Chapter 1

Label-Free Cell Phenotypic Identification of D-Luciferin as an Agonist for GPR35

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Abstract

D-Luciferin (also known as beetle or firefly luciferin) is one of the most widely used bioluminescent reporters for monitoring in vitro or in vivo luciferase activity. The identification of several natural phenols and thieno[3,2-b]thiophene-2-carboxylic acid derivatives as agonists for GPR35, an orphan G protein-coupled receptor, had motivated us to examine the pharmacological activity of D-Luciferin, given that it also contains phenol and carboxylic acid moieties. Here, we describe label-free cell phenotypic assays that ascertain D-Luciferin as a partial agonist for GPR35. The agonistic activity of D-Luciferin at the GPR35 shall evoke careful interpretation of biological data when D-Luciferin or its analogues are used as probes.

Key words Bioluminescence, Dynamic mass redistribution, G protein-coupled receptor, GPR35, D-Luciferin, Resonant waveguide grating biosensor

1 Introduction

Luciferase genes from firefly and other beetles are commonly used as reporter genes to study transcription regulation, and to localize, track, and quantify a myriad of biological events or functions in living cells and animals [1–4]. As they produce the brightest form of bioluminescence, luciferase genes can be used to transfect tumor cells, stem cells, or infectious species, which are then inoculated into research animals such as rats or mice. As a natural substrate for the beetle luciferase reporter systems, D-Luciferin ((S)-2-(6′-hydroxy-2′-benzothiazolyl)thiazoline-4-carboxylic acid, *see* Fig. 1) is injected into animals so real-time, noninvasive monitoring of disease progression and/or drug efficacy in animal model systems using bioluminescence imaging is possible. D-Luciferin is also commonly used for in vitro luciferase and ATP, gene reporter, high-throughput screening, and various contamination assays [5, 6]. Luciferase catalyzes a bioluminescence reaction that uses luciferin, Mg-ATP, and molecular oxygen to produce a characteristic

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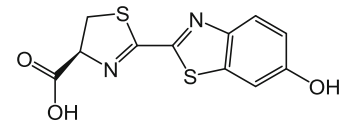

D-Luciferin

Fig. 1 Chemical structure of D-Luciferin

yellow-green emission, which shifts to red light in vivo at 37 °C [7–10]. However, little is known about the biological activity of D-Luciferin.

Using label-free resonant waveguide grating (RWG) biosensor enabled dynamic mass redistribution (DMR) assays [11–16], we had previously discovered several thieno[3,2-b]thiophene-2-carboxylic acid derivatives [17] and natural phenols [18] as agonists for G protein-coupled receptor 35 (GPR35). These findings had motivated us to search for common fluorescent and bioluminescent probe molecules that contain phenol and/or carboxylic acid moieties, two of which are important for the activation of GPR35 [17, 18]. GPR35 is an orphan G protein-coupled receptor (GPCR) whose natural agonist(s) are still controversial [19–22]. Label-free DMR pharmacological profiling of D-Luciferin in HT-29, a colon cancer cell line endogenously expressing GPR35, showed that D-Luciferin is a partial agonist for this receptor with an EC_{50} of 12.9 μM [23]. Thimm et al. later confirmed using a radioligand binding assay that D-Luciferin binds to GPR35 with a K_i of 9.86 μM [24].

This chapter describes protocols as to how to determine the pharmacological activity of D-Luciferin acting at the GPR35 using multiple types of DMR assays. These assays include (1) one-step agonism assay to detect its agonistic activity, (2) one-step co-stimulation assay to determine its partial agonistic activity, (3) two-step antagonism assay to determine its specificity to activate GPR35, (4) two-step desensitization assay to determine the competitive agonism against known GPR35 agonists.

2 Materials

2.1 Tissue Culture Medium and Cell Line

1. Human colorectal adenocarcinoma HT-29 cell line (ATCC® HTB-38™, American Type Cell Culture, Manassas, VA, USA).
2. Tango™ U2OS-GPR35-*bla* cell line (Invitrogen, Grand Island, NY). This cell line stably expresses human GPR35 linked to a TEV protease site and a Gal4-VP16 transcription factor, as well as a β-arrestin/TEV protease fusion protein and the β-lactamase reporter gene under the control of a UAS response element.
3. McCoy's 5A Medium Modified (Invitrogen).

4. Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen).
5. Antibiotic solution 100×: 10,000 U penicillin/mL, 10,000 μg streptomycin/mL.
6. Complete medium for HT-29 cells: McCoy's 5A Medium Modified supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin.
7. Complete medium for U2OS-GPR35-*bla* cells: DMEM medium supplemented with 10% dialyzed FBS, 0.1 μM NEAA, 25 μM Hepes (pH 7.3), 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 200 μg/mL zeocin, 50 μg/mL hygromycin, and 100 μg/mL geneticin.
8. Trypsin/ethylenediaminetetraacetic acid (EDTA) solution 10×: 2.5% Trypsin, 0.2% $4Na^{+}$-EDTA.

2.2 Microplates and Instruments

1. Corning 75 cm^2 tissue culture-treated polystyrene flask with vented cap (T-75) (Corning Incorporated, Corning, NY, USA).
2. Corning 384-well polypropylene compound storage plate.
3. Corning Epic® 384-well microplate tissue culture-treated.
4. 384-well, black-wall, clear bottom assay plates with low fluorescence background (Corning).
5. 8-well chamber slide (Nalge Nunc International, Rochester, NY, USA).
6. Matrix 16-channel electronic pipettor (Thermo Fisher Scientific, Hudson, NH).
7. Epic® system (Corning). This reader is a wavelength interrogation reader system tailored for RWG in microplate (384- and 1536-well). This system consists of a temperature-control unit (26 °C), an optical detection unit, and an on-board liquid handling unit with robotics. The detection unit is centered on integrated fiber optics, and enables kinetic measures of cellular responses with a time interval of ~15 s.
8. Epic® BT system (Corning). This reader is a small-footprint, swept wavelength interrogation-based imaging system [25]. This system has a spatial resolution of 90 μm and a temporal resolution of 3 s.
9. BioTek cell plate washer ELx405 (BioTek Instruments Inc., Winooski, VT, USA).
10. 1.5 mm thick glass cover-slip (Corning).
11. Zeiss confocal microscope Axiovert 40 (Carl Zeiss Microscopy GmbH, Jena, Germany).
12. Tecan Safire II microplate reader (Tecan, Männedorf, Switzerland).

2.3 Reagents and DMR Assays

1. Pamoic acid and D-Luciferin (Sigma Chemical Co., St. Louis, MO, USA).
2. Zaprinast (Enzo Life Sciences, Farmingdale, NY, USA).
3. YE210 (6-bromo-3-methylthieno[3,2-b]thiophene-2-carboxylic acid) (Corning).
4. SP05140, SPB05142 (CID2745687; methyl-5-[(tert-butylcarbamothioyl-hydrazinylidene)-methyl]-1-(2,4-difluorophenyl)-pyrazole-4-carboxylate), and SPB05143 (Ryan Scientific, Inc., Mt. Pleasant, SC).
5. Hank's Balanced Salt Solution (HBSS): 1× with calcium and magnesium, but no phenol red.
6. HEPES buffer: 1 M HEPES, pH 7.1.
7. Assay buffered vehicle solution: 1× HBSS, 10 mM HEPES, pH 7.1.
8. Anti-GPR35 antibody (T-14, intracellular domain) (Santa Cruz biotechnology, Santa Cruz, CA, USA).
9. Alexa Fluor® 594-labeled donkey anti-goat IgG (H+L) (Invitrogen).

2.4 Data Analysis Software

1. Microsoft Excel (Microsoft Inc., Seattle, WA, USA).
2. Epic® Offline Viewer (Corning).
3. GraphPad Prism 5 Software (Graph Pad Software Inc., La Jolla, CA, USA).
4. NIH ImageJ (http://imagej.nih.gov/ij/).

3 Methods

3.1 Cell Culture

This section describes protocols to culture HT-29 cells in biosensor microplates for DMR assays, to culture U2OS-GPR35-*bla* cells in cell assay microplates for Tango β-arrestin translocation gene reporter assay, and to culture HT-29 in 8-well chamber slide for receptor internalization assay.

3.1.1 HT-29 Cell Culture in Biosensor Microplate

1. Passage HT-29 cells in T-75 flasks using 1× trypsin/EDTA. Generally, cells are split every 5 days, and a 1:10 split is used to provide maintenance culture. The confluent cells obtained in one T-75 flask are sufficient for experimental culture in at least one biosensor microplate. The cells between 3 and 15 passages are preferred for DMR assays (*see* **Note 1**).
2. Harvest confluent cells using 1× trypsin-EDTA solution. Centrifuge the cells down, remove the supernatant, and re-suspend the cell pellet in freshly prepared complete medium for HT-29.

