

Microglia-specific fluorescent probes for live cell imaging

Cheryl Leong, Sung Chan Lee, Jiyeon Ock, Xin Li, Peter See, Sung Jin Park, Florent Ginhoux,
Seong-Wook Yun, Young-Tae Chang

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Materials & Methods

Synthesis and characterization of CDr10b: The aminophenyl bodipy (10 mg, 32 μ mole) and 3-ethoxy-4-methoxybenzaldehyde (8.6 mg, 48 μ mole) were dissolved with acetonitrile (5 mL) in 20 mL vial and 3 eq. of pyrrolidine and acetic acid were added to reaction mixture. The reaction vial was heated to 60 °C by heating block reactor for 3 hrs. After reaction completion, NaHCO₃ saturated solution (50 μ L) and chloroacetylchloride 20 μ L were added to reaction mixture. Crude compound was purified by column chromatography and obtained product was 15 mg.

¹H-NMR (CD₃OD + CDCl₃) δ 8.36 (s, 1H), 7.71 (m, 3H), 7.55 (d, J = 16.0 Hz, 1H), 7.39 (d, J = 8.5 Hz, 2H), 7.33 (d, J = 16.0 Hz, 1H), 7.17 (m, 2H), 6.88 (d, J = 9.0 Hz, 1H), 6.72 (s, 1H), 6.38 (bs, 2H), 4.25 (s, 2H), 4.18 (m, 2H), 3.92 (s, 3H), 1.63 (s, 3H), 1.50 (t, J = 7 Hz, 3H), ¹³C-NMR (CDCl₃) δ 165.35, 158.33, 150.99, 148.28, 145.69, 140.13, 138.52, 136.95, 134.65, 129.95, 129.49, 128.76, 122.30, 119.42, 119.12, 115.96, 111.15, 110.86, 64.30, 55.57, 42.66, 29.31, 15.01, 14.22; ESI-MS m/z (M + H) calc'd: 550.18, found 550.20, 530.20 (M - F)

Synthesis and characterization of CDr10a: ¹H-NMR (CD₃OD + CDCl₃) δ 8.46 (s, 1H), 7.76 (m, 3H), 7.65 (d, J = 16.0 Hz, 1H), 7.43 (d, J = 8.5 Hz, 2H), 7.32 (d, J = 16.0 Hz, 1H), 7.19 (m, 2H), 6.89 (d, J = 9.0 Hz, 1H), 6.75 (s, 1H), 6.39 (bs, 2H), 4.20 (m, 2H), 3.95 (s, 3H), 2.04 (s, 3H), 1.71 (s, 3H), 1.49 (t, J = 7 Hz, 3H), ¹³C-NMR (CDCl₃) δ 165.45, 159.33, 151.99, 147.28, 146.69, 140.33, 138.55, 136.98, 134.65, 129.95, 129.49, 128.76, 123.30, 119.52, 119.82, 114.96, 112.15, 110.86, 64.30, 55.58, 42.66, 29.51, 15.01, 14.22; ESI-MS m/z (M + H) calc'd: 516.22, found 516.20.

Synthetic Materials & Method: All reactions were performed in oven-dried glassware under a positive pressure of nitrogen. Unless otherwise noted, starting materials and solvents were purchased from Aldrich and Acros organics and used without further purification. Analytical TLC was carried out on Merck 60 F254 silica gel plate (0.25 mm layer thickness) and visualization was done with UV light. Column chromatography was performed on Merck 60 silica gel (230–400 mesh). NMR spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer. Chemical shifts are reported as δ in units of parts per million (ppm) and coupling constants are reported as a J value in Hertz (Hz). Masses of all the compounds were determined by LC-MS of Agilent Technologies with an electrospray ionization source. The Phenomenex C18 (50 mm \times 4.6 mm, 5 μ m) column was used for purity confirmation with 0.1% TFA containing acetonitrile/water gradient (5% to 100% in 10 min) elution conditions. Spectroscopic measurements were performed on a fluorometer and a UV/Vis instrument, Synergy 4 of bioteck company and Gemini XS fluorescence plate reader. The slit width was 1 nm for both excitation and emission. Relative quantum efficiencies were obtained by comparing the areas under the corrected emission spectra. The following equation was used to calculate quantum yield.

$$\Phi_x = \Phi_{st}(I_x/I_{st})(A_{st}/A_x)(\eta_x^2/\eta_{st}^2)$$

where Φ_{st} is the reported quantum yield of the standard, I is the integrated emission spectrum, A is the absorbance at the excitation wavelength, and η is the refractive index of the solvents used. The subscript x denotes unknown and st denotes the standard. Rhodamin B was used as a standard and the sample concentration was 10 μ M in DMSO.

pH response: pH response from pH 2 to 12 was measured using 10 μ M of **CDr10b**, **CDr10a** or **pHrodo** (Life technologies) in 50% DMSO/50% pH buffer at the known excitation and emission wavelengths using a fluorescence plate reader.

Cell culture: Primary brain cultures were generated from the whole brains of postnatal day 1-3 (P1-3) FVB/N mice by trypsinization and trituration methods in accordance with the animal handling regulations of our institution. Briefly, fresh brains were incubated with 0.25% trypsin for 1 hour at 37°C, followed by neutralization with Fetal Bovine Serum (FBS). A single cell suspension was generated by sequential trituration using pipette tips of decreasing diameter. Finally, the cell suspension was filtered using a 40-micron strainer. Cells were grown in DMEM/F12 (Gibco) with 10% FBS and 1% penicillin-streptomycin (Gibco). BV2 and MEF cells were cultured in DMEM + 5% FBS and DMEM + 20% FBS respectively with 1% penicillin streptomycin (Gibco).

High throughput screening:

Primary neurons and primary astrocyte enriched cultures were generated using the adherence isolation protocol by Jana et.al⁸. Primary microglia enriched cultures were generated by adherence methods and harvested using the mild trypsinization method as described by Saura et.al⁹. Each cell type isolated by these methods was 80-90% pure when determined by immunocytochemistry. Neuron, astrocyte and microglia enriched cultures were plated in duplicate on to 384 well plates and incubated with 500 nM of fluorescent compound for 1 hour before image acquisition using the ImageXpress Micro™ high throughput. A total of 5,000 compounds from a series of DOFL BODIPY libraries were screened.

Immunocytochemistry: Primary neural cells were fixed in 4% PFA and permeabilized with 0.1% Triton-X (Sigma). Cells were identified using the following markers at the respective dilutions: microglia – Iba (Wako) 1:500, astrocytes - GFAP (DakoCytomaton) 1:1,000, neurons - β -III-tubulin (Sigma) 1:1,000 and oligodendrocytes (Millipore) 1:500. For secondary antibody staining, anti-mouse Alexa Fluor 546 IgG, anti-rabbit Cy5 IgG and anti-mouse Alexa Fluor 488 IgM (Invitrogen) 1:1000 were used. Images were scored using intensity analysis software MetaXpress® and confirmed by image-based manual analysis for the identification of hit compounds. For isolectin staining, isolectin-B4 FITC conjugate (Sigma), 20 μ g/ μ l. was applied to live cells and co-stained with compound respectively.

