Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

A Fluorescent Probe for Imaging Symmetric and Asymmetric Cell Division in Neurosphere Formation

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Inventory

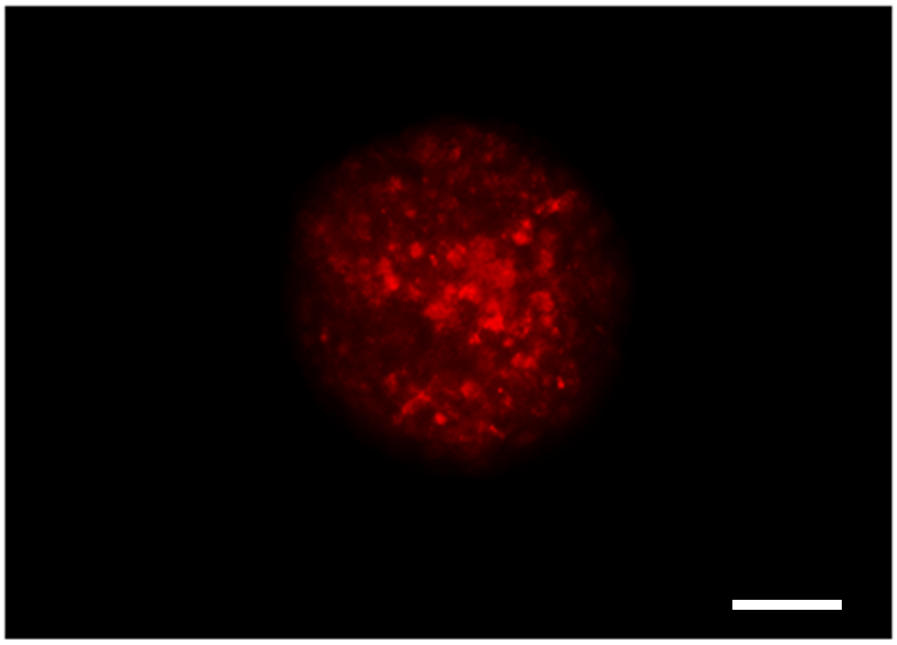


Fig. S1. CDy5-stained neurosphere. Epifluorescence micrograph of a living neurosphere generated in the presence of **CDy5**. More brightly stained cells are distinguishable. Scale bar, 50 µm.

