR139 is higher than that in the thalamus, amygdala and spinal cord^[2,3], which suggests a significant role of GPR139 in the CNS.

GPR139 was first reported as a Gq-coupled receptor^[2,3]. Matsuo *et al* overexpressed GPR139 in 293-EBNA cells and found that it was capable of activating serum response factor mediated transcription. Additionally, this reaction could be inhibited by a G_{q/11} selective inhibitor^[2]. This observation was confirmed through the discovery of a series of GPR139 agonists using calcium mobilization assays^[7,8]. Susens *et al* identified the signal transduction pathway using both Ca²⁺ mobilization and luciferase-reporter-gene assays. They proposed that GPR139 was coupled to an inhibitory G-protein and mediated by phospholipase C^[3]. However, Hu *et al* identified GPR139 as a Gs-coupled receptor because overexpressed GPR139 in HEK293 cells could increase basal intracellular cAMP con-

centrations^[6]. Previous studies have shown that Gq-coupling is the main signaling pathway of GPR139 and might activate other pathways^[8]. Furthermore, it was noted that GPR139 appears to be a monomer in HEK-293 cells and a dimer in CHO-K1 cells^[3].

In this study, we described an HTS assay to screen antagonists to GPR139 based on intracellular calcium influx and identified a series of small molecule hits that blocked the activity of GPR139 induced by compound **1**. All of the compounds showed reasonable potencies (close or below 2 μmol/L), of which two compounds (NCRW0001-C02 and NCRW0005-F05) possessed the same core region consisting of 3,3-difluoro-4-phenylazetidin-2-one. A preliminary structure-activity study suggested that substitution of electron-donating groups on the phenyl group was beneficial for antagonistic effects. These compounds showed little similarity to the structures of antagonists previously reported. Our findings thus offer novel structures and provide promising tools in the study of both the signaling pathway(s) and physiological significance of this orphan receptor.

In summary, we conducted an HTS study based on intracellular calcium influx and identified five small-molecule antago-

nists that blocked the activity of GPR139 agonist compound 1. Our discovery supports the claim that GPR139 is a $G\alpha_q$ -coupled receptor.

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