3. Seed 3.2×10^4 cells freshly suspended in 50 μL of the complete medium into each well of an Epic® 384-well biosensor microplate using a 16-channel electronic pipettor (*see* **Note 2**).
4. Incubate cells in the laminar flow hood for ~20 min at room temperature to minimize the commonly occurring edge effect.
5. Place the microplate inside an incubator and culture cells for ~24 h at 37 °C under air/5 % CO_2. After overnight culturing the cells usually reach ~95 % confluency and are ready for DMR assays.

3.1.2 U2OS-GPR35-bla Cell Culture in Microplate

1. Passage U2OS-GPR35-*bla* cells in T-75 flasks. The cells between 3 and 15 passages are used for Tango assay (see below).
2. Harvest confluent cells using 1× trypsin-EDTA solution. Centrifuge the cells down, remove the supernatant, and re-suspend the cell pellet in the complete medium for this cell line.
3. Seed 10,000 cells in 50 μL of the complete medium/well in 384-well, black-wall, clear bottom assay plates with low fluorescence background using a 16-channel electronic pipettor.
4. Incubate cells in the laminar flow hood for ~20 min at room temperature.
5. Place the microplate inside an incubator and culture cells for ~24 h at 37 °C under air/5 % CO_2.

3.1.3 HT-29 Cell Culture in 8-Well Chamber Slide

1. Seed 10,000 cells in 100 μL of the complete medium/well into each well of an 8-well chamber slide using an electronic pipettor.
2. Place the slide inside an incubator and culture cells for ~24 h at 37 °C under air/5 % CO_2 for overnight.

3.2 DMR Agonist Assay

This section describes the one-step protocol to detect potential agonistic activity of ligands in a given cell line. Since the DMR signal of a ligand is obtained after the cells reach confluency, it primarily reflects the effect of the ligand on the cells. The confluent cells typically give rise to a steady baseline during the assay duration (~hours). A ligand that triggers a detectable DMR is considered to have an agonistic activity in the cells. Since the DMR signal is an integrated whole cell response, the similarity in DMR signature is often useful to hypothesize that two ligands that trigger similar DMR in a cell line may activate the same pathway, sometime via the same target [26–29].

Figure 2 compares the dose responses of D-Luciferin with three known GPR35 agonists, pamoic acid [30], zaprinast [31], and YE210 [17]. All compounds triggered a dose-dependent DMR signal that shares similar characteristics; that is, all are biphasic, consisting of an early positive DMR (P-DMR) event and a late negative-DMR (N-DMR) event. Based on their P-DMR

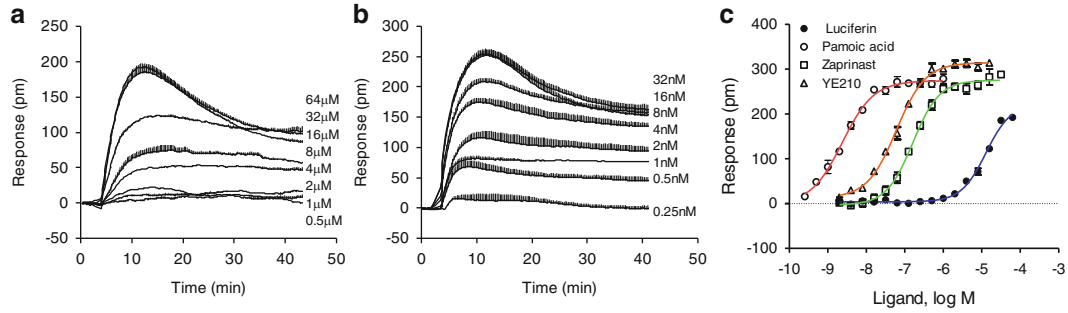

Fig. 2 The DMR characteristics of ligands in HT-29 cells. (**a**) The dose-dependent kinetic DMR signals of D-Luciferin; (**b**) the dose-dependent kinetic DMR signals of pamoic acid; and (**c**) the P-DMR amplitudes of four ligands as a function of ligand dose. All data represent mean ± s.d. ($n = 4$, except for $n = 8$ for zaprinast). This figure is reproduced from ref. 23 through the Creative Commons Attribution License

amplitudes, the DMR dose-responses of all compounds fitted well with a monophasic sigmoidal curve, leading to an EC_{50} of 0.0024, 0.064, 0.16, and 12.9 μM for pamoic acid, YE210, zaprinast, and D-Luciferin, respectively.

