Identification of a Novel Small-Molecule Agonist for Human G Protein–Coupled Receptor 3

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ABSTRACT

G protein–coupled receptor 3 (GPR3) is an orphan G protein–coupled receptor (GPCR) predominantly expressed in mammalian brain and oocytes. GPR3 plays important roles in these two organs and is known as a Gαs-coupled receptor–activated constitutively in cells. However, the signal transduction pathway and pharmacological function of GPR3 remain unclear because of the lack of a specific ligand. By use of a human embryonic kidney 293 cell line stably expressing FLAG-GPR3-green fluorescent protein, a chemical screening for GPR3 ligands was performed using homogeneous time-resolved fluorescence cAMP assay. Diphenyleneiodonium chloride (DPI) was identified as a novel agonist of GPR3 with weak or no cross-reactivity with other GPCRs. DPI was further characterized to activate several GPR3-mediated signal transduction pathways, including Ca2+ mobilization, cAMP accumulation, membrane recruitment of β-arrestin2, and receptor desensitization. Parallel studies revealed that the activity of DPI is much more pronounced than sphingosine 1-phosphate, a previously reported GPR3 agonist. Our study identified a novel and specific agonist of GPR3, which provides a useful tool for further study of this orphan GPCR.

INTRODUCTION

G protein–coupled receptors (GPCRs), which constitute the largest and the most versatile family of membrane receptors, respond to a wide range of extracellular stimuli and activate intracellular signal transduction pathways, which ultimately lead to cellular responses. Upon agonist stimulation, GPCRs activate a variety of heterotrimeric G protein signaling pathways, including Goαs, Goi, Goq, and Go12/13. The downstream signal of Goαs activation is to elevate intracellular cAMP, a second messenger produced by adenylate cyclase, whereas activation of Goi leads to an opposite effect. Goq activation leads to production of diacylglycerol and inositol phosphate, followed by the release of calcium ion from endoplasmic reticulum to cytoplasm. β-Arrestins are versatile adapter proteins that form complexes with most GPCRs following agonist stimulation and receptor phosphorylation (Ferguson et al., 1996; Mehlmann et al., 2004; Lefkowitz and Shenoy, 2005). They play a central role in the processes of homologous desensitization and sequestration of GPCR, which leads to the termination of GPCR activation.

The G protein–coupled receptor 3 (GPR3, also referred to as GPCR21) is an orphan receptor predominantly expressed in the brain, ovaries, and testes (Iismaa et al., 1994; Mehlmann et al., 2004; Tanaka et al., 2007). GPR3, GPR6, and GPR12 share greater than 50% identity and 65% similarity at the amino acid level and make up a family of constitutively active Gαs-coupled GPCRs that elevate intracellular cAMP constantly. The physiologic function of GPR3 was initially studied in oocytes. Mehlmann et al. (2004) found that GPR3 in the oocyte is an important link in communication between the somatic cells and ovarian follicle oocyte and is crucial for the regulation of meiotic prophase arrest. This phenomenon was soon confirmed by other studies (Mehlmann, 2005; Deng et al., 2008; Kovanci et al., 2008), although the specific signaling that activates GPR3 remains elusive. GPR3 has also been associated with premature ovarian aging in mice (Ledent et al., 2005). However, a recent study with premature ovarian failure patients indicated that mutations in GPR3 might not be a common cause of this problem (Zhou et al., 2010).

HUMAN G PROTEIN–COUPLED RECEPTOR 3; HBSS, Hanks’ balanced salt solution; HEK, human embryonic kidney; HTRF, homogeneous time-resolved fluorescence; IBMX, 3-isobutyl-1-methyl-xanthine; iso, isoproterenol; NOX, NADPH oxidase; RFP, red fluorescent protein; S1P, sphingosine 1-phosphate; TIRF, total internal reflection fluorescence.
GPR3 also plays important roles in neuronal development and functions. GPR3 has been reported to arrest cell cycle, inhibit proliferation, and induce differentiation in cerebellum granule neurons during postnatal development (Tanaka et al., 2007, 2009). GPR3 is also involved in neuropathic pain after peripheral nerve injury and regulates morphine-induced antinociception (Ruiz-Medina et al., 2011). More importantly, GPR3 has been proposed to potentiate γ-secretase activity and stimulate the production of β-amyloid both in vitro and in vivo, which might lead to the pathology of Alzheimer’s disease (Thathiah et al., 2009). The regulation of γ-secretase activity by GPR3 was recently discovered to be dependent on β-arrestin2 rather than Goα signaling (Thathiah et al., 2013).

