Electronic Supplementary Material (ESI) for Chemical Communications

< Supporting Information >

Exploiting the mechanism of cellular glucose uptake to develop an image-based high-throughput screening system in living cells

Ala Jo,[a] Jongmin Park[b] and Seung Bum Park*[a, b]

[a]Department of Chemistry, and [b]Department of Biophysics and Chemical Biology,

Seoul National University, Seoul 151-747, Korea.

Corresponding Author E-mail : sbpark@snu.ac.kr

1. General information and materials

^{1}H and ^{13}C NMR spectra were recorded on a Agilent 400-MR DD2 [Agilent, USA], and chemical shifts were measured in ppm downfield from internal tetramethylsilane (TMS) standard. Product mass analyses were performed on LC/MS system equipped with a reverse phase column (C-18, 50 x 2.1 mm, 5 μm) and photodiode array detector using electron spray ionization (ESI) or atmospheric pressure chemical ionization (APCI). Reverse phase HPLC analysis was performed on a VPODS C-18 column (150 x 4.6 mm) at a flow rate of 1.0 mL/min for analysis, and PRC-ODS C-18 column (250 x 20 mm) at a flow rate of 10.0 mL/min for preparation, Shimadzu LC-6AD pump, SPD-10A detector (Japan). HPLC solvents consist of water containing 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B). L-(–)-glucose, 2,4-dinitrophenol, phloretin, acetic anhydride, sodium methoxide (0.5 M solution in methanol), benzoyl chloride, pyridine, DMF, DMSO and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. *N*,*N*-Diisopropylethylamine, triethylamine, and 2-bromoethanol were purchased from TCI. Ez-cytox kit was purchased from Daeil Co. and was used for the cell viability test. Hoechst 33324 and 6-NBDG were purchased from Invitrogen.

Fluorescence microscope, HTS equipment, and analysis program for biological cell imaging experiment

We carried out fluorescence microscopy studies with Olympus Inverted Microscope Model IX71, equipped for epi-illumination using a halogen bulb [Philips]. Emission signal of each experiments were observed at a spectral setting: red channel using a 510–550 band pass exciter filter, a 570 nm center wavelength chromatic beam splitter, a 590 nm-long pass barrier filter [Olympus]. Emission signal of each experiments were detected with 12.5M pixel recording digital color camera [Olympus]. Quantification of fluorescence images was analyzed by Image-Pro Plus® 6.2 program and all graphs were figured by GraphPad Prism 5. The quantified data are the mean of fluorescent signal measurements of 40–50 cells from at least three different independent experiments. HTS was performed by InCell analyzer 2000 [GE Healthcare] and fluorescence images were analyzed by InCell analyzer 1000 workstation 3.6 program. For the Ez-cytox-based cell cytotoxicity test, the absorbance of 96-well plate was measured by BioTek Synergy HT Microplate reader.

2. General procedure for biological experiments

Cell culture

C2C12 (mouse myoblast) cells were obtained from ATCC [American Type Culture Collection]. C2C12 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution at 37 ℃ in an atmosphere of 5% CO_2.

Competition test in C2C12 myoblasts

To measure the competitive cellular uptake of GB2 and L-GB2, C2C12 myoblasts cultured on a cover glass bottom dish were incubated for 30 min at 37 ℃ in the presence of individual fluorescent glucose bioprobes, GB2 and L-GB2 in DMEM containing 55 mM D-glucose, 11 mM D-glucose, or no glucose. The final concentration of fluorescent glucose analogs was adjusted to 10 μM. After washing with cold PBS, fluorescence images were obtained at an excitation wavelength of 540 nm using a fluorescence microscope (Olympus IX71).

Inhibition of endocytosis in C2C12 myoblast cells

C2C12 myoblasts cultured on a cover glass bottom dish were incubated for 30 min with GB2 or L-GB2. The final concentration of fluorescent glucose analogs was adjusted to 10 μM. During the incubation, temperature was adjusted to 37 ℃ or 4 ℃. After washing with cold PBS, fluorescence images were obtained at an excitation wavelength of 540 nm using a fluorescence microscope (Olympus IX71).