1. Prepare 4× compound solutions, each with 14 point 2× dilution series, by diluting the stored concentrated solutions with the assay buffered solution (1× HBSS). Prepare 2× dilution series of buffer solutions containing equal amount of dimethyl sulfoxide (DMSO) as intra-plate negative controls (*see* **Note 3**). Transfer all solutions into a 384-well polypropylene compound storage plate using a pipettor to prepare a compound source plate.
2. After culture, wash the confluent cells in the biosensor microplate twice with the HBSS using the BioTek washer, and maintain in 30 μL HBSS to prepare a cell assay plate (*see* **Note 4**).
3. Place the cell assay plate and the compound source plate inside the hotel of the Epic® system for about 1 h (*see* **Note 5**).
4. Record the baseline wavelengths of all biosensors in the cell assay microplate, normalize to zero, and record a 2–5 min baseline (*see* **Note 6**).
5. Pause the reader, and transfer 10 μL 4× of compound solutions from the compound source plate to the cell assay plate using the on-board liquid handler.
6. Run the reader immediately after compound addition (*see* **Note 7**), and record the DMR for about 1 h (*see* **Note 8**).
7. Background-correct all DMR signals (*see* **Note 9**).
8. Plot the maximal DMR response, or the DMR amplitude at specific time point post stimulation as a function of compounds, or compound doses (*see* **Note 10**).
9. Calculate the EC_{50} values of different agonists using nonlinear activation function of the Prism software.

3.3 DMR Co-stimulation Assay

This section describes the one-step protocol to determine the partial agonism of D-Luciferin using co-stimulation assay. Here, concentration-response curves to pamoic acid are performed in the presence of D-Luciferin at different fixed doses; vice versa, the concentration-response curves to luciferin are done in the presence of pamoic acid at different fixed doses. Pamoic acid behaves as a full agonist in DMR assays using HT-29 cells [23]. Results showed that the presence of D-Luciferin did not alter the potency of pamoic acid, but increased the starting signal in a dose-dependent manner (*see* Fig. 3a). Conversely, for low doses of pamoic acid (2 and 20 nM), D-Luciferin dose-dependently led to an increased DMR signal; however, for pamoic acid at 200 nM (a saturating concentration), D-Luciferin at high doses reduced the DMR in a dose-dependent manner (*see* Fig. 3b). These results suggest that the two compounds interact at an overlapping binding site, and D-Luciferin is less efficacious agonist of GPR35 than pamoic acid.

1. Prepare a series of mixtures consisting of D-Luciferin at a fixed dose and pamoic acid at 2× dilution dose series by diluting the stored concentrated solutions of pamoic acid with the D-Luciferin solution. D-Luciferin is fixed to 0, 4, 40, or 200 μM in 1× HBSS.
2. Prepare a series of mixtures consisting of pamoic acid at a fixed dose and D-Luciferin at 2× dilution dose series by diluting the stored concentrated solutions of D-Luciferin with the pamoic acid solution. Pamoic acid is fixed at 0, 8, 80, or 800 nM in 1× HBSS.