Although the constitutive Goα activity of GPR3 has long been recognized to play an important role, little is known about GPR3’s other signaling pathways because of the lack of GPR3 agonist. Sphingosine 1-phosphate (S1P) has been about GPR3 signaling (Thathiah et al., 2013). More importantly, after peripheral nerve injury and regulates morphine-induced antinociception (Ruiz-Medina et al., 2011). Consequently, S1P failed to induce the cAMP accumulation. We further confirmed that DPI activates other GPCR signaling pathways via GPR3 and induces β-arrestin2-mediated internalization and desensitization of GPR3. We also observed that S1P failed to induce the recruitment of β-arrestin2 to the membrane-bound GPR3. Our study identified a novel agonist of GPR3, which may provide a useful tool for further study of this orphan GPCR.

**Materials and Methods**

DPI, forskolin, isoproterenol (iso), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO). Homogeneous time-resolved fluorescence (HTRF) cAMP dynamic 2 assay kits were obtained from Cisbio (Codolet, France). The FuGENE6 transfection reagent was purchased from Roche (Basel, Switzerland). Human GPR3 was polymerase chain reaction–amplified from the genomic DNA of human embryonic kidney 293 (HEK293) cells using the following primer set: 5’-ATGGGGAGAAACCCGGGACC-3’ and 5’-TCATGGAGTTGTAGGTGGCAG-3’. The purified DNA fragments were inserted into pcDNA3 plasmid in-frame with either a FLAG-tag at the N terminus or GFP at the C terminus. β-Arrestin2 was amplified from cDNA of HEK293, using 5’-ATGATGTGGGGTGCAGG-3’ and 5’TACACAGGGTATGAGGTGCAC-G3’. The purified DNA fragments were inserted into pcDNA3 plasmid in-frame with an HR region or C terminus. 

**Cell Culture and Transfection.** HEK293 cells and human osteosarcoma U2OS cells were grown in a monolayer in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere. HeLa cells were maintained in modified Eagle’s medium supplemented with 10% FBS. Calcium phosphate precipitation method was used for the transfection of HEK293 cells, whereas FuGENE6 transfection reagent was used to transfect U2OS and HeLa cells. HEK293 cells stably expressing FLAG-GPR3-GFP and/or Goα16 were grown under the same condition, with the exception of the supplementation of G418 (200 μg/ml) in the medium.

**HTRF cAMP Assay.** HTRF cAMP dynamic 2 assay kits, obtained from Cisbio, are based on a competitive immunoassay using cryptate-labeled anti-cAMP antibody and d2-labeled cAMP. Experiments were performed 48 hours after transfection according to the manufacturer’s instruction. In brief, cells were harvested and suspended to a density of 8 x 10^5 cells/ml in culture medium. The cell suspension (4000 cells in 5 μl) was dispensed into each well of a 384-well OptiPlate (white; PerkinElmer Life and Analytical Sciences, Waltham, MA). Phosphate-buffered saline (5 μl) containing chemical compound with indicated concentrations and the phosphodiesterase inhibitor IBMX with a final concentration of 200 μM were added, and the plate was incubated at room temperature for 30 minutes. Lysis buffer (10 μl) containing d2-cAMP (a fluorescent conjugated cAMP) and anti-cAMP antibody was then added. After 60-minute incubation at room temperature in the dark, HTRF signal was detected with an Envision 2101 plate reader (PerkinElmer Life and Analytical Sciences). Fluorescence emission at 620 and 665 nm was measured, and the ratio between 620 and 665 nm was calculated. The higher the value was, the higher the intracellular cAMP level.

**Calcium Mobilization Assay.** HEK293 cells stably expressing Goα16 were transfected with plasmids encoding FLAG-GPR3 or other GPCRs. Twenty-four hours later, cells were reseeded at a density of 40,000 cells per well into a 96-well plate. The culture medium was changed to Hanks’ balanced salt solution (HBSS) buffer containing 6.3 mM d-glucose, 5 g/ml bovine serum albumin, and 2 μM Fluo-4 AM. After incubation at 37°C for 45 minutes, cells were washed, and new HBSS buffer was added. Chemicals prepared in HBSS buffer were then dispensed into the well using a FlexStation III microplate reader (Molecular Devices, Sunnyvale, CA), and intracellular calcium change was recorded at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. For the receptor desensitization assay, after the first stimulation and measurement, cells were returned to the incubator for 20 minutes to allow recovery before a second stimulation was performed.