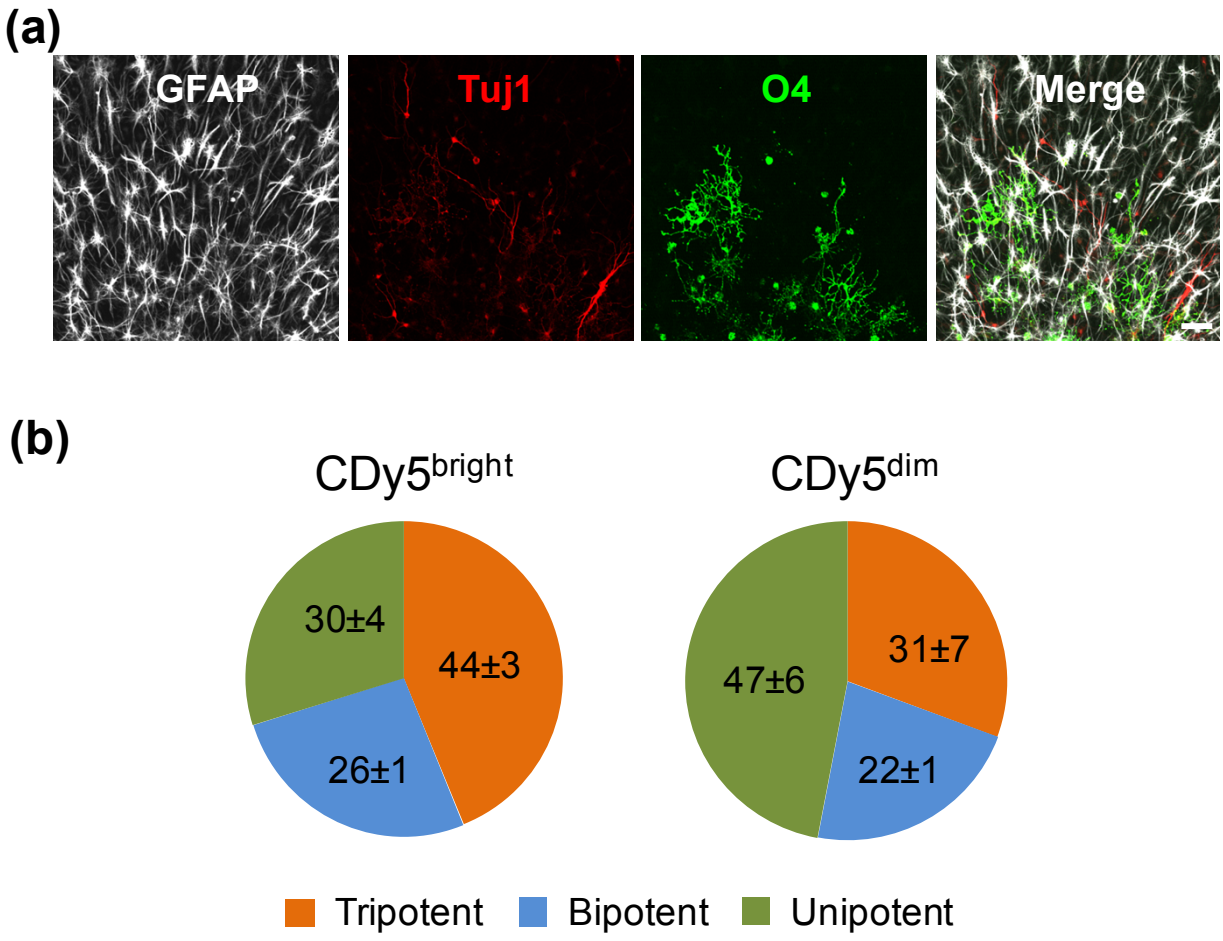


Fig. S2. Multipotency test. Representative immunocytochemistry images of a differentiated tri-potent neurosphere. Astrocytes, neurons and oligodendrocytes were stained using antibodies against GFAP, Tuj1 and O4, respectively. Scale bar, 50 μm. (G) Percentages of neurospheres possessing different differentiation potentials. Neurospheres generated from **CDy5**^{bright} (n=91) and **CDy5**^{dim} (n=86) cells were classified into tri-, bi- and uni-potent depending on the number of positively stained cell types. Data represent mean ± SD of duplicates.

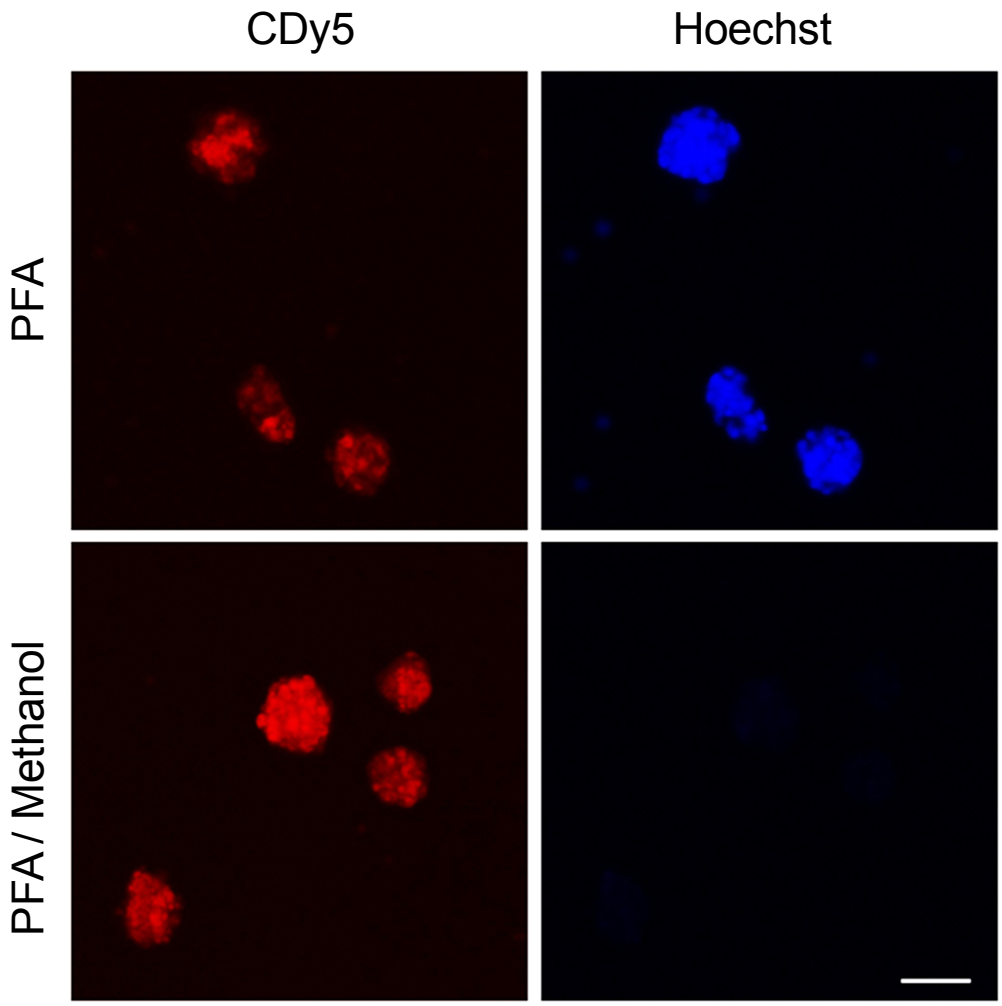


Fig. S3. CDy5 binding mode. Epifluorescence micrographs of **CDy5** and Hoechst 33342 co-stained neurospheres acquired after fixation with paraformaldehyde (upper panels), and paraformaldehyde followed by methanol treatment (lower panels). Scale bar, 50 μm.