Radiolabeled 2-deoxyglucose uptake measurement

C2C12 myoblasts cultured on a 24-well plate were treated with 10 μM of compounds (P33A04, P33D04, P33F06, P33G07 and DMSO) and incubated for 24 h at 37 ℃ with 5% CO_2. After washing with cold PBS, cells were treated with ^{14}C-labeled 2-deoxyglucose (0.1 μCi/well) in Hepes-buffered saline (HBS) and incubated for 10 min at 37 ℃ with 5% CO_2.[1–2] The cells were then washed 3 times with PBS, added 50 μL of RIPA buffer, and kept on ice for 2 h. After cell lysis, centrifuge for 10 min at 15,000 g and collect supernatants, and measured radioactivity using Liquid Scintillation Counter.

Non-radiolabeled 2-deoxyglucose uptake measurement

C2C12 myoblasts cultured on a 6-well plate were treated with 10 μM of compounds (P33A04, P33D04, P33F06, P33G07 and DMSO) and incubated for 24 h at 37 ℃ with 5% CO_2. After washing with cold PBS, cells were treated with 1 mM 2-deoxyglucose in PBS and incubated for 20 min at 37 ℃ with 5% CO_2. After washing three times with PBS, cells were harvested, added 500 μL of 10 mM Tris-HCl buffer, and sonicated with Sonomacher. After heat inactivation at 80 ℃ for 15 min and centrifugation at 15,000 g for 10 min, the supernatants were collected. We measured the amount of 2-deoxyglucose-6-phosphate according to the protocol of 2-deoxyglucose (2DG) Uptake Measurement Kit [COSMO BIO Co., ltd.]

High-throughput screening (HTS) using InCell Analyzer 2000

C2C12 myoblast cells were seeded on black well and clear bottom 96 plate (2×10^3 cells/well) and incubated at 5 % CO_2, 37 ℃ for overnight. Various chemicals from our in-house pDOS library[3] were treated to the designated cells with pin tool with 10 μM final concentration for 24 h. GB2 (5 μM) and Hoechst 33342 (2 μg/mL) were added to individual cells. After 30 min incubation at 37 ℃, the cells were washed twice with PBS containing 20 μM phloretin (pPBS) and prepared for imaging with adding 100 μL of pPBS per well.

Fluorescence images of the resulting plate were taken automatically by InCell Analyzer 2000. The fluorescence images of GB2 were captured with excitation filter at 543±11 nm with 1.3 sec of exposure and emission filter in Red channel. The fluorescence images of Hoechst 33342 were captured with excitation filter at 350±25 nm with 0.3 sec of exposure and emission filter in Blue channel.

***In Vitro* Cytotoxicity Test**

Cell viability was measured by the EZ-cytox assay kit, and the experimental procedure was followed bythe manufacturer's manual. Briefly, cells were cultured into 96-well plates at a density of 3×10^3 cells/well for 24 h, followed by the treatment of individual compounds at various concentrations. After 24 h incubation at 37 ℃, 10 μL of WST-1 solution [2-(4-nitrophenyl)-5-(2-sulfophenyl)-3-[4-(4-sulfophenylazo)-2-sulfophenyl]-2H-tetrazolium disodium salt] was added to each well, and the resulting plates were incubated for an additional 10 min at 37 ℃. Absorbance in 455 nm was measured by microplate reader. The percentage of cell viability was calculated by following formula: % cell viability = (mean absorbance in test wells)/(mean absorbance in control well) × 100. Each experiment was performed in triplicate.

HPLC analysis for monitoring cellular metabolite of GB2

C2C12 myoblast cells were treated with GB2 for 30 min and washing three times with cold pPBS. The resulting cells were treated with RIPA buffer and stored at –78℃ for 30 min. The lysed cells were centrifuged at 15,000 g for 10 min. Then, the supernatant was collected and added with cold acetone to precipitate proteins. After centrifugation at 15,000 g for 10 min, the supernatant was collected and lyophilized to remove all liquids. The resulting residue was solubilize with methanol and subjected to HPLC analysis to identify the GB2 recovered from cytoplasm. The HPLC analysis was performed using UV detector at 550 nm for the selective monitoring of GB2 with the UV absorbance of Cy3 dye.

