Supplementary materials

Discovery of natural phenols as G protein-coupled receptor-35 (GPR35) agonists

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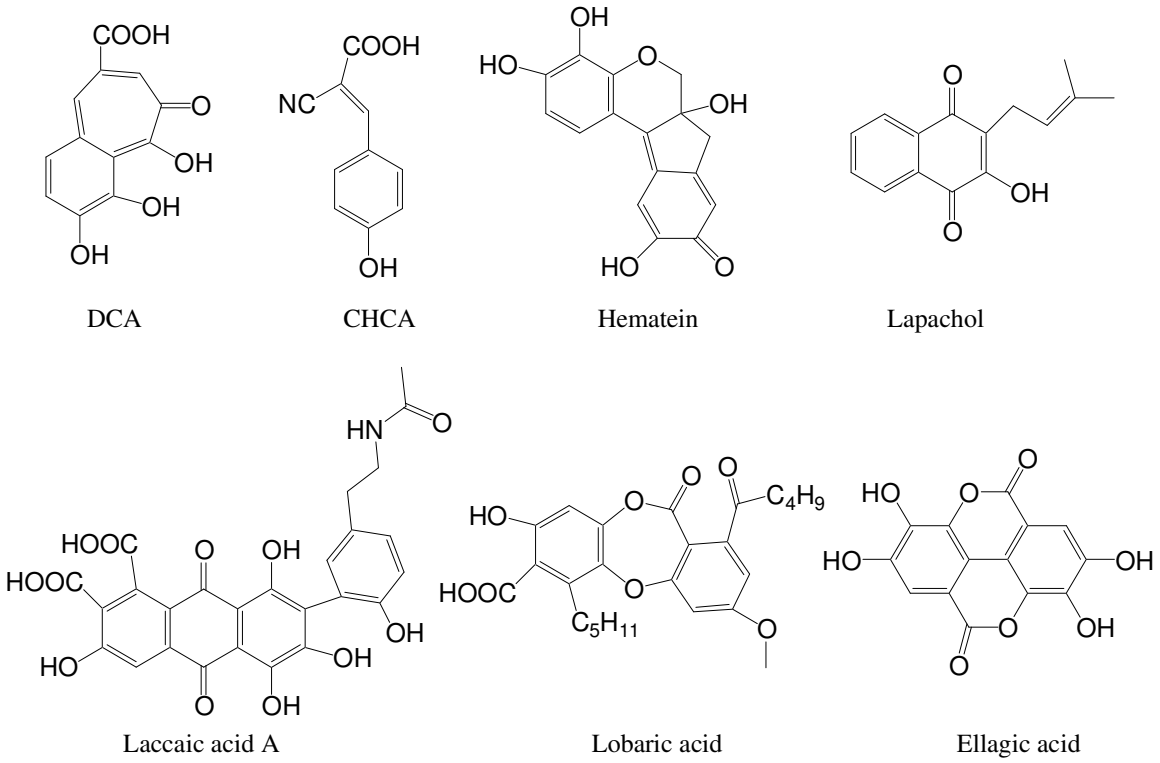
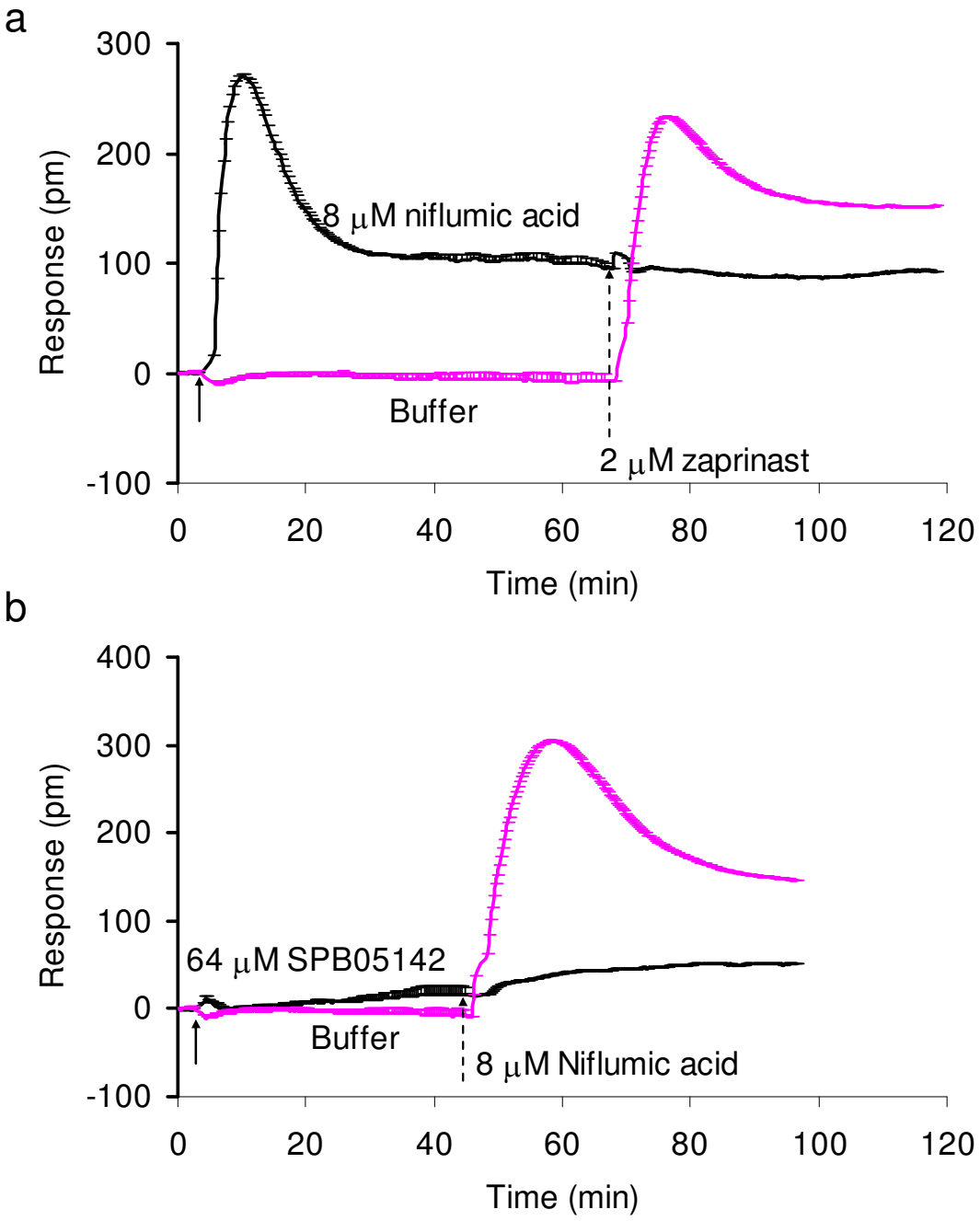
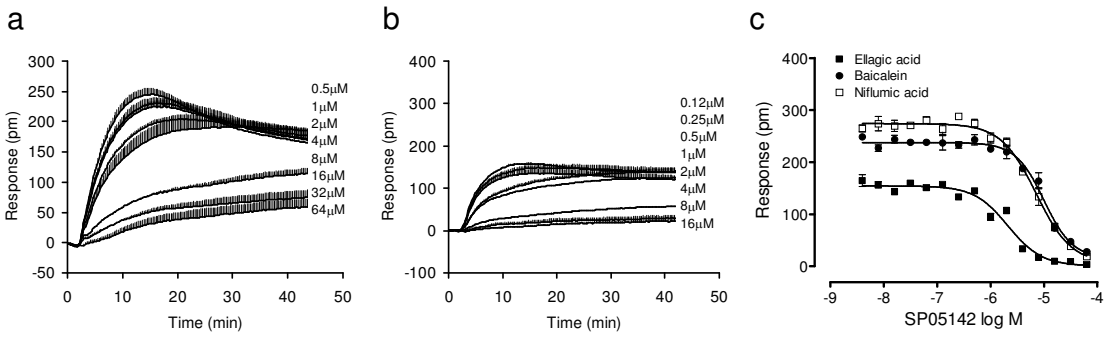