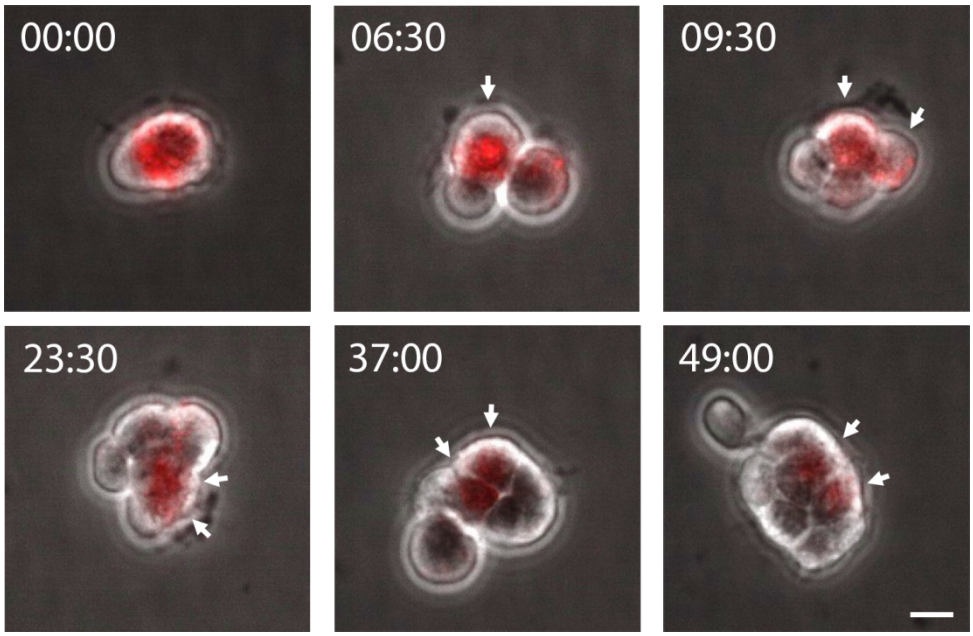


Fig. S4. Time-lapse imaging. Time-lapse live imaging of a **CDy5**-stained cell over 2 days shows a neurosphere generation by both symmetric and asymmetric divisions. Cells remained stained by **CDy5** were marked by white arrows. Time format, hh:mm. Scale bar, 10 μm.

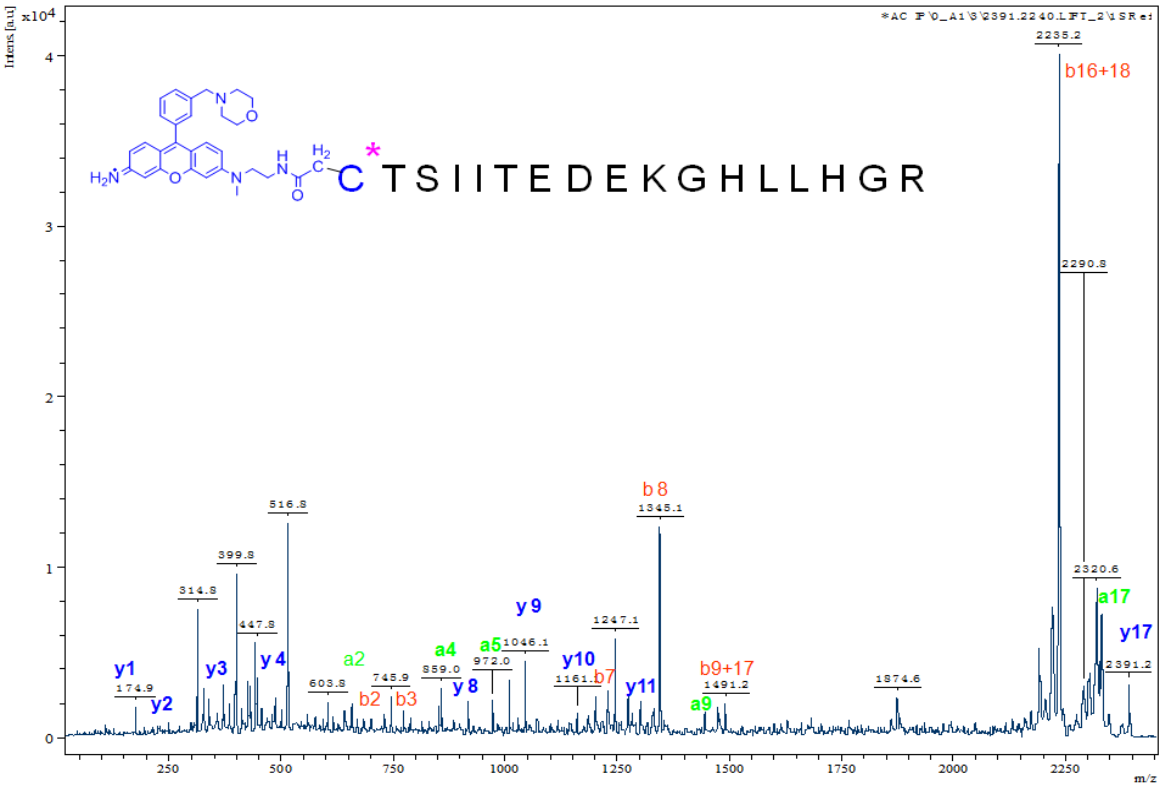


Fig. S5. CDy5 binding site determination by MALDI TOF-TOF MS. MS/MS fragment analysis of tryptic peptide (CTSIITEDEKGHLLHGR) indicates the cysteine is covalently bound by **CDy5**. One missing tryptic cleavage occurred on the lysine residue.