3. Intracellular fate of D-glucose and GB2

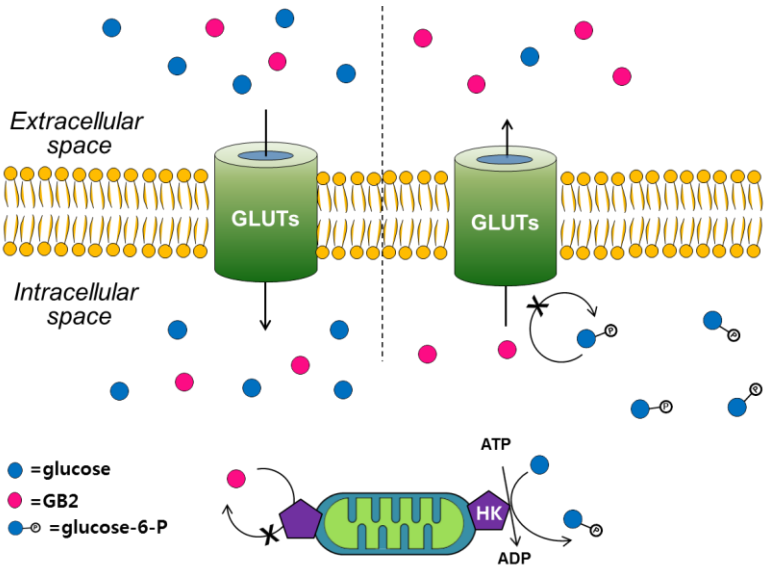


Figure S1. Schematic of the physiological behavior of D-glucose and GB2. As illustrated in Figure S1, hexokinases (HKs) serve not only as the initial gateway for glucose metabolism but also as the retaining mechanism of an essential cellular nutrient inside the cells in the form of glucose-6-phosphate (Glc-6-P). ^{18}FDG and 2-NBDG are known to be phosphorylated by HKs as glucose. Therefore, ^{18}FDG and 2-NBDG can be trapped within cells for an extended period of time, which might be essential to monitor the distribution of the glucose analog, although the phosphorylation of 2-NBDG leads to rapid degradation into non-fluorescent products. In our study, we observed a substantial decrease of fluorescent signals after washing GB2-treated cells with phosphate-buffered saline (PBS). Similar to 6-NBDG, GB2 is not a substrate for hexokinases (Fig. S2); thus, GB2 cannot be retained inside the cell. Therefore, a conventional bioimaging procedure will lead to significant time-dependent loss of fluorescent signals, which might be caused by the escape of non-phosphorylated GB2 from the cells through GLUTs due to its reversed concentration gradient after washing with PBS.

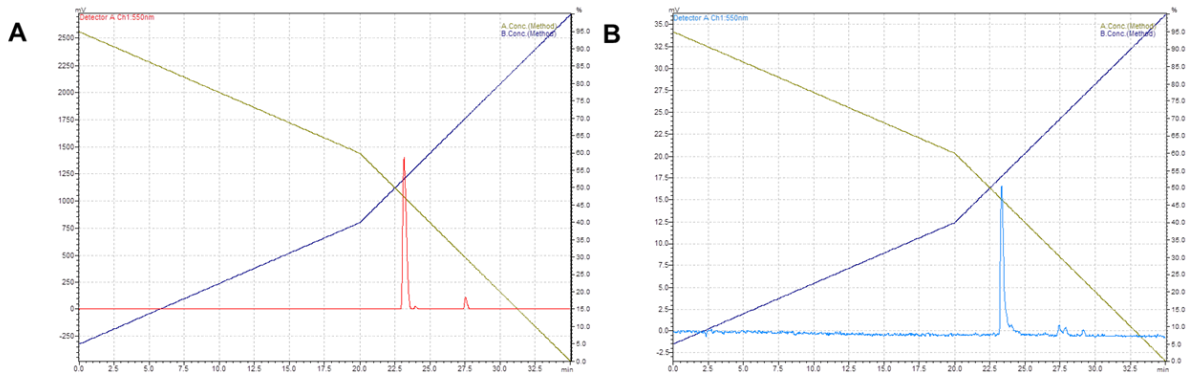


Figure S2. Intracellular fate of GB2 after its cellular uptake. (A) GB2 as an authentic sample. (B) Cellular metabolite of GB2 from C2C12 cell lysate after cellular uptake for 30 min. The recovered fluorescent material from cytoplasm was identical to original GB2, which confirms that GB2 is not the substrate of hexokinases.