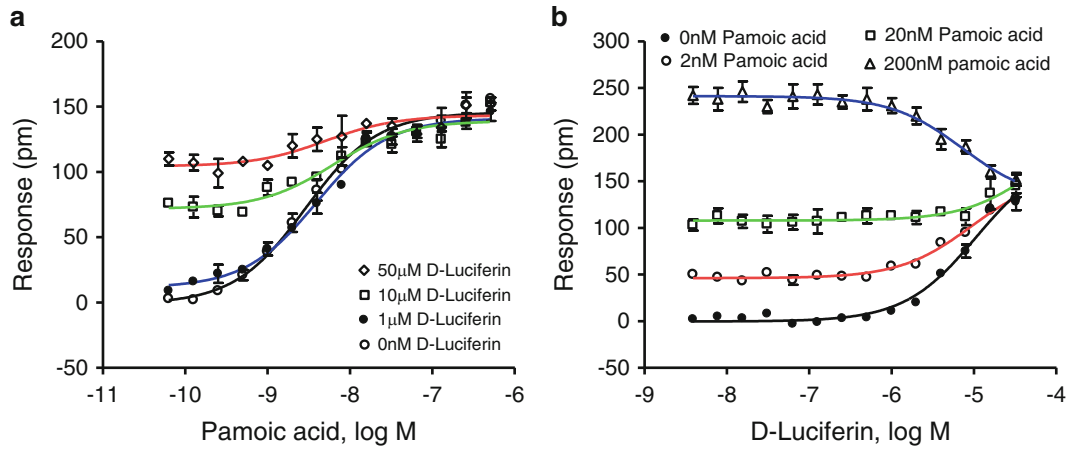

Fig. 3 The DMR arising from co-stimulation with two agonists. (**a**) The dose-dependent responses of pamoic acid in the absence and presence of D-Luciferin at three different fixed doses; the cell seeding density was 25,000 cells/well; and (**b**) the dose-dependent response of D-Luciferin in the absence and presence of pamoic acid at three fixed doses, the cell seeding density was 32,000 cells/well. The ligand P-DMR amplitudes were plotted in mean ± s.d. ($n = 4$). This figure is reproduced from ref. 23 through the Creative Commons Attribution License

3. Transfer all solutions into a 384-well polypropylene compound storage plate using a pipettor to prepare a 4× compound source plate.
4. Repeat the **steps 2–7** in Subheading 3.2 to obtain the DMR arising from pamoic acid in the absence or presence of D-Luciferin at three different fixed doses, or from D-Luciferin in the absence or presence of pamoic acid at three different fixed doses.
5. Calculate the P-DMR amplitudes using the Epic® offline viewer. Fit the dose responses with nonlinear regression activation model using the Prism software to generate EC_{50} values.

3.4 DMR Antagonist Assay

This section describes the two-step protocol to determine the competitive nature of D-Luciferin against three GPR35 antagonists, SP05140, SP05142, and SP05143. Here, the cells are pretreated with a known GPR35 antagonist at different doses for 5 min, followed by stimulation with D-Luciferin at a fixed dose (typically at its EC_{80}, or EC_{100}). SPB05142 is a known GPR35 antagonist [30], but the antagonistic activity of its analogues, SPB05140 and SPB05143, is unknown. Figure 4 shows the ability of three antagonists to block the DMR of 32 μM D-Luciferin. All three antagonists did not result in any detectable DMR in HT-29 cells, but almost completely blocked the DMR of D-Luciferin (*see* Fig. 4a), resulting in an apparent IC_{50} value of 5.0 ± 0.9 μM, 1.5 ± 0.2 μM, and 2.6 ± 0.3 μM ($n = 4$) for SPB05140, SPB05142, and SPB05143, respectively (*see* Fig. 4b), suggesting that D-Luciferin is a GPR35 agonist.

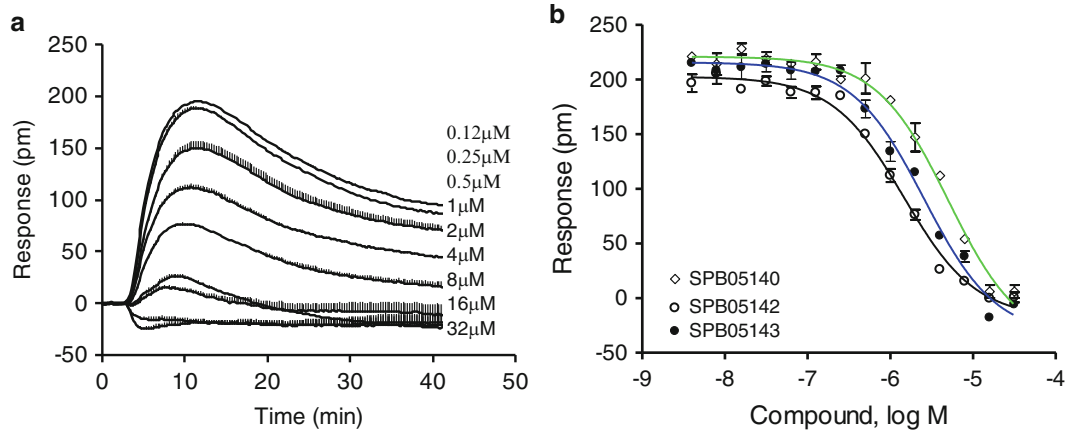

Fig. 4 The inhibition of the DMR of 32 μM D-Luciferin by three GPR35 antagonists in HT-29 cells. (**a**) The real-time kinetic response of D-Luciferin after pretreated with SPB05142 at different doses; and (**b**) the D-Luciferin P-DMR amplitudes as a function of three antagonist doses. The three GPR35 antagonists were SPB05140, SPB05142, and SPB05143 ($n = 4$). This figure is reproduced from ref. 23 through the Creative Commons Attribution License