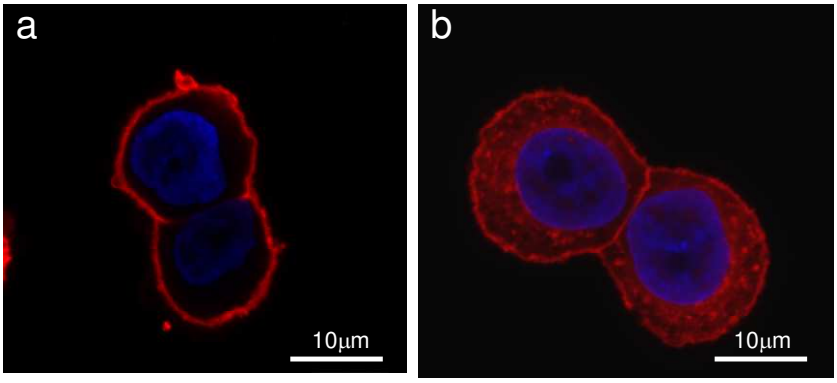
Supplementary Figure 1 Structures of 7 natural product GPR35 agonists identified.



Supplementary Figure 2 DMR desensitization and antagonism assays to characterize GPR35 agonist niflumic acid in HT-29 cells. (a) Two-step real time DMR desensitization assays. The cells were initially stimulated with a GPR35 agonist or the assay buffer, followed by repeated stimulation with zaprinast at a fixed dose (2 μM). The zaprinast response, after re-establishing baseline 2 min before the 2nd stimulation, was used as an indicator for the agonist to cause desensitization. Niflumic acid was used as an example. (b) Two-step real time DMR antagonism assays. The cells were initially pretreated with a GPR35 antagonist (SPB05142) or the assay buffer, followed by stimulation with a GPR35 agonist at a fixed dose (typically its EC_{80} or EC_{100}). The agonist response, after re-establishing baseline 2 min before the agonist stimulation, was used as an indicator for the antagonist to block the DMR of the agonist. Niflumic acid was used as an example. The data represents mean±s.d. from 2 independent measurements, each in 4 replicates (n=8).



Supplementary Figure 3 Dose-dependent inhibition of the DMR signals of representative GPR35 agonists by the antagonist SP05142. (a) Real time DMR of 32μM baicalein in the presence of SP05142 at different doses; and (b) Real time DMR of 2μM ellagic acid in the presence of SP05142 at different doses; (c) The amplitudes 10min poststimulation as a function of SP05142 doses. The data represents mean±s.d. from 2 independent measurements, each in 4 replicates (n=8).



Supplementary Figure 4 Myricetin triggered GPR35 internalization in HT-29 cells. Representative confocal fluorescence images of HT-29 under different conditions: (**a**) treated with the assay vehicle containing 0.1% DMSO; (**b**) treated with 8 μM myricetin. The images were obtained after compound treatment for 1hr, permeabilized, stained with anti-GPR35, followed by fluorescent secondary antibody. Red: GPR35 stains; Blue: nuclei stains with DAPI. Representative images obtained from 2 independent measurements were used.