Fluorescence imaging and processing: Cells were stained with 500 nM of **CDr10b** or **CDr10a** for 1 hour followed by 1 hour of washing in fresh media before image acquisition. All fluorescence microscopy was carried out using the ECLIPSE Ti microscope (Nikon Instruments Inc) on the NIS Elements 3.10 software. For images of organelle localization (mitochondria, lysosomes, endoplasmic reticulum and golgi), we used the following fluorescent dyes from Life technologies: Mitotracker Green, LysoTracker green, ER tracker green and Golgi tracker green. Images were acquired using a LSM 5 DUO (inverted) Laser Scanning Confocal Microscope using a 100X oil objective. Images were deconvoluted using Hyugen's Deconvolution. Co-localization analysis was carried out using the Coloc 2 plugin (Version by Daniel J White, Tom Kazimiers, Johannes Schindelin) in Fiji. Pearson's coefficient values were average from analysis done from 3 sets of images with approximately 5-10 cells per image.

Flow cytometry: Primary brain cell cultures were generated from the brains of P1 CX3CR1-GFP pups as described above. Cells were stained with 500 nM of compound for 1 hour and washed with PBS before acquisition on the BD LSR II analyzer. Cells were acquired using the appropriate filters for GFP and Texas Red wavelengths.

Washing and SDS-PAGE: BV2 microglia were stained with 500 nM of **CDr10b** or **CDr10a** for 1 hour and washed for 1 hour with DMEM + 10% FBS or 100% methanol and imaged at the same exposure time as unwashed cells. For SDS-PAGE, BV2 microglia were stained with 500 nM of **CDr10b** or **CDr10a** for 1 hour followed by cell lysis with Cell Lytic M (Sigma). 20 µg of cell lysate was run on 12 % SDS-PAGE and analyzed using a typhoon fluorescence scanner. The gel was then stained with comassie blue.

Cytotoxicity assays: Cytotoxicity assays were carried out using the MTS reagent kit (Promega) on 24h compound treated BV2 microglia cells in accordance with the manufacturer's instructions.

Real time RT-PCR: Total RNA was isolated from the cells using the RNeasy Mini Kit (QIAGEN Inc.) according to the manufacturer's instructions. Gene expression for the cytokines IL-6, TNF α and GAPDH was measured using the Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystem) on a StepOne™ Real-Time PCR System (Applied Biosystems). mRNA levels of the above mentioned genes were normalized to GAPDH expression levels using the Q-gene relative expression software tool.

The following primer sequences (5' to 3') were used in this study:

| | |
|-----------------------------------|----------------------|
| mGAPDH-F1 | AAGGGCTCATGACCACAGTC |
| mGAPDH-R1 | GGATGCAGGGATGATGTTCT |
| mTNFα-F1 | TAGCCAGGAGGGAGAACAGA |
| mTNFα-R1 | TTTTCTGGAGGGAGATGTGG |

| | |
|-----------------|----------------------|
| mIL-6-F1 | CCGGAGAGGAGACTTCACAG |
| mIL-6-R1 | TCCACGATTTCCAGAGAAC |

Nitric oxide assay: BV2 cells were pretreated for 1 hour with 500 nM of **CDr10b** before addition of 100 ng/ μ l of LPS or 20U/ml (20 ng/ml) of IFN- γ in fresh media. After incubation for 24 hours, 50 μ l of cell culture media was mixed with an equal volume of Griess reagent (Sigma) in a 96 well plate. Light absorbance was measured at 540 nm. Nitric oxide concentration was determined by comparison with a standard curve generated from a titration of sodium nitrite.

Activation intensity measurements by imaging: BV2 cells were activated with 100 ng/ μ l of LPS for the stipulated times. One hour before image acquisition 500 nM of **CDr10b** was added to the cells. Cells were imaged using flow cytometry or the ImageXpress high throughput system at 20X magnification and intensity measurements were calculated by intensity based multi-wavelength scoring.

Live cell tracking: For cell tracking, videos were acquired on the Biostation IM (Nikon) at 10X magnification for 24 hours. For imaging cellular interactions, U251 glioma cells were treated with 10 μ M of camptothecin. After 24 hours, media was replaced and the U251 cells were stained with 500 nM of Cell Tracker Green (Invitrogen) for 1 hour. BV2 microglia were stained for 1 hour with 500 nM of **CDr10b**. After staining, both cell types were washed briefly in fresh media. Stained BV2 microglia were trypsinized and seeded with the U251 cells. Images were acquired over 36 hours on the Biostation IM (Nikon) at 20X magnification at intervals of 20 mins.

Live cell washing: BV2 microglia were stained with 500 nM of compound for 2 hours. Cells were then briefly rinsed with fresh media and imaged using the Biostation IM(Nikon) at 10X magnification for 20 hours at intervals of 15 mins. For the quantification of signal intensity, the Time Series Analyzer plugin (Balaji, J. 2007) was used. Values were averaged from a set of three regions of interest, each consisting of 3-10 cells.

Protein extraction for LC separation: The compound stained BV2 cells were lysed with solution including 0.1% TFA, 5% acetonitrile (ACN). The cells were suspended in the solution and ultrasonicated for 4 min using 40% power, 6 sec on/ 3 sec off cycle. The lysate was centrifuged for 5 min at maximum speed to remove the insoluble debris. The protein concentration of the supernatant was measured by Bradford method.

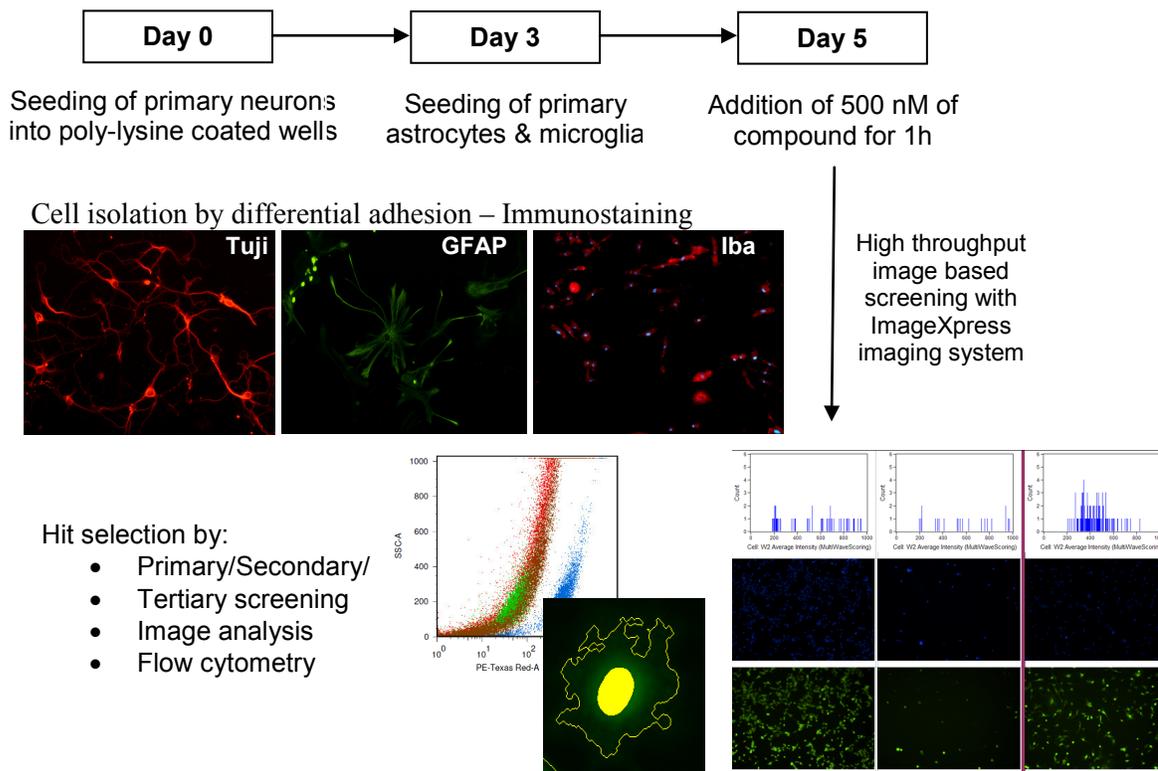
Off-line LC separation and SDS-PAGE separation: Dionex Ultimate 3000 2D LC system was employed to fractionate the cell lysate using Dionex ProSwift RP-2H column. The 40 min HPLC gradient consisted of 100% buffer A (0.1% TFA, 5% ACN) for 2 min; 0–24% buffer B (0.1% TFA, 95% ACN) for 3 min;