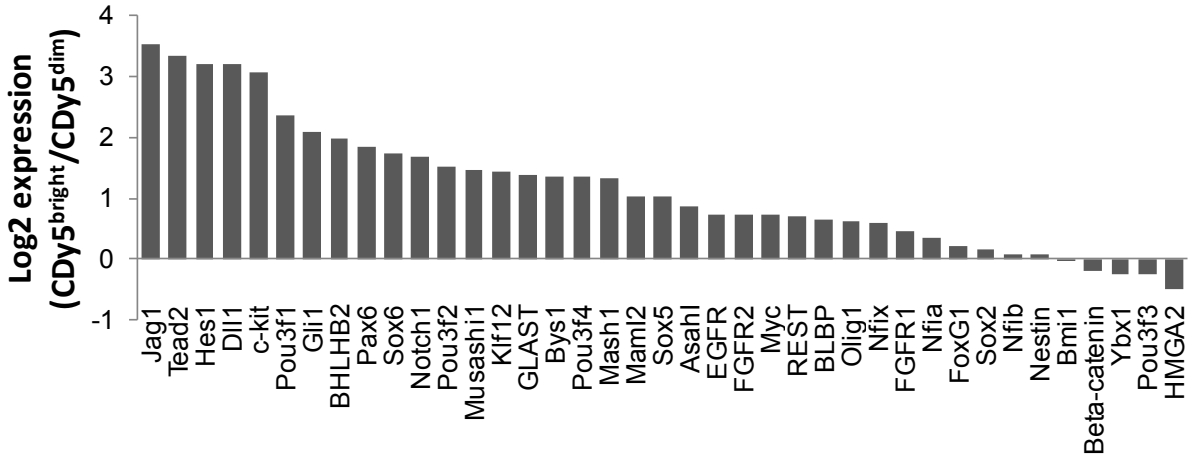


Fig. S6. Quantitative analysis of gene expression in 65 CDy5^{bright} and 69 CDy5^{dim} neurosphere cells by single cell RT-PCR.

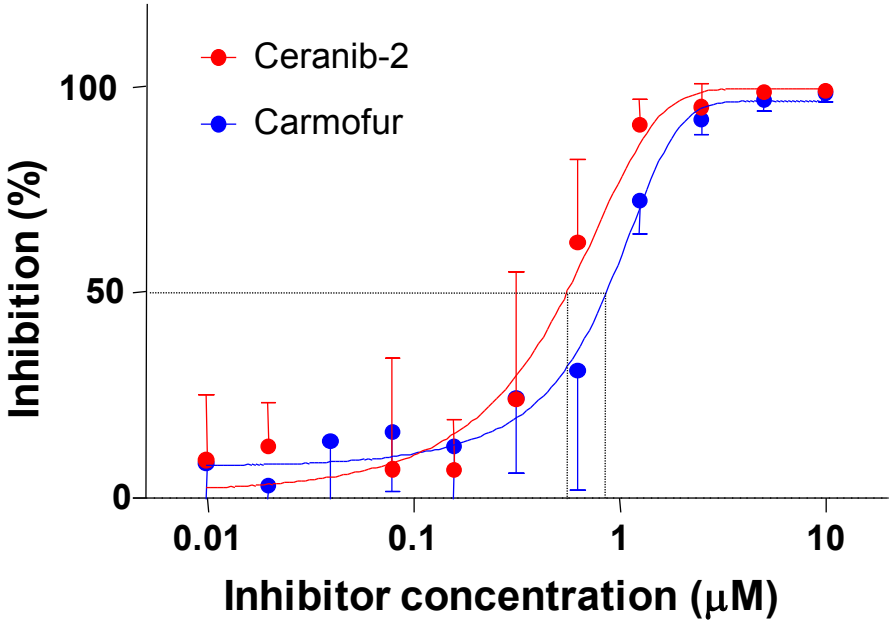
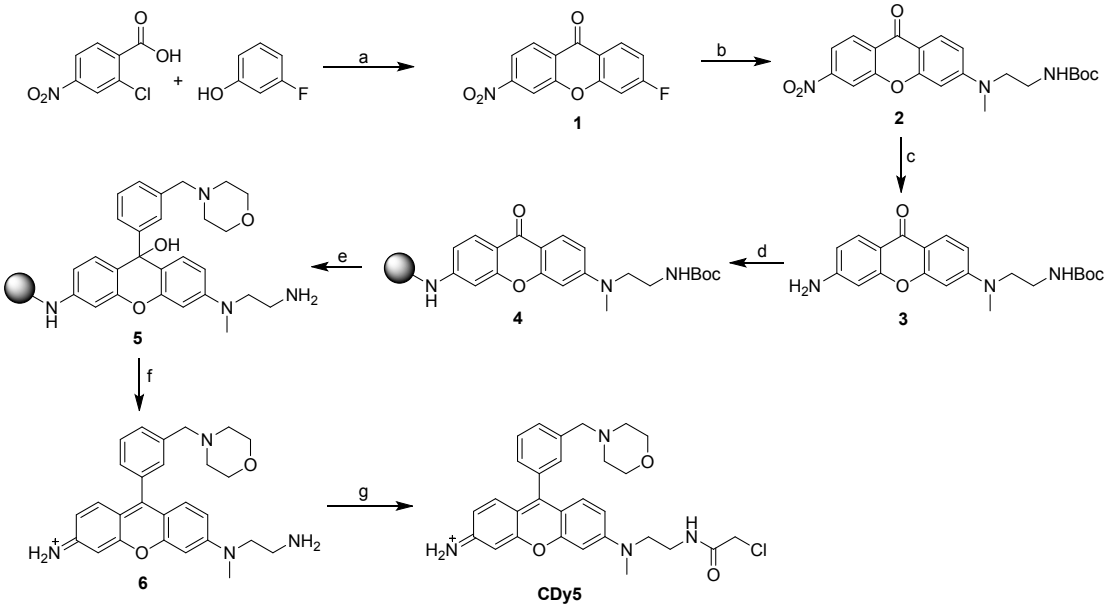