**Receptor Internalization Assay.** U2OS cells were seeded onto glass coverslips in 24-well plates and transfected with plasmids encoding FLAG-GPR3 or other GPCRs. Twenty-four hours after transfection, cells were reseeded into a density of 40,000 cells per well into a 96-well plate. The culture medium was changed to Dulbecco’s modified Eagle’s medium containing 0.5% FBS at 37°C for 2 hours followed by 1-hour incubation in media containing DPI (10 μM) or vehicle (0.1% dimethylsulfoxide (DMSO)). Cells were then washed three times with phosphate-buffered saline and fixed in 4% paraformaldehyde. After counterstaining of nuclei with Hoechst dye 33342, cells were examined using an FV10i confocal microscope (Olympus, Tokyo, Japan).

**β-Arrestin2 Translocation Assay.** HeLa cells were cultured in a 60-mm dish and cotransfected with plasmids encoding FLAG-GPR3-GFP or β-arrestin2–RFP. Twenty-four hours after transfection, cells were reseeded into a 24-well glass-bottom plate (Nest, Shanghai, China) and stimulated with DPI (10 μM), S1P (3 μM), or vehicle control (0.3% DMSO) for the indicated duration. The fluorescent signals of membrane-bound receptor or β-arrestin2 were collected as live images using a total internal reflection fluorescence (TIRF) microscope (Olympus).

**Statistical Analysis.** Data were analyzed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Nonlinear regression analysis was performed to generate dose-response curves and calculate concentration for 50% of the maximal effect (EC50). Data are presented as means ± S.E.M. Two-tailed Student’s t tests were performed, and P < 0.05 was considered to be statistically significant.
Results

Establishing GPR3-Expressed Cell Lines with Constitutive Gaq-Coupled Signaling. To establish a screen system to identify GPR3-specific ligands, we tested the cAMP level influenced by overexpression of GPR3 in HEK293 cells using HTRF cAMP assay. Forskolin, a known adenylate cyclase activator used as a positive control to evaluate the assay system, indeed led to an elevated intracellular cAMP compared with DMSO vehicle control (Fig. 1A). Compared with the vector control, either transiently or stably expressed GPR3 in HEK293 cells significantly increased cAMP level, which can be further elevated by forskolin (Fig. 1A). To develop a high-throughput screening assay, a HEK293 cell line stably expressing GFP-tagged GPR3 was established (GPR3-E1). In addition to the constitutive Gaq activity of GPR3 observed in this stable line (Fig. 1A), the GPR3-GFP fusion protein was located both on the cell plasma membrane and in the cytosol (Fig. 1B). This phenomenon is consistent with a recent report that showed constitutive endocytosis of GPR3 transiently expressed in HEK293 (Lowther et al., 2013). Therefore, the GPR3-E1 clone possesses all known GPR3 characteristics and is suitable for the subsequent screening assay to identify the GPR3 ligand.

Identification of DPI as a Novel Agonist for GPR3.

With the GPR3-E1 clone, approximately 40,000 compounds were screened for agonists of GPR3 using the HTRF cAMP assay. Twenty-three candidate chemicals from the primary screening were identified to cause at least a 2-fold increase of intracellular cAMP level in the GPR3-E1 clone (Fig. 2A).

Forskolin from the library was also identified, supporting the reliability of our assay (Fig. 2A). These compounds were further tested in a 10-fold series dilution, and four compounds of the 23 compounds induced dose-dependent activation of adenylate cyclase in the GPR3-E1 clone (Fig. 2, B and C). Among them, the compound 34214 (DPI), a known inhibitor of NADPH oxidase (NOX) (Aldieri et al., 2008), showed the best agonist activity with an EC50 of 1 μM in cAMP assay (Fig. 2D). The elevation of cAMP induced by DPI appeared to be GPR3-dependent because, in HEK293 cells without GPR3 transfection, no increase of cAMP was observed (Fig. 2D).