24-62% buffer B for 25 min; 62-100% buffer B for 2 min followed by 100% buffer A for 3 min; 100% buffer A for 5 min. In total 40 fractions were collected. The chromatography was recorded by UV detector at 214nm and by fluorescence detector at 610nm. The fractions that carry the fluorescent signal were selected to concentrate to dryness using vacuum centrifuge. The fractions collected and dried from the LC separation were separate by 4-15% SDS-PAGE gel or 12% SDS-PAGE gel. The gel was scanned by Typhoon 9400 variable mode imager (Amersham Biosciences, NJ). The gel band carrying fluorescent signal was cut out for in-gel digestion.

In-gel digestion: The proteins that trapped in the gel pieces was reduced by dithiothreitol (DTT) and alkylated by iodoacetamide (IAA). Tryptic digestion was performed by using porcine trypsin (Sequencing Grade Modified, Promega, Wisconsin) overnight. The tryptic peptides were extracted by 5% formic acid in 50% ACN and vacuum dried by vacuum centrifuge.

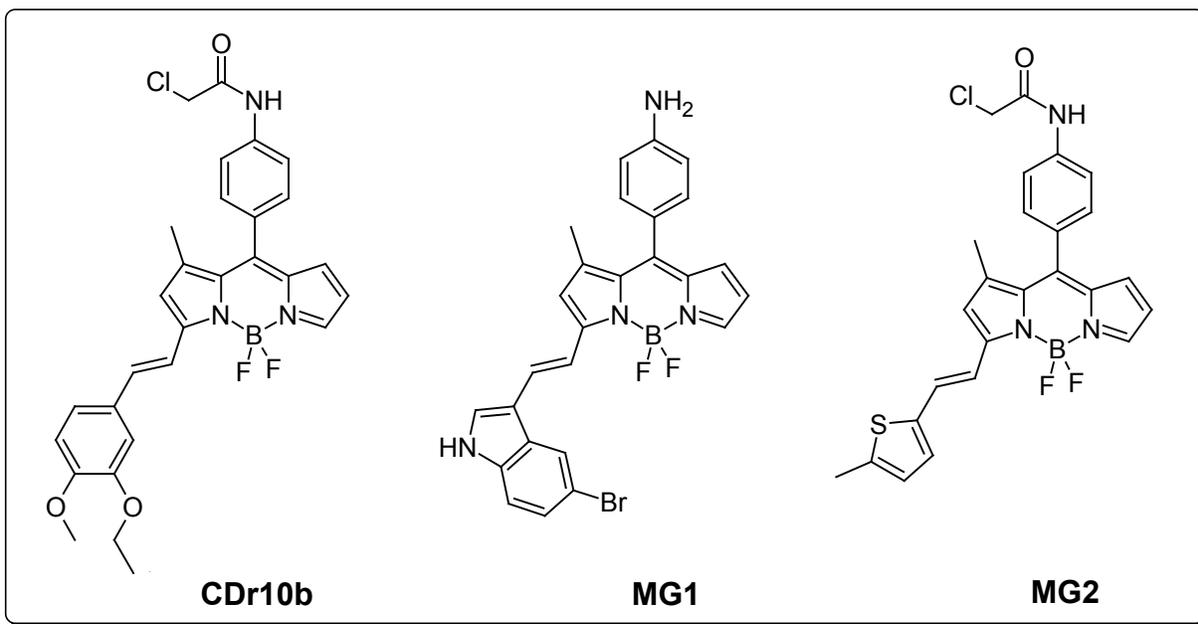
Data analysis: The peak list was submitted to Mascot server for searching against the uniprot_sprot database. The Mascot dat file was processed by in-house program to select those peptides with score above 20 and peptides with score above identity score. The peptide number for each protein was calculated. Proteins were grouped according to the similarity of the sequence.