Fig. S7. Neurosphere assay to determine the effect of acid ceramidase inhibition on neurosphere formation. Acid ceramidase inhibitors Carmofur and Ceranib-2 significantly inhibited neurosphere formation. Data are represented as mean ± SD (n=5).



Scheme S1. **Synthesis of CDy5.** *Reagents and conditions*: (a) K_2CO_3, CuI, DMF, 130ºC, 16h and Con.H_2SO_4, 80ºC, 1h; (b) tert-butyl 2-(methylamino) ethylcarbamate, DMSO, 90ºC, 8h; (c) Pd/C, hydrazine, 90ºC, 2h; (d) 2-chlorotrityl chloride resin, pyridine, DCM-DMF, r.t, 4h; (e) Grignard reagent, THF, 60ºC, 16h; (f) 1% TFA in DCM, r.t., 15 min; (g) chloroacetyl chloride, pyridine, DCM, r.t., 30min, 0ºC.

Compound 1 **–** To a solution of 2-chloro-4-nitrobenzoic acid (3.0 g, 14.88 mmol) in DMF (40 mL) was added 3-fluorophenol (2.47 g, 16.38 mmol), potassium carbonate (3.08 g, 16.38 mmol) and copper powder (102 mg, 1.61 mmol). After heating at 130 ºC overnight, the reaction mixture was cooled to r.t., and slowly poured into ice-cold 1N HCl solution (300 mL). The solution was stirred until the brown solid was formed. The solid was filtered off and washed with cold water to yield a brown solid (3.1 g). The crude solid was dissolved in conc. sulfuric acid (20 mL), and heated at 80ºC for 1 hr. After cooling to r.t., the reaction mixture was poured into ice (350 mL) and stirred for 1h. The precipitated solid was filtered, washed with cold water and dried under vacuum to render 1. ^{1}H-NMR (CDCl3) δ 8.50 (d, J = 8.48, 1H), 8.37 (m, 2H), 8.20 (dd, J = 1.75, 1H), 7.21 (m, 2H) MS (ESI): m/z 260.18 (M+1).

Compound 2 **–** Compound 1 (1.0 eq.) was dissolved in DMSO (0.2 M) and the tert-butyl 2-(methylamino) ethylcarbamate amine was added (2 eq.) in one portion. The reaction mixture was heated to 90ºC and

stirred for 8 h. The solution was then cooled to r.t. and water was added. The precipitate was collected and washed with diethyl ether and water to render 2. ^{1}H-NMR (CDCl3) δ 8.42 (d, J = 8.48, 1H), 8.23 (d, J = 1.75, 1H), 8.11 (dd, J = 9.06, 2.04, 2H), 6.80 (dd, J = 2.04, 1H), 6.55 (d, J = 2.05, 1H), 4.79 (br, NH), 3.62 (m, 2H), 3.36 (m, 2H), 3.12 (s, 3H), 1.42 (s, 9H) MS (ESI): m/z 414.42 (M+1).

Compound 3 **–** A solution of compound 2 (826 mg, 2 mmol) in ethanol (20 mL) was purged with nitrogen for 10 min. Hydrazine monohydrate (0.485 mL, 10 mmol) and 10% Pd/C (83 mg) were added. The mixture was refluxed under nitrogen for 2 hrs. Then, after removing Pd/C by filtration, the crude mixture was concentrated under vacuum to yield compound 3, which was used directly in the loading reaction with 2-chloro-trityl chloride resin without further purification.

Compound 4 **–** Compound 3 (0.75 mmol) was dissolved in dichloromethane (10 mL) and added to 2-chlorotrityl chloride resin (0.5 mmol) suspended in dichloromethane (1 mL) and pyridine (3 mmol). After stirring for 4 hrs, the resin was filtered off and washed with DMF (X5), methanol (X10), and dichloromethane (X10), and dried under vacuum to provide the loaded compound 4.