DPI has been used as a NOX inhibitor. We wondered whether the inhibitory effect of DPI on NOX contributes to the activation of GPR3. In the LOPAC library we screened, we found that compound 33979 [AEBSF; 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride] and compound 34958 (resveratrol), two known inhibitors of NOX (Kleniewska et al., 2012), failed to enhance the cAMP level in our assay (Fig. 2E). These data suggested that the activation of GPR3 by DPI was NOX inhibition independent.

DPI Shows Good Selectivity for GPR3. S1P has been reported to activate GPR3, GPR6, and GPR12 (Uhlenbrock et al., 2002). However, several other studies demonstrated that S1P failed to activate GPR3 in our system (Fig. 3A). Our results showed that DPI did induce cAMP accumulation, whereas S1P did not activate GPR3 in our system (Fig. 3A). We speculated that the endogenous expression of other Gaq-coupled S1P receptors, such as S1P1, S1P2, S1P3, and S1P5 (Siehler and Manning, 2002), might have interfered with the cAMP measurement. GPR3, GPR6, and GPR12 share high similarity in amino acid sequence, so we tested DPI in HEK293 expressing GPR3, GPR6, or GPR12. As demonstrated in Fig. 3B, DPI activated adenylate cyclase only in cells expressing GPR3, but not in cells expressing GPR6 or GPR12.

In addition to the cAMP accumulation, several other pathways possibly downstream of GPR3 were tested with DPI. In parallel, using β2AR as a receptor control, we further examined the specificity of DPI on other GPCRs. By use of a cell system with overexpressed Ga10, GPCR activity can be switched to Gαq-coupled signaling, and agonist activity can be monitored using Ca2+ mobilization assay (Zhang and Xie, 2012). In agreement with previous observations, DPI induced Ca2+ mobilization from endoplasmic reticulum to cytoplasm through GPR3 but not β2AR (Fig. 3C). In contrast, isoproterenol, a β2AR-specific agonist, stimulated Ca2+ mobilization in β2AR-expressed cells, indicating a normal function of this receptor in cells (Fig. 3D). Similar results were obtained when the cAMP level was tested in these cells. In contrast to isoproterenol, DPI only stimulated Gaq-coupled signaling of GPR3 but not β2AR (Fig. 3, E and F). The selectivity assay was expanded to other GPCRs using cAMP or Ca2+ mobilization assay, as shown in Table 1. DPI displayed almost no activity on all 17 receptors tested. These data indicate that DPI is a highly specified agonist for GPR3.

DPI Induces Membrane Translocation of β-Arrestin2, Receptor Internalization, and Desensitization of GPR3.

β-Arrestin2 is known as a negative regulator of G protein-mediated signaling. As an adaptor molecule, β-arrestin2 is recruited to membrane-bound GPCRs after agonist stimulation and mediates receptor internalization and desensitization.
overexpression of GPCR–kinase 2 and β-arrestin2 decreased cell surface GPR3 and cAMP levels (Lowther et al., 2013; Thathiah et al., 2013). S1P, the previously reported GPR3 agonist, failed to enhance the interaction between GPR3 and β-arrestin2 (Yin et al., 2009), suggesting that S1P might not be an agonist for GPR3. To identify novel agonists for GPR3, a high-throughput screening was carried out with the GPR3-E1 clone and HTRF cAMP assay. Compounds distributed above the dashed line possess at least 2-fold stimulation activity on GPR3 (Fig. 2A). The dose-response of compounds 21136, 34214 (DPI), 34302, and 34955 was determined (Fig. 2B). The structure of these compounds is shown in Fig. 2C. DPI was found to be a novel agonist for GPR3 (Fig. 2D). Inhibition of NOX failed to activate GPR3 (Fig. 2E). NOX inhibitors AEBSF (10 μM) and resveratrol (10 μM) were added to the GPR3-E1 clone and cAMP assay was performed. Representative results from three independent experiments are shown. Data are the mean ± S.E.M. (n = 3). ***P < 0.001 versus DMSO treatment.