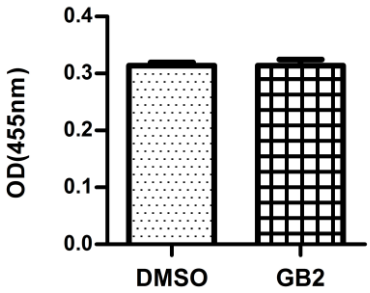


Figure S3. Cell viability test for GB2. After the incubation with GB2 at 10 μM concentration for 30 min, C2C12 cells were washing twice with PBS. Cell viability was measured by the EZ-cytox assay kit. The graph shows the average value of duplicate experiments and the error bars indicate SEM. The morphology of C2C12 cells was not affected by the treatment of GB2.

4. Application of phloretin to GB2-based bioimaging in living cells

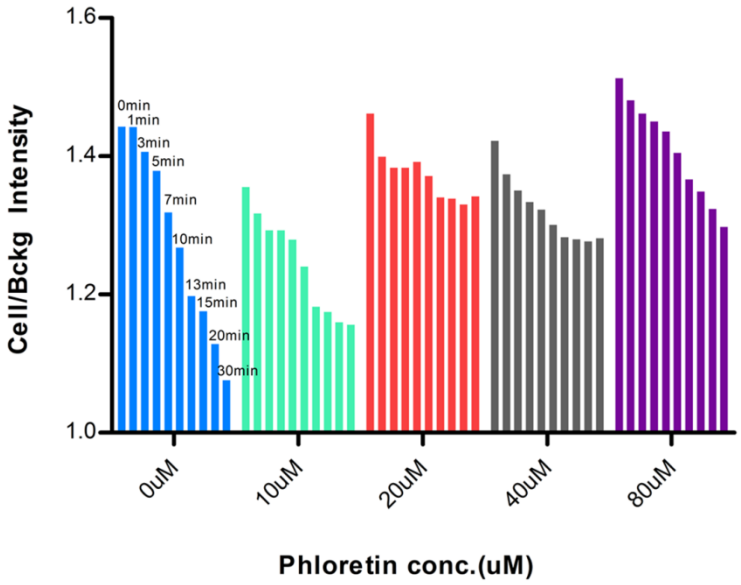


Figure S4. Optimization of the phlorein treatment condition in living cells. After the incubation with GB2 for 30 min, C2C12 cells were washing twice with PBS containing various concentration (none, 10 μM, 20 μM, 40 μM. 80 μM) of phloretin. The fluorescence intensity within cytoplasm was continuously monitored over 30 min in the presence of various concentration of phloretin. The treatment of 20 μM phloretin effectively blocked the efflux of GB2 under this experimental condition.

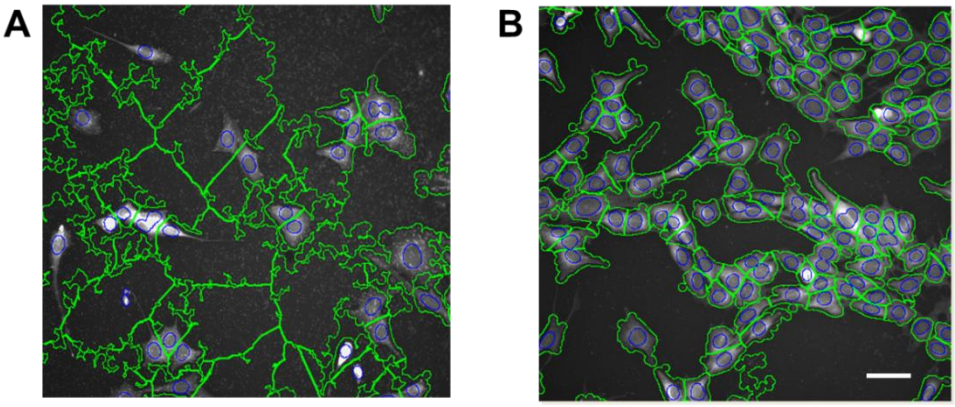


Figure S5. Effect of phloretin on the quantitative analysis of cellular fluorescence intensity in the high-throughput screening system. After the treatment of GB2 and subsequent washing with (A) PBS or (B) pPBS, fluorescence images were taken by InCell analyzer 2000 and fluorescence images were automatically analyzed by InCell analyzer 1000 workstation 3.6 program. Blue line represents the automatically established boundary for cellular nucleus and green line represents automatically established boundary for individual cells. The scale bar represents 20 μm. The imaging condition A failed the automatic recognition of cell boundary due to the extensive efflux of GB2.