a



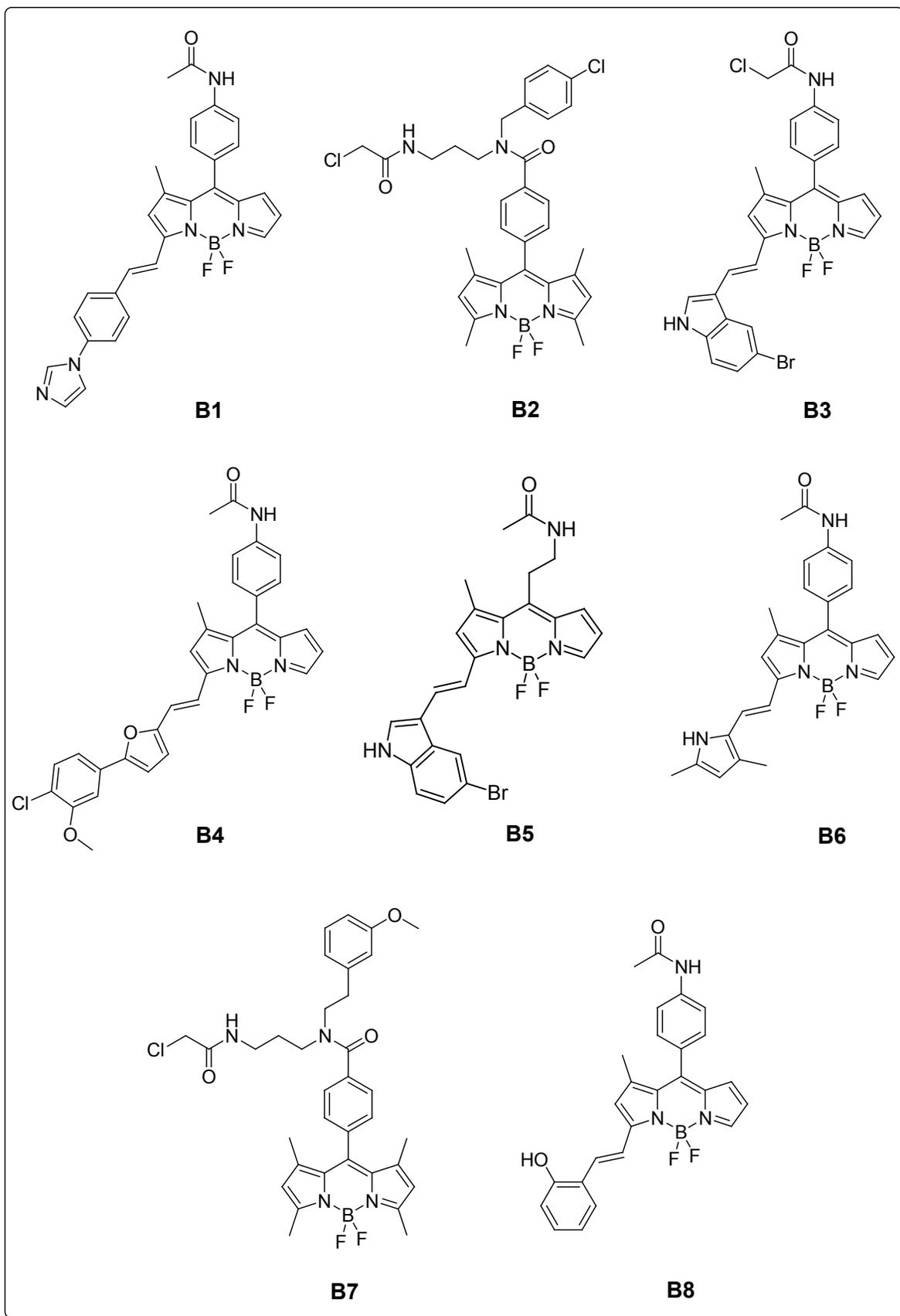
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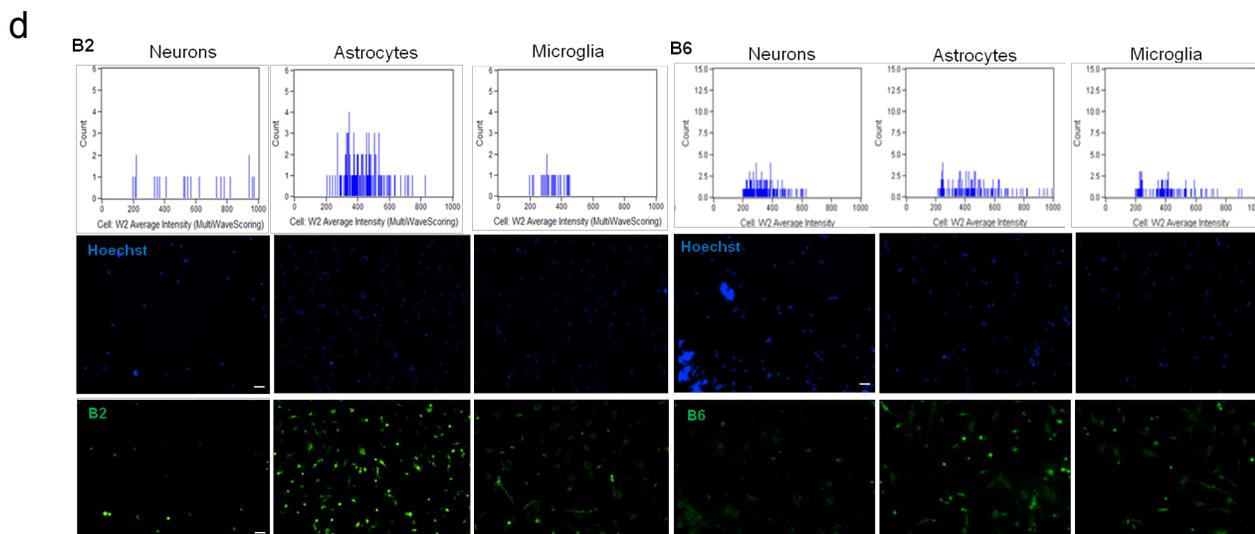
Microglia hit compounds



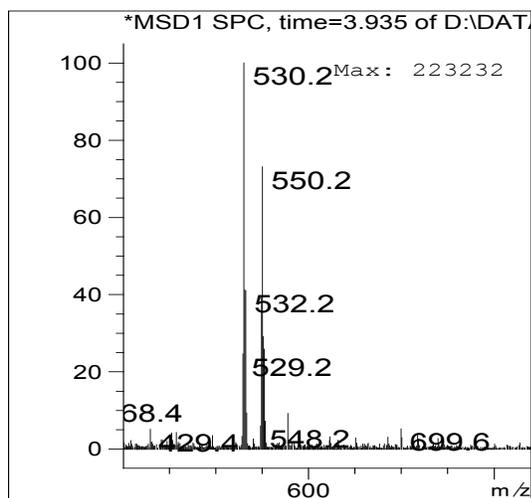
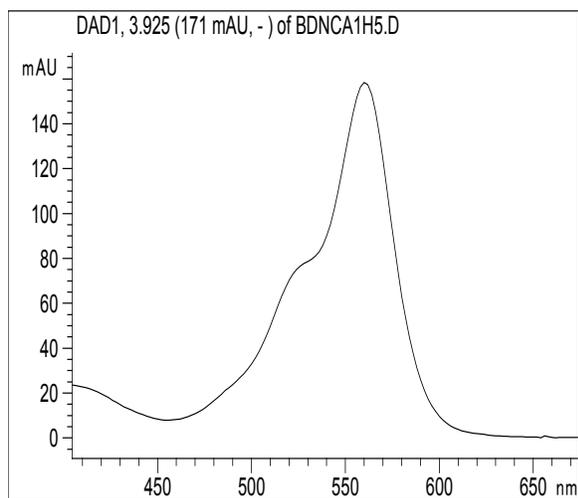
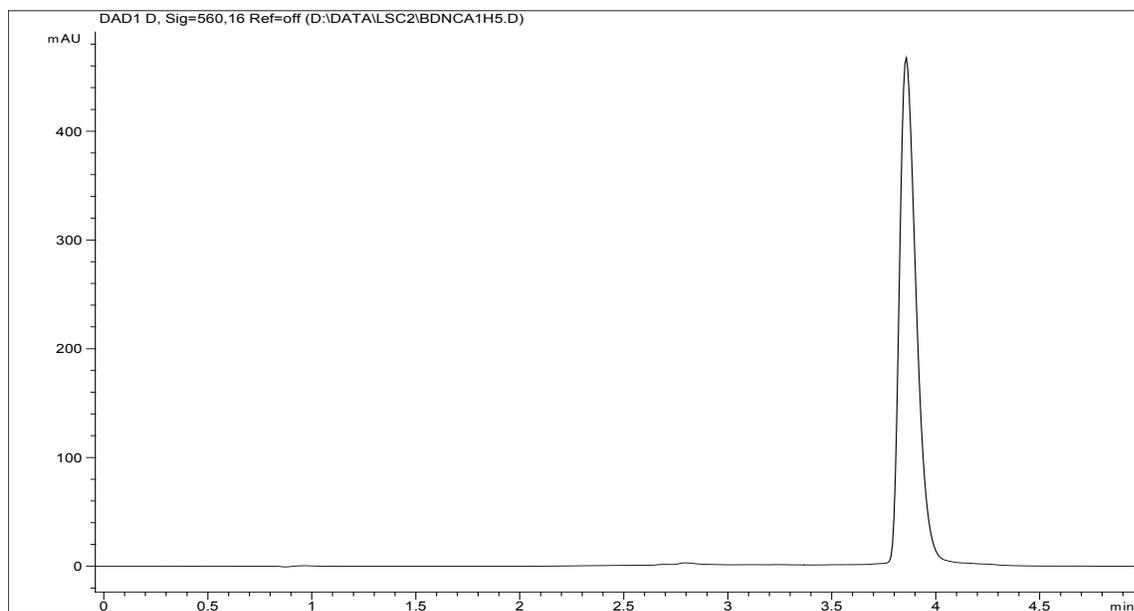
C