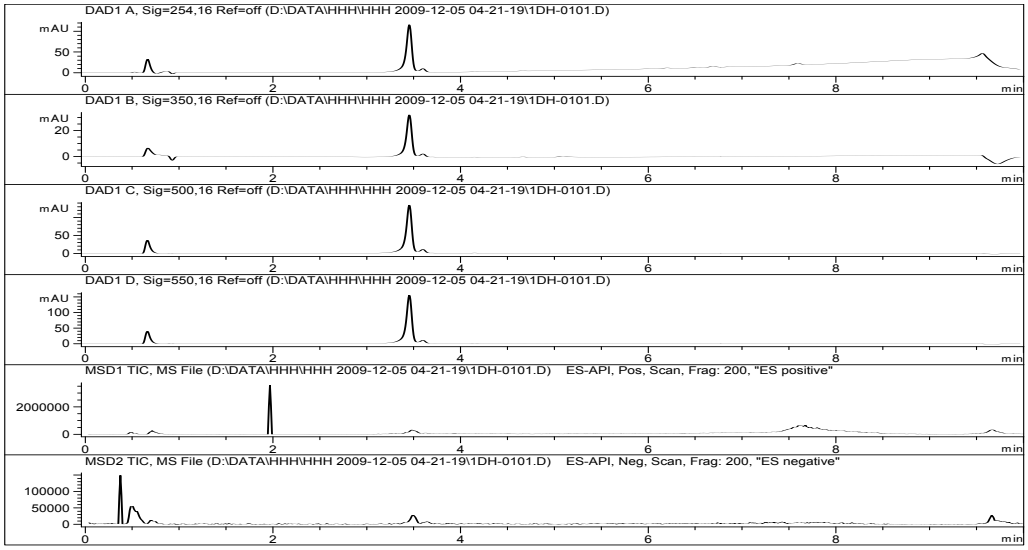
Compound 6 **–** To a solution of the compound 4 (100 mg, 0.1 mmol) in freshly distilled THF (5 mL), a solution of [3-(4-morpholinylmethyl) phenyl] magnesium bromide 0.25M was added and shaked overnight at 60^{o}C. The resin was filtered off and washed with dichloromethane (X5), DMF (X5), methanol (X5), and dichloromethane (X5). The resin was then dried and treated with 1% TFA in dichloromethane (3 mL) for 15 min. The filtrate compound 6 was collected and used in the next step without further purification.

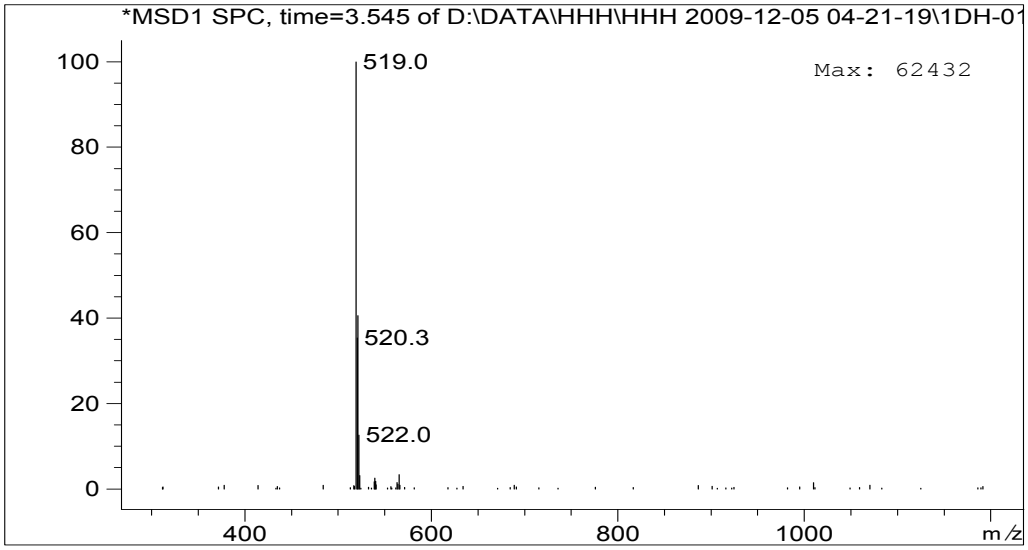
CDy5* –** Compound 6 was dissolved in dichloromethane (4 mL) and cooled in an ice bath. To the solution was added pyridine (0.5 mL), followed by chloroacetic anhydride (100 mg). After 30 min, the reaction mixture was diluted with DCM, washed with 1N HCl, aq. $NaHCO_3$, and brine sequentially, dried over anhydrous sodium sulfate, concentrated, and purified by silica gel column chromatography to give ***CDy5 (5 mg, 0.047 mmol).

Data S1. Characterization of CDy5.

^{1}H-NMR (MeOH-d_4) δ 9.37 (br s, NH), 7.82 (m, 2H), 7.65 (s, 1H), 7.59 (d, J=7.02Hz, 1H), 7.31 (m, 2H), 7.13 (m, 2H), 6.89 (s, 1H), 6.86 (s, 1H), 5.33 (NH, 1H), 4.49 (s, 2H), 3.96 (m, 3H), 3.81 (m, 3H), 3.55 (t, J=6.14Hz, 2H), 3.34 (m, 5H) ^{13}C-NMR (MeOH-d_4) δ 161.62, 160.06, 159.02, 158.61, 158.24, 134.52, 134.12, 133.38, 133.23, 132.52, 132.26, 130.80, 118.27, 115.47, 114.90, 114.21, 98.45, 97.85, 64.78, 61.16, 52.82, 52.26, 44.71, 42.87, 39.60, 38.12ppm. HRMS (ESI): Calculated for $C_{29}H_{31}ClN_4O_3^+$(M+H)$^+$: 519.03 Found: 519.2179

LC-MS(ESI)