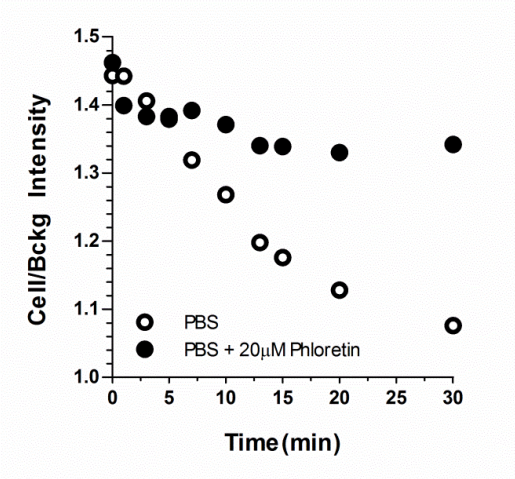


Figure S6. Continuous monitoring of GB2 fluorescence intensity within the cytoplasm over 30 min in the presence and absence of phloretin (20 μM).

5. Image-based HTS assay protocol and pilot screening

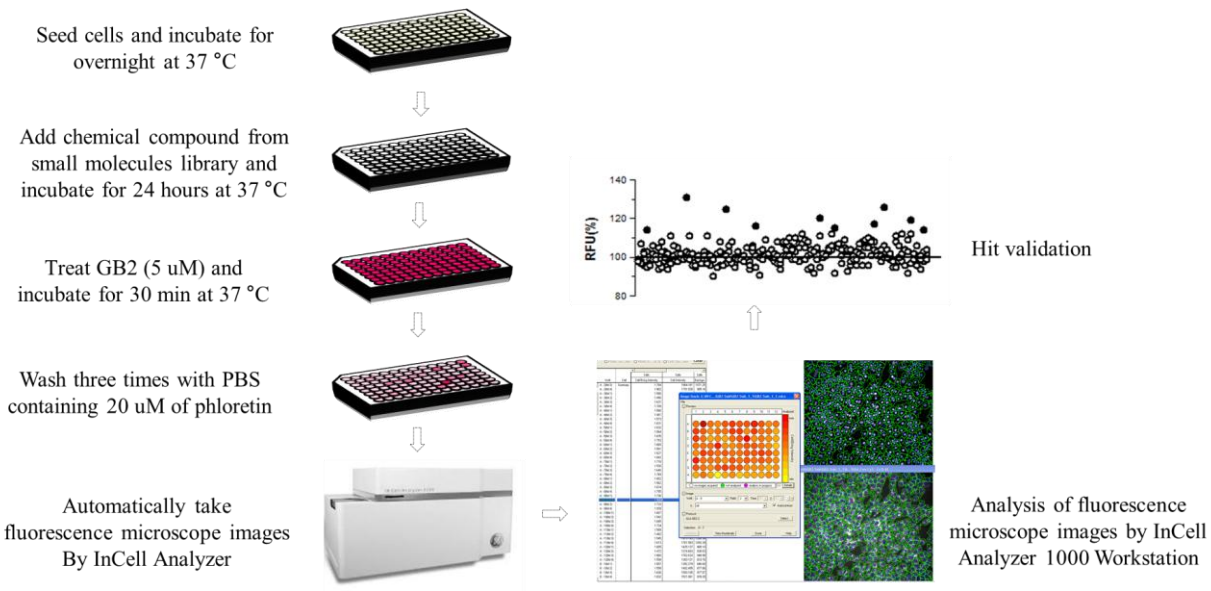


Figure S7. Construction of image-based high-throughput screening (HTS) assay. Screening protocol for image-based HTS using GB2 to monitor cellular glucose uptake.