Non-microglia selective compounds

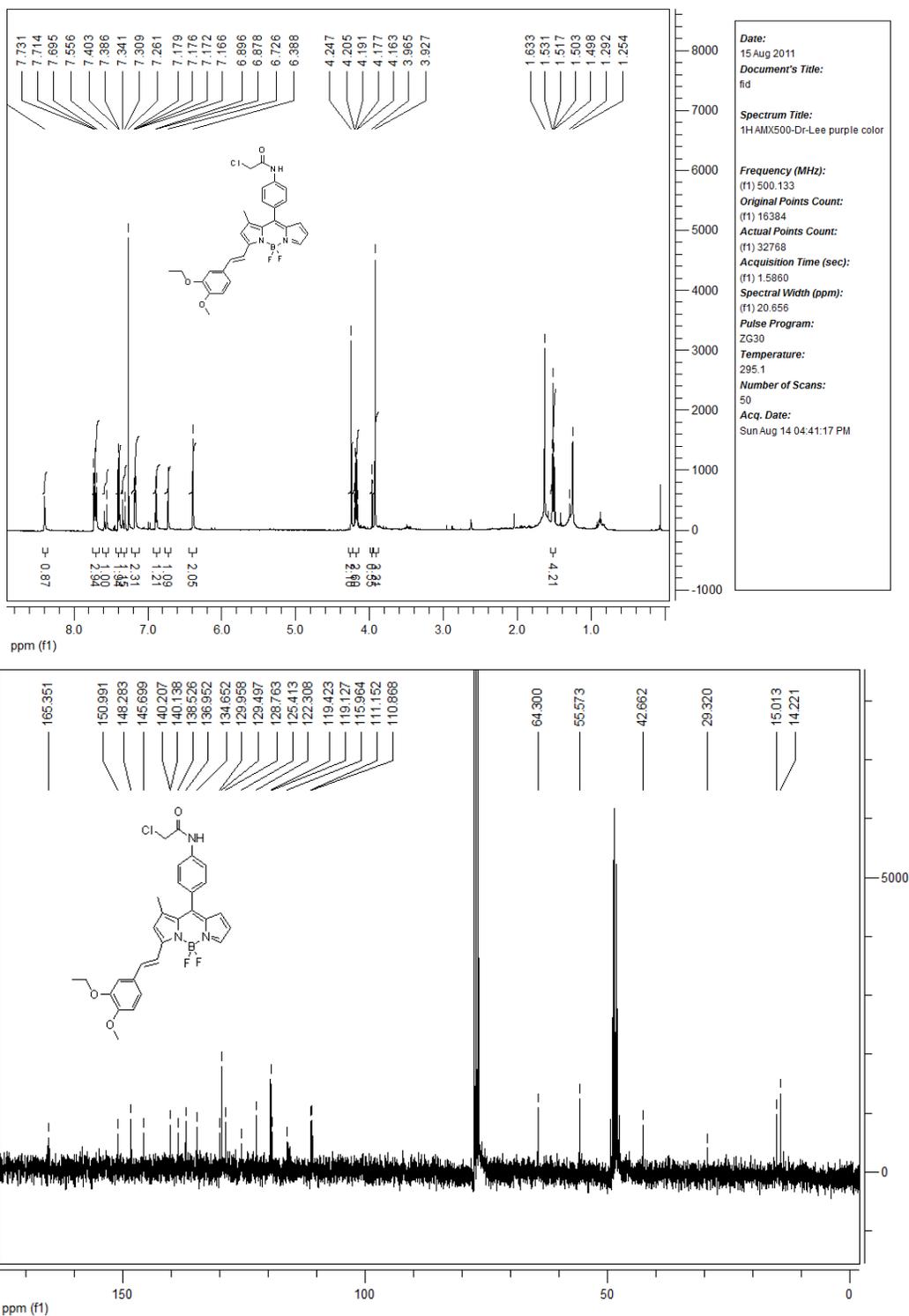




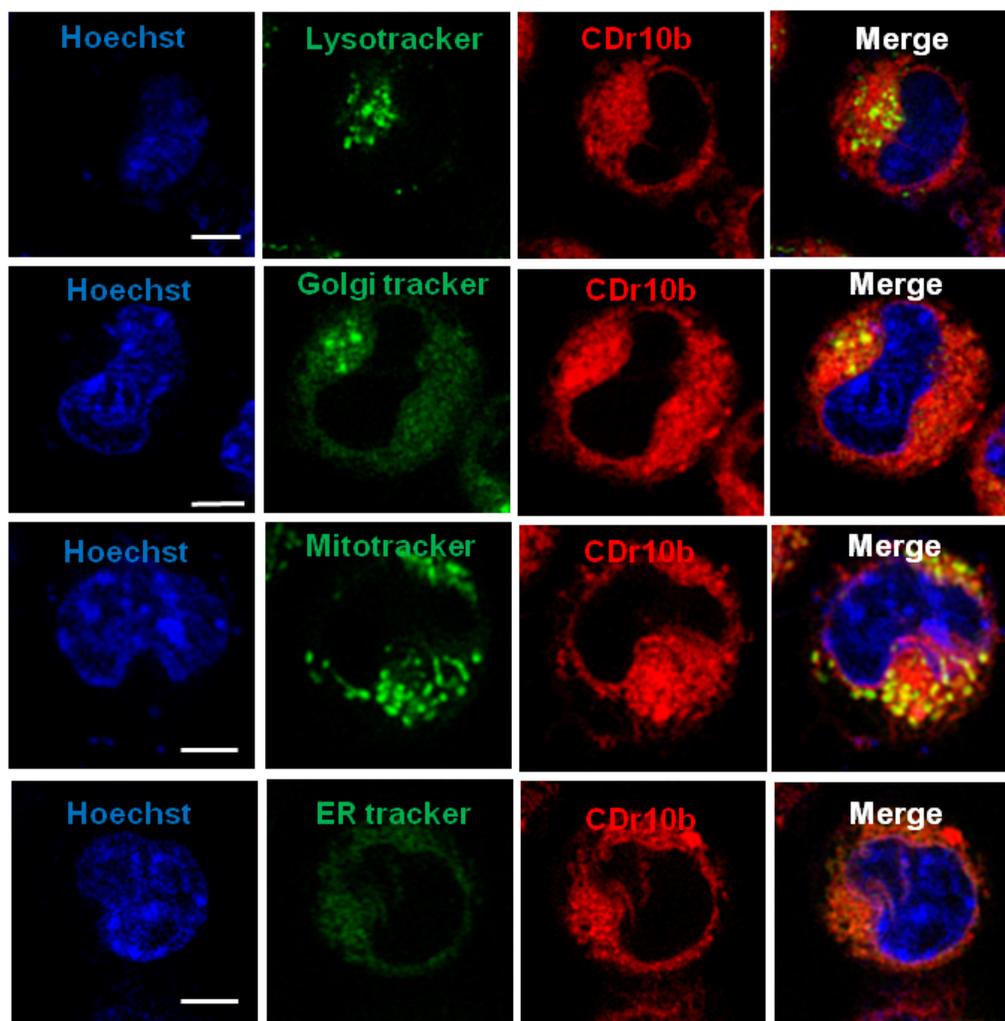
Supplementary Figure 1. Screening format overview and microglia hit structures. (a) Schematic work flow. Image based primary neural screening which employs the use of primary neural cells isolated from P1-3 mouse pup brain preparations. Cells isolated by the differential adhesion methods were found to be 80-90% positive for our cell types of interest. Cells were treated in parallel with compounds from our DOFL and screened using an image-based high throughput screening system. (b) Structures of microglia hit compounds – **CDr10b**, **MG1** and **MG2**. (c) Representative structures of non-microglia hit compounds from our DOFL libraries based on the BODIPY scaffold (d) Representative screening images and histogram analysis of 2 representative non-microglia selective compounds, B2 and B6 from primary neurons, astrocytes and microglia . Scale bar, 20 μm .