1. After both cell and compound plates reach equilibrium inside the reader, pretreat the cells with antagonists at different doses for 5 min.
2. Establish a 2 min baseline.
3. Stimulate the cells with D-Luciferin at 32 μM.
4. Record DMR for about 1 h.
5. Background-correct all DMR signals using intra-plate negative controls.
6. Plot the P-DMR amplitudes of 32 μM D-Luciferin as a function of antagonist doses to determine their IC_{50} values using nonlinear regression.

3.5 DMR Desensitization Assay

This section describes the two-step protocol to detect the ability of D-Luciferin to desensitize the cells responding to the repeated stimulation with known GPR35 agonists. This assay is similar to the antagonist assay, except that the two steps are often separated by 1 h. Results showed that D-Luciferin dose-dependently caused HT-29 cells desensitized to the repeated stimulation with 250 nM zaprinast, leading to an apparent IC_{50} of 6.8 μM (*see* Fig. 5), which was close to the EC_{50} of D-Luciferin (12.9 μM). This result suggests that both D-Luciferin and zaprinast activate the same receptor, GPR35.

1. Prepare cell assay and compound source plates similar to the **steps 1** and **2** in Subheading 3.2.
2. Incubate cell and compound plates inside the reader for about 1 h.

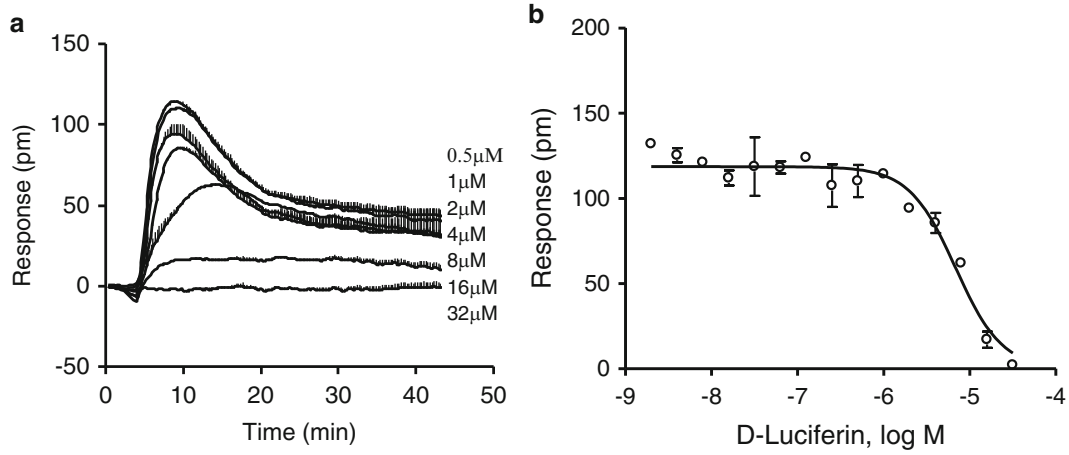

Fig. 5 The desensitization of HT-29 to 250 nM zaprinast after 1 h pretreatment with D-Luciferin at different doses. Only the zaprinast-induced DMR signals were shown: (**a**) the real-time kinetic response of zaprinast; and (**b**) the zaprinast P-DMR amplitudes as a function of D-Luciferin concentration ($n = 4$). This figure is reproduced from ref. 23 through the Creative Commons Attribution License

3. Establish a 2 min baseline.
4. Pause the reader, and add D-Luciferin dose series solutions.
5. Run the reader immediately, and record the DMR of D-Luciferin for about 1 h.
6. Stop the reader, and reestablish a second 2 min baseline.
7. Pause the reader, and add solutions of 250 nM zaprinast.
8. Run the reader immediately, and record the DMR of zaprinast for about 1 h.
9. Background-correct all DMR signals using intra-plate negative controls.
10. Plot the P-DMR amplitudes of 250 nM zaprinast as a function of D-Luciferin dose to determine its IC_{50} to desensitize the cells responding to zaprinast using nonlinear regression.