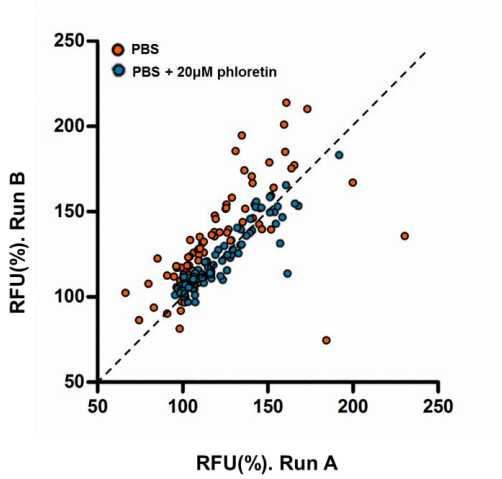


Figure S8. Reproducibility of GB2 based image-based HTS assay was validated by the cross-confirmation of 2 independent screenings performed in both cases (with or without phloretin). Suppression of GB2 efflux using pPBS significantly enhanced the reproducibility of the HTS assay. Using our image-based HTS protocol, we then performed the pilot screening in duplicate by using 88 representative compounds from our in-house pDOS library[2] in the absence or presence of phloretin to demonstrate the accuracy and reproducibility of our HTS assay. Two independent assay data in both cases (with or without phloretin) were utilized to confirm the assay reproducibility by showing their linearity. As shown in Figure S8, we observed improved linearity between the 2 independent assay data when the image-based HTS was performed in PBS containing 20 μM phloretin (pPBS). These results indicate that our phenotype-based screening system meets the criteria of a practical HTS assay for drug discovery owing to its robustness, reproducibility, and accuracy.

6. Image-based comparison of GB2 and 6-NBDG

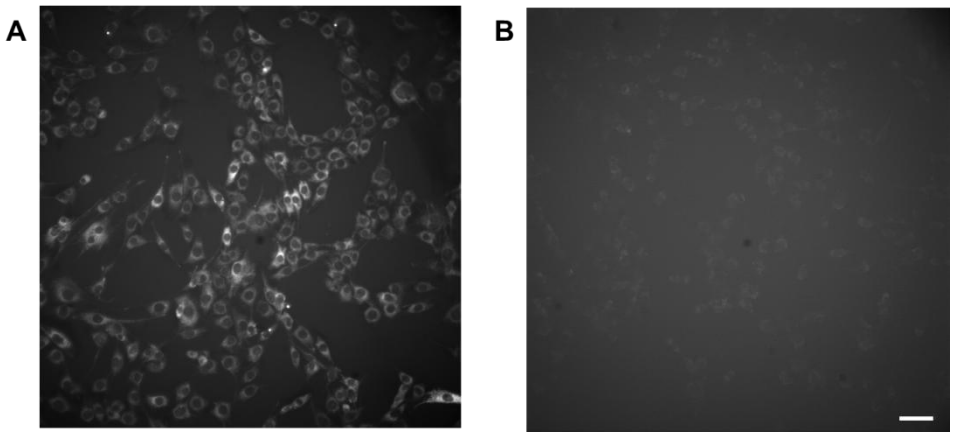


Figure S9. Comparison of GB2 and 6-NBDG with our image-based HTS assay system. Cellular image of C2C12 cells after treatment with (A) GB2 (5 μM) and (B) 6-NBDG (20 μM) for 30 min, followed by washing twice with PBS containing 20μM phloretin. Fluorescence microscope images were captured using InCell Analyzer 2000 with same exposure time. The scale bar represents 20 μm.

7. Linearity between the concentration of GB2 and the fluorescence

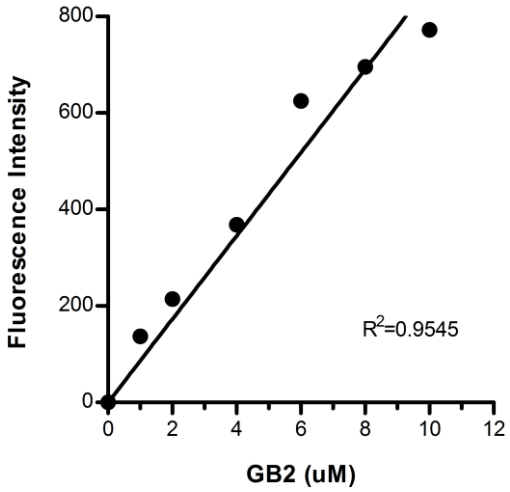


Figure S10. Linearity between the concentration of GB2 and the fluorescence. Fluorescence intensities were measured at an excitation wavelength of 540 nm and at an emission wavelength of 560 nm within water. Fluorescence intensities were measured by using a fluorescence spectrophotometer (Varian).