Experimental Procedures

Neurosphere preparation

Mouse brains harvested from E14.5 fetuses were digested with 0.25% trypsin/1mM EDTA solution for 30 minutes at 37°C. The tissues were triturated sequentially with a 10 ml pipette, 1 ml blue tip and 0.2 ml yellow tip in the medium containing 10% FBS. The dissociated cells were washed 3 times with PBS by repeated resuspension and centrifugation and filtered through a 40 μm strainer. The obtained single cells were plated in a DMEM/F12 medium containing 10 ng/ml bFGF, 20 ng/ml EGF and B27 without vitamin A and cultured for 5-6 days without changing medium. All animal experiment procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Neurosphere staining with CDy5

Dissociated neurosphere cells were cultured in a complete neurosphere culture medium containing 2 μM of **CDy5** for 6 days. They were dissociated for single cell imaging, live cell time-lapse imaging and clonal neurosphere imaging. For imaging at a later time, the neurospheres were fixed by 4% paraformaldehyde for 5 minutes and stored in PBS at 4°C.

Neurosphere assay

Neurosphere cells collected by FACS were resuspended in neurosphere culture medium and plated in triplicate in 6-well culture plates at a density of 3,000 cells per well. The cells were then cultured in an incubator without agitation. After 6 days culture the numbers of neurospheres were counted manually under a microscope. To assess the effects of **CDy5** and AC inhibitors on neurosphere formation, dissociated neurosphere cells were plated in 12-well culture plates at a density of 1,000 cells per well and cultured in the presence of 2 μM and 4 μM **CDy5** or 0.01 to 10 μM of AC inhibitors. For vehicle control DMSO was added to be 0.1%. The IC_{50} values were calculated using GraphPad Prism software.

Neurosphere differentiation and immunocytochemistry

Single neurospheres were plated onto glass coverslips coated with laminin and poly-L-Lysine and cultured in bFGF/EGF depleted neurosphere culture medium containing 5% fetal bovine serum. The differentiated cells were fixed with 4% paraformaldehyde and stained using primary antibodies: Tuj1 (Covance), glial fibrillary acidic protein (Dako), and O4 (Millipore) and appropriate secondary antibodies: Alexa Fluor 488 goat anti-mouse, Alex Fluor 594 goat anti-mouse and Alexa Fluor 647 donkey anti-rabbit (Life Technologies).

Confocal microscopy and 3D image reconstruction

CDy5-stained neurosphere cells were cultured for 2 days in a 96-well plate and stained with Hoechst 33342 and CellTracker™ Green CMFDA (Life Technologies) for confocal microscopy using an Olympus FV1000 LSCM equipped with Olympus Plan-Apochromat 60x water lens. The z-stack images were acquired every 0.2 µm with xy resolution of 320 x 320 pixels in sequential mode. The emission band for **CDy5** was at 550-600 nm. Each color image was converted to 8-bit gray for reconstruction of 3D image using Bioptonics Viewer software.

Protein Extraction

Neurospheres stained with **CDy5** were collected by centrifugation at 453 x g for 3 minutes and the pellet was washed three times with cold PBS before resuspension in a lysis buffer containing 40 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS (Sigma), 10 µl/ml protease inhibitor cocktail (EDTA free, GE healthcare), 50 µg/ml DNase I and 50 µg/ml RNase A. For extraction of only cytosolic soluble proteins the cells were lysed in a buffer containing only 40 mM Tris, protease inhibitors cocktail, DNase I and RNase A. The cell extract was homogenized by ultrasonication for 30 second and then incubated for 30 minutes at room temperature. The supernatant was collected after centrifugation at 20,000 x g for 45 minutes at 4 ^{o}C. The protein concentration was determined by Bradford protein assay reagent (Bio-Rad).

Two dimensional gel electrophoresis

The protein sample of 1 mg was diluted in 340 µl of rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% IPG buffer (GE healthcare) and loaded to 18 cm ReadyStrip™ IPG strips pH 3-10NL or pH 5-8NL (Bio-Rad) by passive rehydration. It was separated first by isoelectric focusing for 60,000 Vhrs at 20 °C on a PROTEAN IEF Cell (Bio-Rad). Then the IEF strips were reduced in an equilibration buffer I containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS and 2% DTT at room temperature for 10 minutes and alkylated with a SDS-PAGE equilibration buffer II containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide and a trace of bromophenol blue at room temperature for an additional 10 minutes. The equilibrated IEF strips were embedded in 0.5% low melting temperature agarose dissolved in Tris-glycine-SDS buffer on top of the second dimension 12% SDS-PAGE gel. After electrophoresis for 5 hours at 30 mA, the gel was scanned on a Typhoon 9400 scanner (GE healthcares) for 2D fluorescence image. A duplicate gel was stained using PlusOne™ Silver Staining Kit (GE healthcare) according to the manufacturer's protocol.

In-gel trypsin digestion

The excised gel was washed with water and cut into small pieces of approximately 1 mm^3 in size. The gel pieces were rinsed with 50% acetonitrile/25mM ammonium bicarbonate buffer (pH 7.8) 3 times, dehydrated in 100% acetonitrile and dried by speed vac. They were digested with 10ng/µl trypsin gold, (Mass Spectrometry grade, Promega) in 25 mM ammonium bicarbonate buffer (pH 8.0) overlaid with 10 µl of 25 mM ammonium bicarbonate buffer for 16 hours at 37 °C. The peptides were extracted sequentially with 20 mM ammonium bicarbonate buffer followed by 50% acetonitrile in 0.1% trifluoroacetic acid (TFA). The pooled peptides were speed vac dried and dissolved in 7 µl of 0.1% TFA.