3.6 Receptor Internalization Assay

This section describes the protocol to stain GPR35 to visualize the location of GPR35 before and after stimulation with zaprinast or D-Luciferin. Results showed that similar to zaprinast, D-Luciferin resulted in remarked internalization of endogenous GPR35 in HT-29 cells (*see* Fig. 6).

1. After overnight culture, add agonist solutions to the cells and incubate at 37 °C for 1 h. Equal amount of DMSO solution is used as the negative control.
2. Fix the cells with 4% formaldehyde in 1× PBS for 15 min.
3. Permeabilize the cells with a buffer containing 4% goat serum, 0.1% bovine serum albumin (BSA), 0.1% Triton X100 in 1× PBS for 2 h. The use of serum and BSA is to block nonspecific binding of antibody.

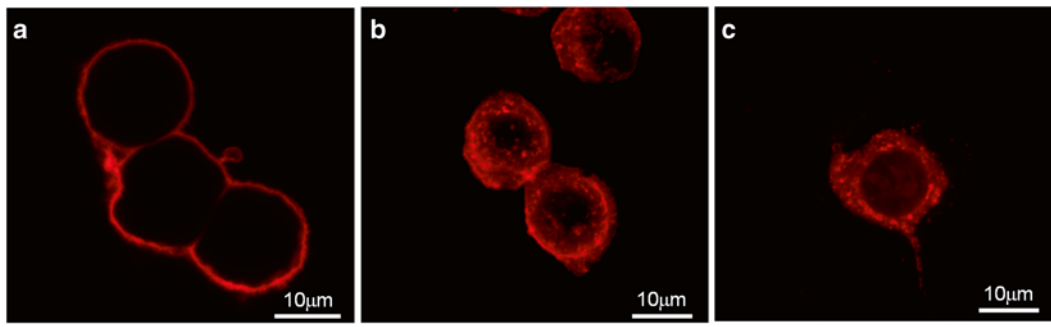

Fig. 6 Confocal fluorescence images of HT-29 after different treatments. (**a**) the assay vehicle containing 0.1% DMSO; (**b**) 32 μM D-Luciferin; (**c**) 10 μM zaprinast. The images were obtained after compound treatment for 1 h, permeabilized, stained with anti-GPR35, followed by fluorescent secondary antibody. *Red*: GPR35 stains. Representative images obtained from two independent measurements were used. This figure is reproduced from ref. 23 through the Creative Commons Attribution License

4. Wash the cells with PBS for 5 min, and incubate with the anti-GPR35 (1:500) in 3% BSA/PBS buffer for 24 h (*see* **Note 11**).
5. Incubate with the secondary antibody Alexa Fluor® 594 donkey anti-goat IgG (H + L) (1:500) in 3% BSA/PBS for 1 h at room temperature.
6. Wash the cells once with PBS, and seal the cells with 1.5 mm thick glass cover-slip.
7. Store the dried slide at 4 °C until imaging.
8. Perform confocal imaging with Zeiss confocal microscope Axiovert 40.
9. Analyze the images using NIH Image J software.

3.7 Tango β-Arrestin Translocation Assay

This section describes the protocol to perform Tango β-arrestin translocation assay using U2OS-GPR35-*bla* cells. This assay measures gene reporter activity arising from GPR35 activation. Agonist binding to GPR35 causes recruitment of protease tagged β-arrestin to GPR35 at the cell plasma surface. The protease then cleaves a protease cleavage site that links a transcription factor with the receptor C-terminus, thus releasing the transcription factor, which then translocates to the nucleus and activates the expression of β-lactamase. Results showed that zaprinast, pamoic acid, and D-Luciferin all led to a dose-dependent response in U2OS-GPR35-*bla* cells, yielding an EC_{50} of 5.0 μM, 9.4 μM, and 277 μM, respectively (*see* Fig. 7). Interestingly, in this assay zaprinast acted as a full agonist, pamoic acid a strong partial agonist, D-Luciferin a weak partial agonist.