8. Cell viability test for primary hit compounds

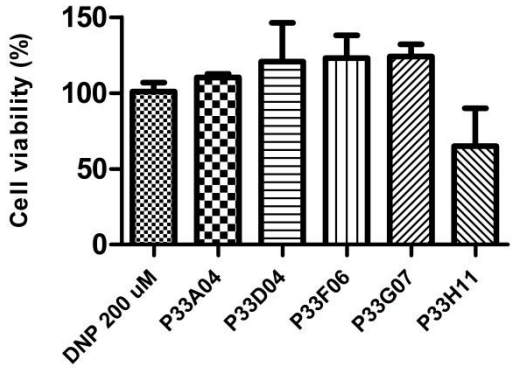
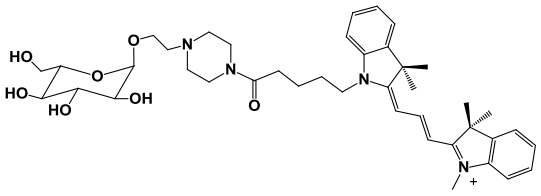


Figure S11. Cell viability test of primary hit compounds in C2C12 myoblasts. Individual chemicals were treated to C2C12 cells for 24 h. Cell viability was normalized to DMSO control as 100 %. The graph shows the average value of duplicate experiments and the error bars indicate SEM.

9. Synthetic procedure and full characterization of L-GB2

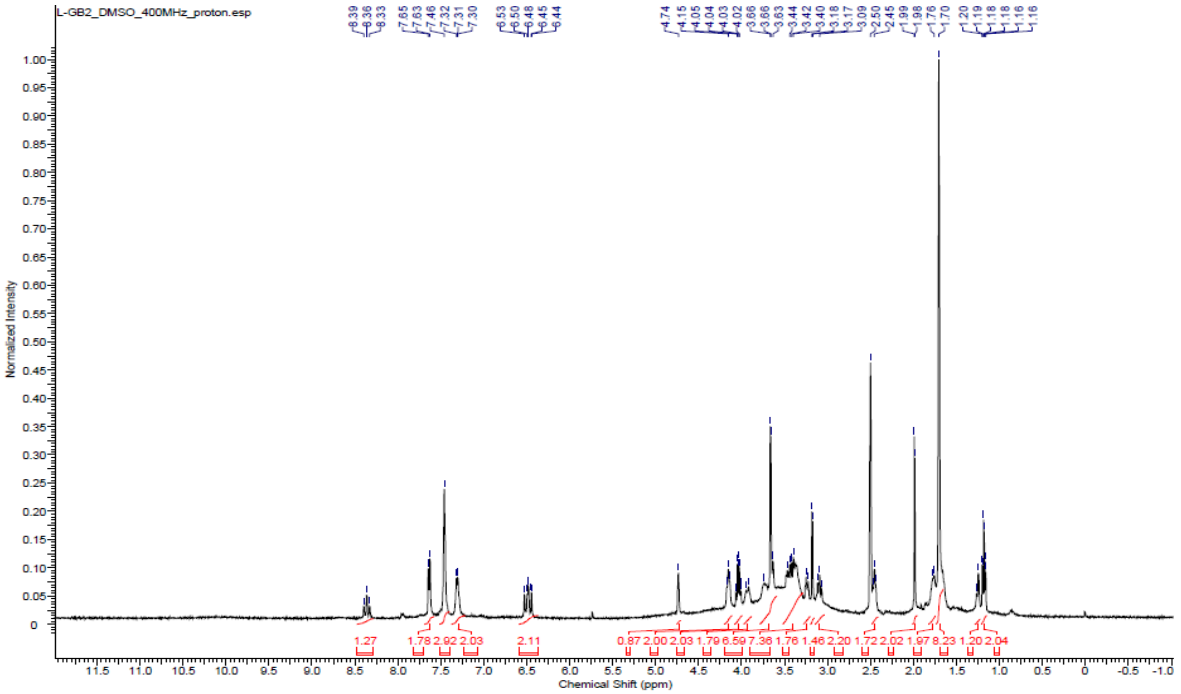
L-GB2 was prepared using the same procedure described in the previous report for GB2 synthesis.[4]