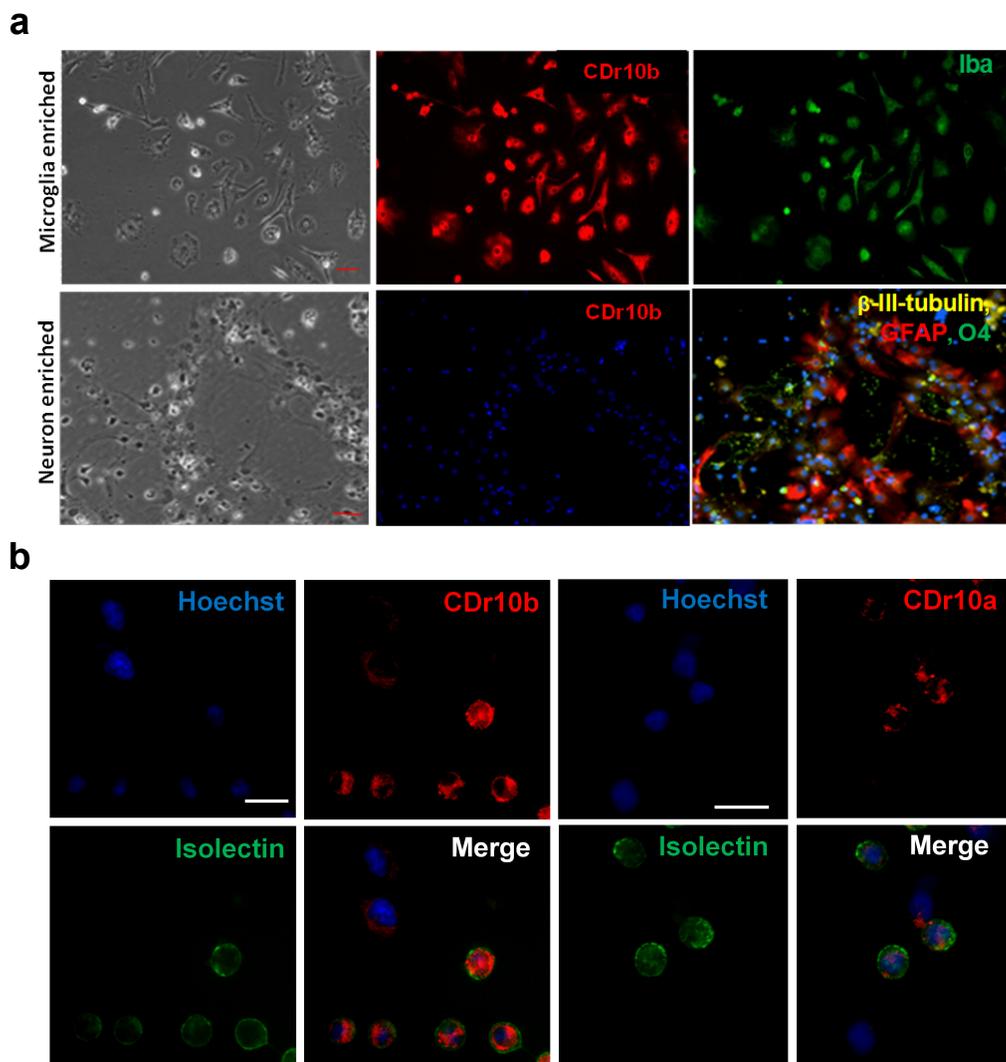
Supplementary Figure 2. LC-MS spectrum of CDr10b. HPLC condition: column (5 μ m, C18, 50 x 4.6 mm), 0.1 % TFA acetonitrile/water gradient from 5 to 100 % with 5 min running time.