LC MALDI TOF-TOF MS/MS analysis

Tryptic peptides in 6.4 µl was injected into Dionex Ultimate 3000 capillary HPLC system equipped with Acclaim® PepMap™ µ-Guard columns. Column temperature was maintained at 25 °C, micropump flow rate was 4 µl/min and acetonitrile gradient from 5 to 60% in 0.05% TFA was applied within 1 hour.

Fractions (10 sec /spot) were directly spotted onto Prespotted AnchorChip target plate 384 with LC coupled Proteineer Fc (Bruker Daltonics) according to manufacturer's protocol. The peptide MALDI MS and MS/MS was analysed using UltraFlex III TOF-TOF (Bruker Daltonics) with WarpLC 1.2 and the Compass 1.2 software package including FlexControl 3.0 and FlexAnalysis 3.0 with PAC peptide calibration standards. The peak lists were submitted to in-house Mascot server (http://phenyx.bii.a-star.edu.sg/search_form_select.html) using BioTools 3.2 (Bruker Daltonics) and searched against SwissProt database (517100 sequences) with peptide mass tolerance of 100 ppm and 1 missed cleavage allowed, considering of variable modifications of carbamidomethyl of cysteine and oxidation of methionine.

2D Western blot

A protein sample of 1.5 mg extracted from **CDy5**-stained neurosphere was separated by 2D SDS-PAGE. The proteins were transferred from a part of the gel (5x8 cm) containing the major fluorescence spots onto a PVDF membrane. The membrane was blocked with in PBS containing 0.05% Tween 20 and 5% skim milk for 1 hour and incubated with goat anti-acid ceramidase polyclonal antibody (T-20) (1:500 dilution, Santa Cruz, sc-28486) which was detected using donkey anti-goat IgG-Alexa 647. Fluorescent signals from **CDy5** and the antibody were detected on a Typhoon 9.4 scanner and analyzed using ImageQuant 5.2 software (GE healthcare).

Pull-down assay

The cytosolic soluble protein sample of 1 mg extracted from **CDy5**-stained neurosphere was adjusted to a pH 7.5 and a concentration of 2mg/ml with 1N HCl for a final volume of 0.5 ml. It was mixed 1:1 in volume with 2x IP buffer containing 2% Triton X-100, 300 mM NaCl, 2 mM EDTA, 1% NP-40, 0.2% SDS, 10 mM DTT and 2x protease inhibition cocktail and then heated at 95 ^{o}C for 2 minutes. The supernatant obtained by centrifugation was incubated with 2 µg goat anti-acid ceramidase antibody at 4 ^{o}C overnight with agitation. The sample was incubated with 1.5 mg of Protein G Dynabeads (Invitrogen) at 4 ^{o}C for 2 hours on a rotating mixer and then washed with IP buffer followed by PBS and 0.15 M NaCl containing protease inhibitor. The protein was eluted in 30 µl of 2x Laemmli buffer by heating at 95 ^{o}C for 5 minutes

and subjected to 12% SDS-PAGE. Fluorescent signals from **CDy5** was detected on a Typhoon 9.4 scanner.

Single cell RT-PCR

Individual **CDy5**$^{\text{bright and dim}}$ cells were sorted by FACS and collected directly into 96-well plate containing 10 µl of RT-PreAmp master mix containing 5 µl CellsDirect 2x reaction mix (Invitrogen), 2.5 µl 0.2x assay pool (Applied Biosystems), 0.5 µl SuperScript® III RT/Platinum® Taq mix (Invitrogen) and 2 µl TE buffer (Qiagen) per well. Cells were frozen at -80 ºC and thawed to induce lysis. cDNAs were generated from sequence-specific reverse primers by a reverse transcription at 50 °C for 20 minutes followed by enzyme inactivation at 95 °C for 2 minutes. The cDNA was pre-amplified by 18 cycles of denaturation at 95 °C for 15 seconds and annealing/synthesis at 60 °C for 4 minutes. This pre-amplified RT-PCR products were quantified by real-time PCR using a 48.48 dynamic array (Fluidigm) on the BioMark™ System (Fluidigm). Ct values higher than 28 were considered undetectable and the value of 28 was used as its Ct for calculation in such cases. In total, data for 48 genes including house-keeping gene *β-Actin* in 96 **CDy5**$^{\text{bright}}$ and 96 **CDy5**$^{\text{dim}}$ cell samples were obtained. Data from the cells in which *β-Actin* expression was not detectable or above mean ± 3xSD and the genes whose expressions were not detectable in more than 50% of the cells both in **CDy5**$^{\text{bright and dim}}$ groups were excluded from the statistical analysis. Finally, 39 gene expressions in 65 CDy$^{\text{bright}}$ and 69 **CDy5**$^{\text{dim}}$ cells were analyzed. Ct values of the gene of interest for a specific cell were normalised by subtracting the Ct value of *β-Actin* for the same cell. Principal component analysis was applied to all cells using the Ct values. The principal scores of each cell were plotted using MATLAB software.