DPI shows good selectivity for GPR3. A comparison of receptor activation by DPI (10 μM) and S1P (10 μM) in HEK293 transfected with GPR3 or pcDNA3.0 control is shown in Fig. 3A. DPI exhibited much stronger affinity for GPR3 compared to S1P. DPI was also found to be selective for GPR3 over GPR6 and GPR12 (Fig. 3B and D). DPI-induced calcium changes were measured in HEK293/Ca1.3 cells expressing GPR3 or β2AR (Fig. 3C and E). DPI-induced calcium changes were also measured in HEK293/Ca1.3 cells expressing GPR3 or β2AR (Fig. 3F). Data are the mean ± S.E.M. (n = 3). ***P < 0.001 versus GPR3 transfected cells with DMSO treatment.
a real GPR3 agonist. Therefore, we tested whether DPI could recruit β-arrestin2 to the cell membrane upon GPR3 stimulation. GPR3 and β-arrestin2–RFP fusion proteins were coexpressed in HeLa cells. Cells were treated with DPI, S1P, or vehicle control, and the membrane localization of β-arrestin2 was investigated with a TIRF microscope and live cell imaging system. Before treatment, the distribution of β-arrestin2–RFP appeared to be inside the cells, and no obvious speckles could be observed on the cell membrane surface (Fig. 4A). Ten seconds after DPI stimulation, β-arrestin2–RFP was recruited to the plasma membrane in a speckled pattern. This recruitment became saturated at 60 seconds after DPI addition and lasted for at least 5 minutes (Fig. 4A). Such phenomenon was not observed in either DMSO- or S1P-treated cells.

Next, we tested whether DPI could cause internalization of GPR3. As demonstrated in Fig. 1, GPR3 is constitutively activated and distributed on the cell membrane as well as into the cytoplasm in HEK293 cells. The cytoplasmic localization might represent the spontaneous continuous internalization of this receptor (Fig. 1B). This phenomenon is even obvious in U2OS cells. The distribution of the receptors was mostly in the cytoplasm of U2OS cells (Fig. 4B). Further DPI treatment induced the formation of fluorescent intracellular granules resembling endosomes, indicating the accelerated internalization of GPR3-GFP fusion protein by DPI stimulation (Fig. 4B).

By use of a Ca²⁺ mobilization assay, we tested whether DPI could cause desensitization of GPR3. In contrast to DMSO control, DPI induced intracellular Ca²⁺ elevation during the first round stimulation of GPR3 (Fig. 4C, first arrow). After 20-minute incubation, the cells were stimulated with DPI again. Only those cells prestimulated with DMSO displayed a significant increase of Ca²⁺ signal, whereas cells prestimulated with DPI displayed a reduced Ca²⁺ response (Fig. 4C, second arrow), suggesting the desensitization of GPR3 after DPI stimulation.

**Discussion**

On the basis of sequence similarity, GPR3, GPR6, and GPR12 constitute a GPCR subfamily that constitutively activates the adenylate cyclase (Uhlenbrock et al., 2002). All three receptors are orphan GPCRs predominantly expressed in the central nervous system and genital system in both men and women. Hence, we investigated the specificity of DPI as a GPR3 agonist by studying the effect of DPI on a panel of GPCRs.

### Table 1

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<th>Assay Type</th>
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<td>GPR12</td>
<td>cAMP assay</td>
<td>NR</td>
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<td>cAMP assay</td>
<td>NR</td>
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—, not applicable, no known agonists; A2AR, adenosine A2a receptor; ADRA, α-adrenergic receptor; BAY68-6583, 2-[6-amino-5-dicyano-4-(4-hydroxyphenyl)pyridan-2-ylmethyl]acetamide; CCL19, chemokine (C-C motif) ligand 19; CCR, C-C chemokine receptor; CGS 21680, 3-(4-[6-amino-9-[2[[3,4-diethylaminomethyl]phenyl]propionic acid, DPI, prostaglandin D₂ receptor; DRD1, dopamine receptor D₁; EP, prostaglandin E receptor; GCCR, glucagon receptor; KOR, κ-opioid receptor; NR, no response at concentrations up to 100 μM; PGE₂, prostaglandin E₂.
humans and mice. All three receptors were reported to play important roles in promoting neurite outgrowth (Tanaka et al., 2007). Although these receptors share an overlapping ligand spectrum (Ignatov et al., 2003a,b; Mehlmann et al., 2004), they appear to have different ligand binding affinity, which might reflect their differences in physiologic function. Sphingosylphosphorylcholine binds to GPR12 with high affinity (Ignatov et al., 2003a), whereas S1P is a high-affinity ligand for GPR6 (Ignatov et al., 2003b). There were also morphologic, behavioral, and pathologic differences between mice with deficiency in GPR3 or GPR12. GPR3-deficient mice showed premature ovarian failure and early oocyte aging (Mehlmann et al., 2004; Ledent et al., 2005), whereas no similar symptom was found in GPR12-deficient mice. GPR12 knockout mice showed signs of liver and kidney disease, dyslipidemia, obesity, and deficits in brain development demonstrated by impaired locomotion, motor and balance function, and spatial learning abilities (Bjursell et al., 2006). GPR6-deficient mice showed normal breeding, body weight, locomotor behavior, and motor learning in the Rotarod test (Lobo et al., 2007). In our study, we found that DPI acts as a specific ligand for GPR3 rather than GPR6 and GPR12, supporting the existence of divergent functions within these family members. Furthermore, a broad test with other GPCR family also demonstrated that DPI acts only on GPR3 (Table 1). Such a GPR3-specific ligand will be a useful tool in the study of GPR3 family, especially when individual receptor function needs to be distinguished.