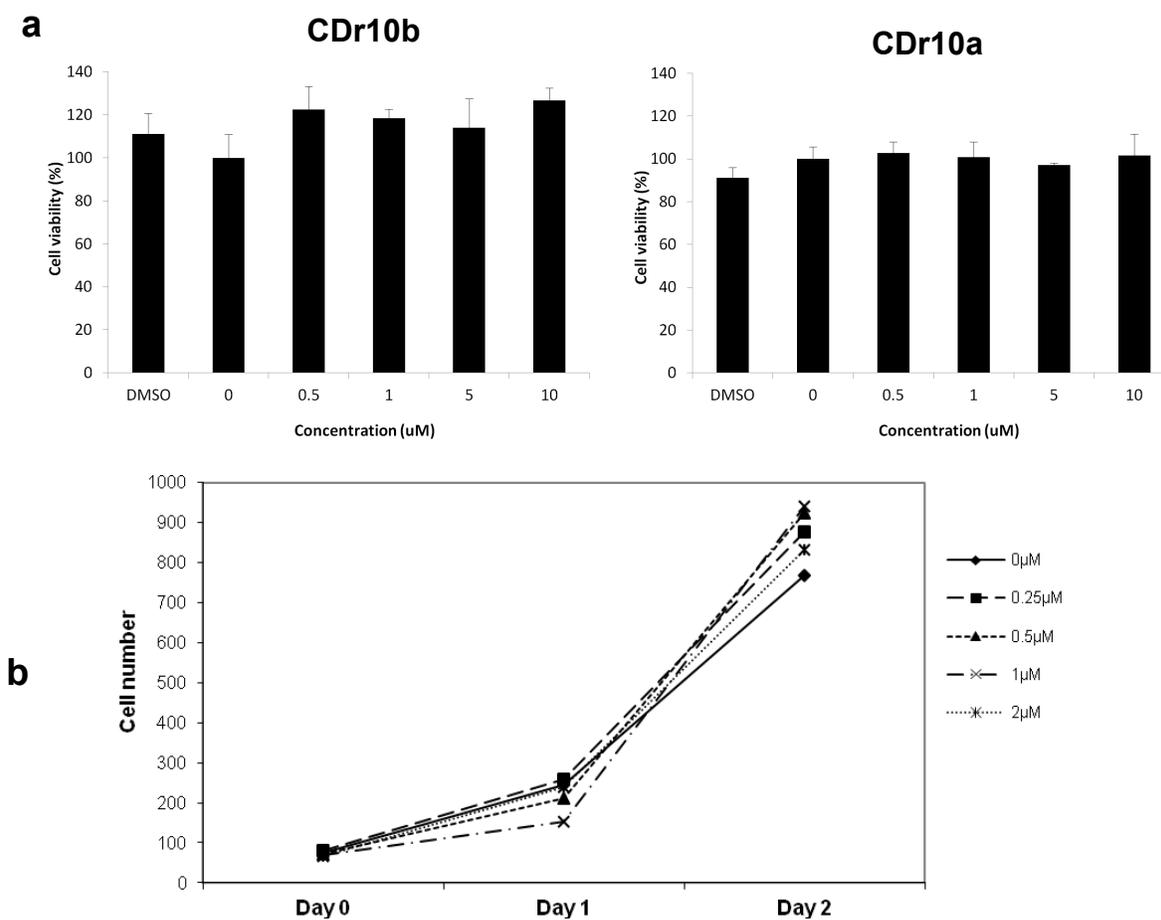
Supplementary Figure 3. 500MHz, 1H, 13C-NMR spectrum of CDr10b(CDCl₃). The upper panel shows UV absorption and the lower panel shows single quadrupole mass spectrums of **CDr10b**.



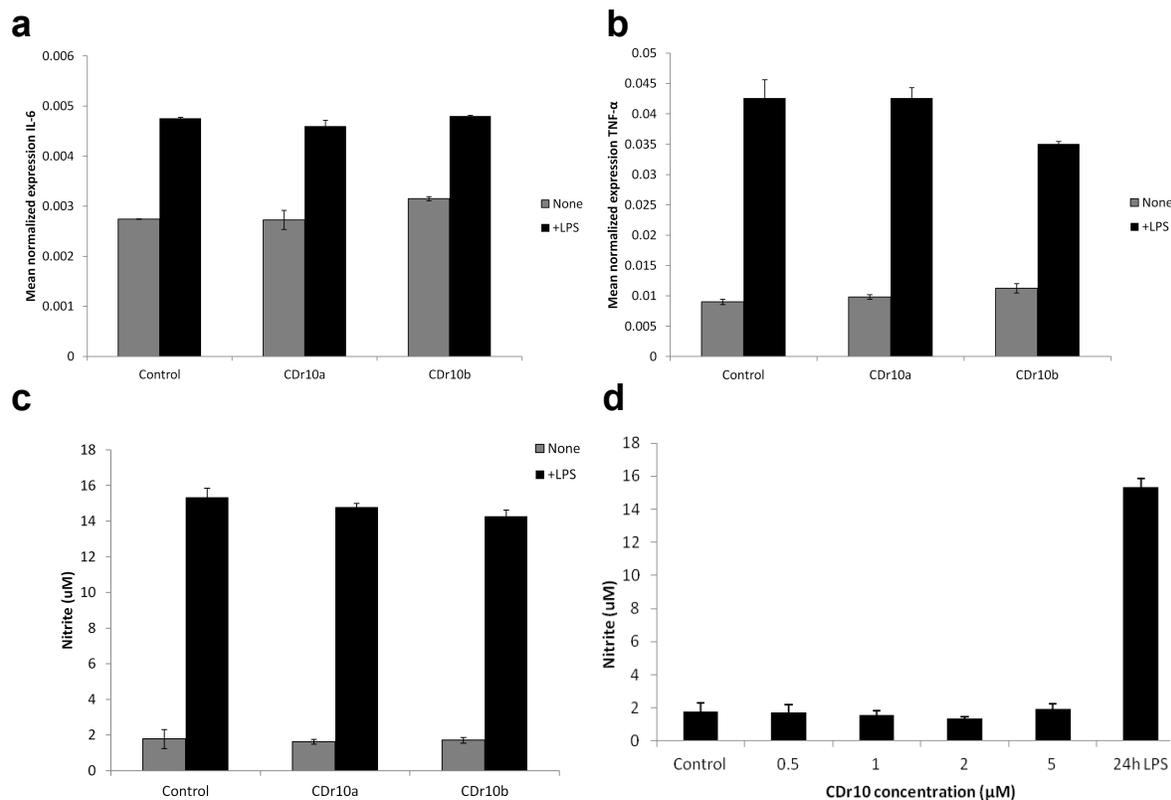
Supplementary Figure 4. CDr10b labeling with organelle trackers. BV2 microglia were co-stained with their respective organelle trackers and **CDr10b** to determine the level of co-localization. Scale bar, 5 μm .



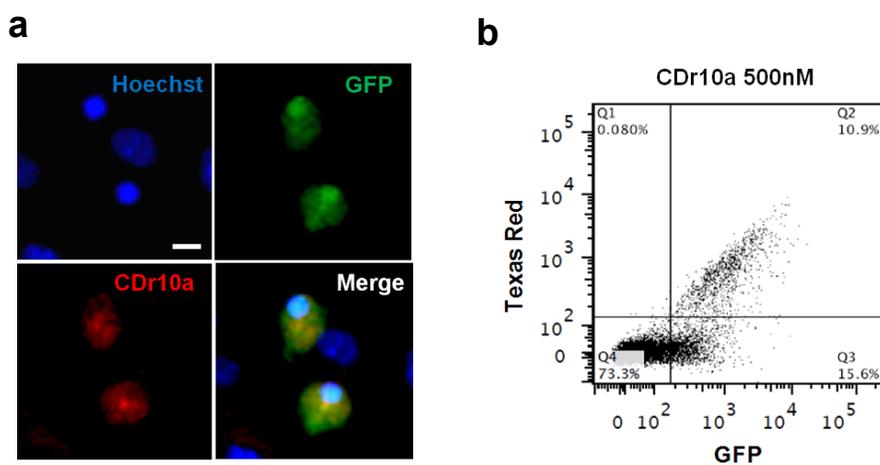
Supplementary Figure 5. Co-labeling of microglia with other markers. (a) Primary microglia enriched cultures (upper panels) and neuron enriched cultures (lower panels) were stained with 500 nM of **CDr10b** and imaged using a Texas red filter. **CDr10b** shows specificity for primary microglia and does not stain other primary neural cells. To confirm for cell type, the cells were fixed and immunostained for the microglia cell surface marker, Iba (green) and the corresponding neuron (β -III-tubulin - yellow), astrocyte (GFAP - red) and oligodendrocyte (O4 -green) markers. Scale bar, 50 μ m. (b) Isolectin B4 (Green) staining of BV2 microglia co-cultured with U251 glioma cells with CDr10b and CD10a (red). Scale bar, 20 μ m.