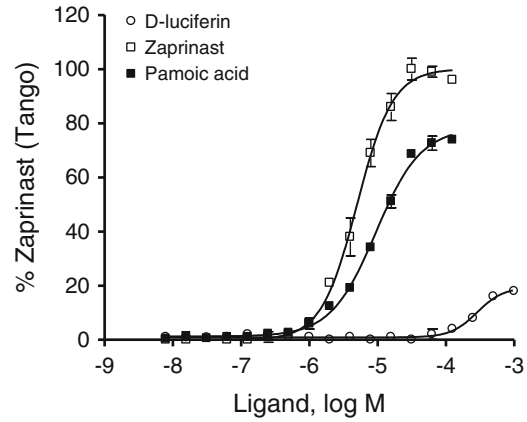

Fig. 7 D-Luciferin caused β-arrestin translocation via GPR35. The dose-dependent responses of three ligands as measured using Tango™ β-arrestin translocation gene reporter assays. The data represents mean ± s.d. from two independent measurements, each in duplicate ($n = 4$). This figure is reproduced from ref. 23 through the Creative Commons Attribution License

1. After overnight culture in 384-well, black-wall, clear bottom assay plate with low fluorescence background, treat the cells with ligands for 5 h in a humidified 37 °C/5 % CO_2.
2. Add the cell permeable LiveBLAzer™ FRET B/G substrate to the cells.
3. Incubate for 2 h, and measure the coumarin:fluorescein ratio using Tecan Safire II microplate reader.
4. Background-correct all responses using intra-plate negative controls.
5. Normalize all coumarin:fluorescein ratios to the zaprinast maximal responses using an intra-plate referencing protocol; that is, a dose response of zaprinast is obtained within the same plate, and a compound response is then normalized to the maximal response of zaprinast which is set to be 100 %.
6. Determine the potency and efficacy of D-Luciferin.

4 Notes

1. Cells can undergo genetic drifting during passaging. Given the high sensitivity of DMR assays, the DMR of a ligand may be sensitive to passages of cells under culture.
2. The biosensor microplate is tissue culture treated and ready to use. However, aged biosensor microplate may become hydrophobic, resulting in air bubbles during cell seeding and possible cell death. Pre-incubation with the complete medium followed by one-time rinse often solves this problem. When air bubble is formed and trapped in the bottoms of the wells, one can use the pipettor to manually remove air bubble(s) after seeding.
3. Many compounds are stored in DMSO. DMSO is a solvent with dual effects on biosensor cell assays; namely, bulk index effect due to its high refractive index, and nonspecific effect on cells due to its biological activity. Thus, it is important to minimize the DMSO concentration in the final compound solution (typically <0.1 v/v%).
4. Many cell types are sensitive to mechanical disturbance during the wash. Receptor signaling is also often sensitive to buffer conditions (e.g., salts, growth factors in medium, pH, etc.). Thus, it is important to optimize the wash protocol so gentle and thorough wash is warranted to obtain optimal assays.
5. RWG biosensor measures changes in local refractive index in living cells upon stimulation. Given that refractive index is sensitive to temperature, any temperature mismatch between the two solutions could cause noticeable interference. A pre-equilibrium step is important to minimize the thermal mismatch effect.

6. Confluent cells generally give rise to a steady baseline. However, washing and thermal equilibrium steps may cause baseline drifting. So, baseline monitoring for a short period of time is essential to high quality data acquisition. Typically, the drifting that is <10 picometer (pm in wavelength shift) within 5 min is considered to be steady and acceptable.
7. The activation of certain receptors may lead to rapid on-set response. Thus, it is important to minimize the blackout period between the baseline and the first recording after compound addition. The blackout period is ideally <1 min.
8. The DMR recording duration is dependent on applications. For drug pharmacology profiling and receptor biology study, it is typically about 1 h. For long-term effect of drugs, it can be long as several days. The temporal resolution of DMR recording can also be variable and adjustable (typically in the range of 3 s to 1 min).
9. Background could be aroused from multiple sources, such as temperature mismatch, plate movement, mechanical disturbance during compound addition, and buffer composition mismatch. Most of background signals can be corrected by using intra-plate negative controls, which contain equal amount of DMSO used for compound solutions; or DMSO dose series equal to the compound dose series. Alternative solvents such as ethanol or water can be used if necessary.
10. DMR is a real-time kinetic response, thus enabling the extraction of multiple kinetic parameters for analyzing drug pharmacology [32–36]. These parameters include the transition time from one to another event, and the amplitudes, duration, and kinetics of each event. For a panel of agonists for the same receptor, multi-parameter analysis is useful to examine biased agonism.
11. The specificity of anti-GPR35 was confirmed by the control peptide from the supplier. Staining showed that the control peptide completely blocked the staining of HT-29 cells with the anti-GPR35 antibody.

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