L-GB2

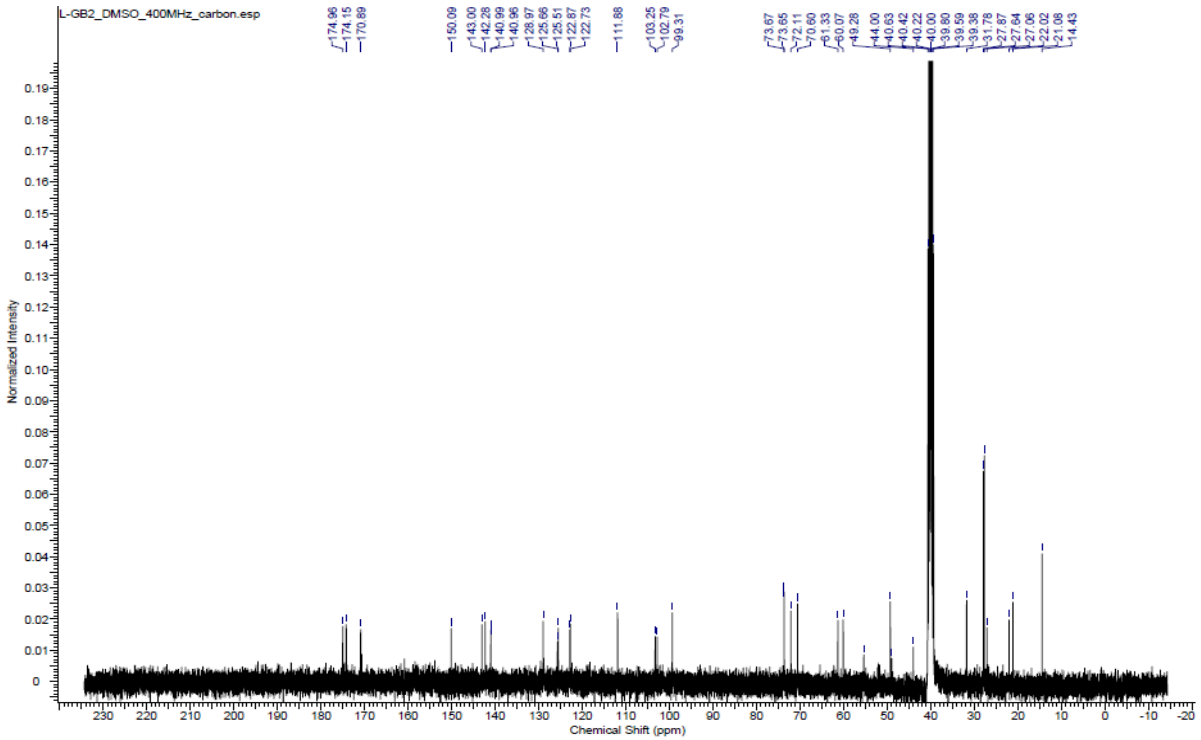


^{1}H NMR [400 MHz, $(CD_3)_2SO$] δ 8.39 (t, *J* = 12 Hz, 1H), 7.64 (d, *J* = 12 Hz, 2H), 7.46 (s, 3H), 7.32–7.30 (m, 2H), 6.53–6.44 (m, 2H), 4.74 (s, 1H), 4.17–4.14 (m, 2H), 4.07–4.01 (m, 2H), 3.95–3.92 (m, 2H), 3.74–3.63 (m, 7H), 3.47–3.40 (m, 8H), 3.25–3.23 (m, 2H), 3.18 (d, *J* = 4 Hz, 2H), 3.09 (t, *J* = 8 Hz, 2H), 2.45 (m, 2H), 1.99 (d, *J* = 4 Hz, 2H), 1.77 (d, *J* = 4 Hz, 2H), 1.70 (s, 9H), 1.27–1.24 (m, 2H), 1.20–1.16 (m, 2H); ^{13}C NMR [100 MHz, $(CD_3)_2SO$] δ 174.96, 174.15, 170.89, 150.09, 143.00, 142.26, 140.99, 140.96, 128.97, 125.66, 125.51, 122.87, 122.73, 111.88, 111.84, 103.25, 102.79, 99.31, 73.67, 73,65, 72.11, 70.60, 61.33, 60.07, 55.38, 49.28, 49.25, 48.95, 44.00, 31.78, 27.87, 27.64, 27.06, 22.02, 21.08, 14.43; HRMS (FAB$^+$): calculated for $C_{41}H_{57}N_4O_7$ $[M]^+$: 717.4227; found : 717.4251.

^{1}H NMR of L-GB2 (DMSO-d_6, 400 MHz)



^{13}C NMR of L-GB2 (DMSO-d_6, 100 MHz)



10. Reference

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4. H. Y. Lee, J. J. Lee, J. Park, S. B. Park, *Chem. Eur. J.*, 2011, ***17***, 143–150.