Materials and Methods

Compounds and Reagents

Baicalein, apigenin, ellagic acid, luteolin, niflumic acid and quercetin were obtained from Tocris Chemical Co. (St. Louis, MO). Zaprinast was obtained from Enzo Life Sciences (Plymouth Meeting, PA). Hematein, laccacid acid A, lapachol, lobaric acid, morin hydrate, myricetin, 3,7-dihydroxylflavone, 3-hydroxyflavone, fisetin, kaempferol, quercetin pentamethyl ether, gossypetin, gossypin, hieracin, diosmetin, chrysin, primuletin, 7-hydroxyflavone, α-cyano-4-hydroxycinnamic acid (CHCA) and 7-deshydroxypyrogallin-4-carboxylic acid (DCA) were obtained from Sigma Chemical Co. (St. Louis, MO). SPB05142 was obtained from Ryan Scientific, Inc. (Mt. Pleasant, SC). YE210 and Epic® 384-well biosensor microplates were obtained from Corning Inc. (Corning, NY, USA). The library consist-

ing of 880 natural products was obtained from Prestwick Chemicals. Compounds were formatted into 96-well daughter sets at 10 mM in 100% DMSO and active working sets stored at −80°C until required. All screens were done at 10 μM for each compound.

Cell Culture

Human colorectal adenocarcinoma HT-29 was obtained from American Type Cell Culture (Manassas, VA, USA). The cells were cultured in McCoy's 5a Medium Modified supplemented with 10% fetal bovine serum, 4.5g/liter glucose, 2 mM glutamine, and antibiotics at 37°C under air/5% CO_2. Tango™ GPR35-bla U2OS cells were purchased from Invitrogen. The cells were cultured according to the protocols recommended by the supplier. Briefly, the cells were passed using McCoy's 5A medium (Invitrogen 16600-082) supplemented with 10% dialyzed fetal bovine serum, 0.1 μM NEAA, 25 μM Hepes (pH 7.3), 1mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml zeocin, 50 μg/ml hygromycin, and 100 μg/ml geneticin in a humidified 37°C/5% CO_2 incubator.

Dynamic mass redistribution (DMR) screens and profiling

Epic® system (Corning Inc., Corning, NY), a wavelength interrogation reader system tailored for resonant waveguide grating biosensors in microtiter plates, was used for dynamic mass redistribution (DMR) assays. 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 ~15sec. For whole cell DMR assays, a common cell preparation procedure was used – that is, cells were directly seeded in Epic® plates and cultured overnight to form confluent monolayer in the corresponding cell culture medium; and the cells were then washed twice using plate washer, and maintained with Hank's balanced salt solution (1x HBSS) and further incubated inside the system for 1hr before measurements.

For DMR agonism screen, a 2-min baseline was first established after the cell preparation step. Immediately after the compound addition using the onboard liquid handler, the cellular responses were recorded in real time for about 1hr. The amplitudes at 10min poststimulation, recorded as the shift in resonant wavelength in picometer (pm), were used as a parameter for identifying agonist hits. A compound whose DMR signal amplitude is between 100 and 400pm was considered to be a hit, considering the DMR characteristics of zaprinast in HT-29 cells. For DMR desensitization screens, cells were initially treated with compounds for 1 hr, followed by stimulation with zaprinast at a fixed dose (1 or 2μM). The cellular responses induced by zaprinast were recorded in real time, and used to identify compounds that cause desensitization (**Supplementary Figure 2**).

All EC_{50} or IC_{50} described in the main text were calculated based on the amplitudes of DMR signals at 10 min post agonist stimulation. For primary screens, compounds, each in duplicate set, were assayed at 10 μM with 0.1% DMSO. Cellular responses induced by the assay buffer with 0.1% DMSO were used as negative controls. All DMR signals were background corrected. Cellular responses induced by 10 μM zaprinast were used as positive controls. The assay robustness, as measured by intra-plate and inter-plate Z'-factor, was calculated and found to be greater than 0.65 for both DMR agonism and DMR antagonism screens.

Three DMR assays were used to ascertain the specificity of GPR35 agonists identified; these assays are DMR agonism assay to detect the DMR induced by the agonist compound, DMR desensitization assay to determine the ability of GPR35 agonist compounds identified to cause receptor desensitization, and DMR antagonism assay to determine the ability of the known GPR35 antagonist SPB05142 to block the DMR of GPR35 agonist compounds.