GPR3 has been reported to play important roles in neuronal development and functions. During postnatal development, GPR3 inhibits the proliferation of cerebellar granule cells (Tanaka et al., 2007). Overexpression of GPR3 promoted whereas RNA interference of GPR3 blocked neurite outgrowth of the rat cerebellar granule neuron (Tanaka et al., 2007). More importantly, GPR3 has been proposed to promote γ-secrectase activity and elevate the β-amyloid level, which makes GPR3 a hot candidate target for developing new therapeutic approaches for Alzheimer’s disease (Thathiah et al., 2009). Because, however, of the lack of GPR3-specific ligands, further study of GPR3 signaling was hindered. In the present study, we were able to develop a high-throughput system to screen GPR3-specific agonist and identified DPI as a specific agonist of GPR3. In parallel, we compared the activity of S1P, the previously reported GPR3 agonist, with DPI and found that S1P failed to trigger GPR3-mediated cAMP accumulation as well as translocation of β-arrestin2 from cytoplasm to membrane. Our observations are in agreement with previous publications reporting that S1P failed to activate GPR3 in a β-arrestin PathHunter assay (Yin et al., 2009) and to elevate cAMP level in GPR3-expressed Chinese hamster ovary (Valverde et al., 2009). Therefore, S1P is not a good GPR3 agonist, and the newly identified compound, DPI, will be useful for further study of the signal transduction pathway and pharmacological function of GPR3. DPI could activate GPR3 in all classic GPCR assays. By use of TIRF technology, we can observe clearly that DPI stimulated GPR3-induced β-arrestin2 membrane translocation and receptor internalization. Interestingly, the regulation of γ-secretase activity by GPR3 was recently discovered to be dependent on β-arrestin2 rather than Goα signaling (Thathiah et al., 2013). Therefore, DPI can be a suitable tool for further study of GPR3 signaling in Alzheimer’s disease in cellular models as well as in mouse models. Because DPI can cause desensitization of GPR3, whether such desensitization can cause any of the biologic consequences needs to be further investigated.

Although we identified DPI as an agonist for GPR3 in our screening, how DPI activates GPR3 and whether DPI acts as an anestrogen or an uesthoric ligand remain elusive. DPI has been used as an inhibitor of NOX for years. However, in our screening, other NOX inhibitors failed to show any activity on GPR3, which ruled out the possibility of involvement of NOX signaling in GPR3 activation. Further researches concerning the structure–activity relationships of DPI as well as identifying native ligands of GPR3 are important for the deeper understanding of this orphan receptor.

In summary, we discovered DPI as a novel GPR3 agonist, which is a useful tool in GPR3 research in many aspects, such as GPR3 signaling in neuron development and GPR3 pathology in Alzheimer’s disease. In addition, the establishment of a high-throughput screen system with the stable cell line expressing the constitutively active GPR3 provides a powerful assay for identification of new agonists and antagonists of human GPR3.

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Authorship Contributions

Participated in research design: Ye, R. Zhang, Xie.
Conducted experiments: Ye, Z. Zhang, Wang, Hua.
Contributed new reagents or analytic tools: Xie.
Performed data analysis: Ye, Wang, R. Zhang, Xie.
Wrote or contributed to the writing of the manuscript: Ye, R. Zhang, Xie.

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