Tango™ β-arrestin translocation gene reporter assays

Tango™ GPR35-*bla* U2OS cells were obtained from Invitrogen. This cell line stably expresses two fusion proteins: human GPR35 linked to a TEV protease site and a Gal4-VP16 transcription factor, and β-arrestin/TEV protease fusion protein. The cell line also stably expresses the β-lactamase reporter gene under the control of a UAS response element. The cells were cultured according to the protocols

recommended by the supplier. Briefly, the cells were passed using McCoy's 5A medium (Invitrogen 16600-082) supplemented with 10% dialyzed fetal bovine serum, 0.1 μM NEAA, 25 μM Hepes (pH 7.3), 1mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml zeocin, 50 μg/ml hygromycin, and 100 μg/ml geneticin in a humidified 37°C/5% CO_2 incubator.

The activation of GPR35 by agonists leads to the recruitment of β-arrestin/TEV protease fusion proteins to the activated GPR35. As a result, the protease cleaves the Gal4-VP16 transcription factor from the receptor, which then translocates to the nucleus and activates the expression of β-lactamase. Briefly, 10000 cells per well were seeded in 384-well, black-wall, clear bottom assay plates with low fluorescence background (Corning), and cultured in Dulbecco's modified eagle medium (Invitrogen,10569-010) supplemented with 10% dialyzed fetal bovine serum, 0.1 μM non-essential amino acids, 25 μM Hepes (pH 7.3), 100 U/ml penicillin, and 100 μg/ml streptomycin. After overnight culture, the cells were stimulated with ligands for 5 hrs in a humidified 37°C/5% CO_2, and then loaded with the cell permeable LiveBLAzer™ FRET B/G substrate. After the two hour incubation the coumarin to fluorescein ratio was measured using Tecan Safire II microplate reader (Männedorf, Switzerland). In the absence of β-lactamase expression (i.e., no GPR35 activation), cells generate green fluorescence. In the presence of β-lactamase expression upon receptor activation, the substrate is cleaved and the cells generate blue fluorescence. The coumarin to fluorescein ratio was used as a normalized reporter response.

For primary screens, compounds, each in duplicate set, were assayed at 10 μM with 0.1% DMSO. Cellular responses induced by the assay buffer with 0.1% DMSO were used as negative controls. Cellular responses induced by 8 μM zaprinast were used as positive controls. The assay robustness, as measured by intra-plate and inter-plate Z'-factor, was calculated and found to be greater than 0.55 for Tango screens. Specifically, the intra-plate Z' was found to be 0.68, 0.55, 0.61, 0.72, 0.87, 0.77, 0.79, and 0.79 for plates 1 to 8.

Receptor internalization assays

HT-29 cells were plated on an 8-well chamber slide (Nalge Nunc International, Rochester, NY, USA) with a seeding density of 10,000 cells per well and incubated at 37˚C for 24 hrs. Next day, cells were stimulated with a compound or equal amount of DMSO at 37˚C for 1 hr. Afterwards, cells were fixed with 4% formaldehyde in 1 x PBS for 15 min, followed by blocking and permeabilization in a buffer containing 4% goat serum, 0.1 % bovine serum albumin (BSA), 0.1% Triton X100 in 1 x PBS for 2 hrs. After 5 min wash with PBS, fixed cells were incubated with the anti-GPR35 (1:500) (anti-GPR35, T-14, intracellular domain) (Santa Cruz biotechnology, Santa Cruz, CA, USA) in 3% BSA/PBS buffer for 24 hrs, followed by incubation with secondary antibody Alexa Fluor® 594 donkey anti-goat IgG (H+L) (1:500) (Invitrogen) in 3% BSA/PBS for 1 hr at room temperature. Cells were finally washed once with PBS and sealed with 1.5 mm thick glass cover-slip (Corning, NY). Dried slides were stored at 4˚C until imaging. Confocal imaging was performed with Zeiss confocal microscope Axiovert 40. 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. The collected images were analyzed using MacBiophotonics Image J software (http://www.macbiophotonics.ca/downloads.htm).