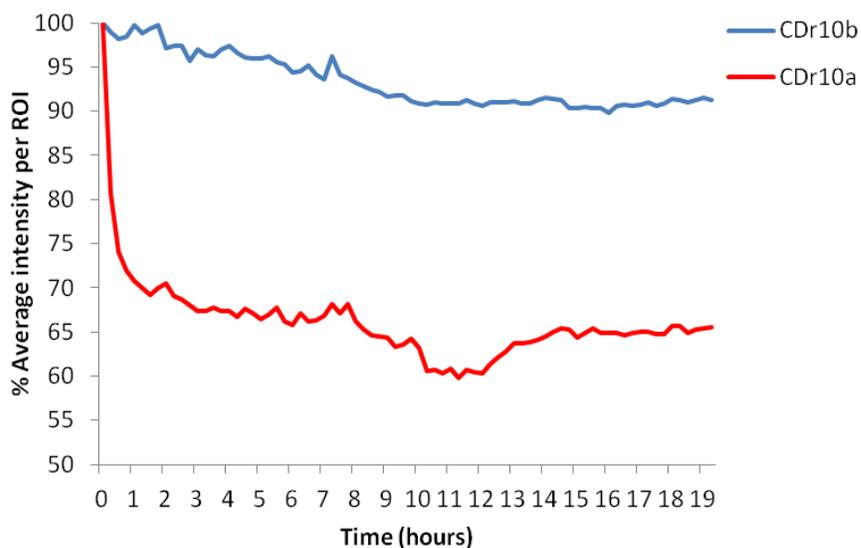
Supplementary Figure 6. Microglia viability assay. (a) Cell viability was accessed using an MTS assay (Promega) on BV2 cells after 24 hours incubation with the respective amounts of **CDr10b** and **CDr10a**. (b) The effect of **CDr10b** (500 nM) on cell proliferation was analyzed by cell counting of live and dead cells using SYTOX Green and Hoechst 33342 nuclei staining (Invitrogen) at 0, 24 and 48 hours. The number of live cells was calculated by subtracting dead cells (SYTOX Green positive cells) from all cells (Hoechst staining).



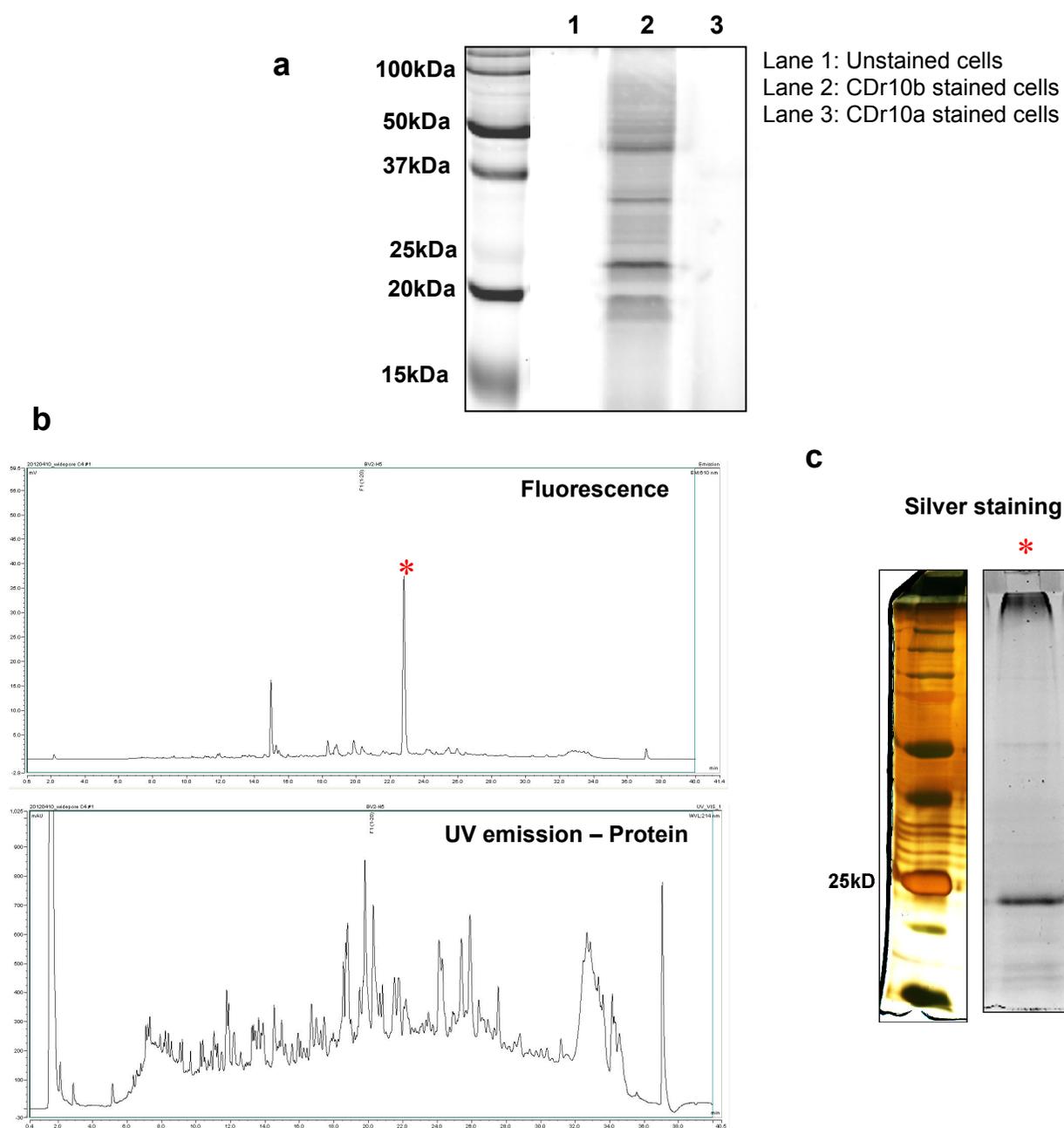
Supplementary Figure 7. Microglia functional assay. CDr10a and CDr10b was accessed for potential activating effects on the microglia by measuring cytokine expression and nitric oxide production. (a, b) BV2 cells were pretreated for 1 hour with 500 nM of compound before addition of 100 ng/ml of LPS in fresh media. After 6 hours, total RNA was isolated from the cells and mRNA expression levels of IL-6 (a) and TNF- α (b) were measured by qRT-PCR. All values were normalized to GAPDH expression levels. (c) For the measurement of nitric oxide concentration, cells were pretreated with 500 nM of compound before addition of 100 ng/ml LPS. After incubation for 24 hours, nitric oxide production was measured using the Griess assay and concentrations determined by comparison to a standard curve. (d) BV2 cells were pretreated for 1 hour with CDr10b (0.5-5 μ M), followed by the addition of LPS 24 hours before nitric oxide measurement. BV2 cells treated with LPS for 24 hours were used as a positive control.



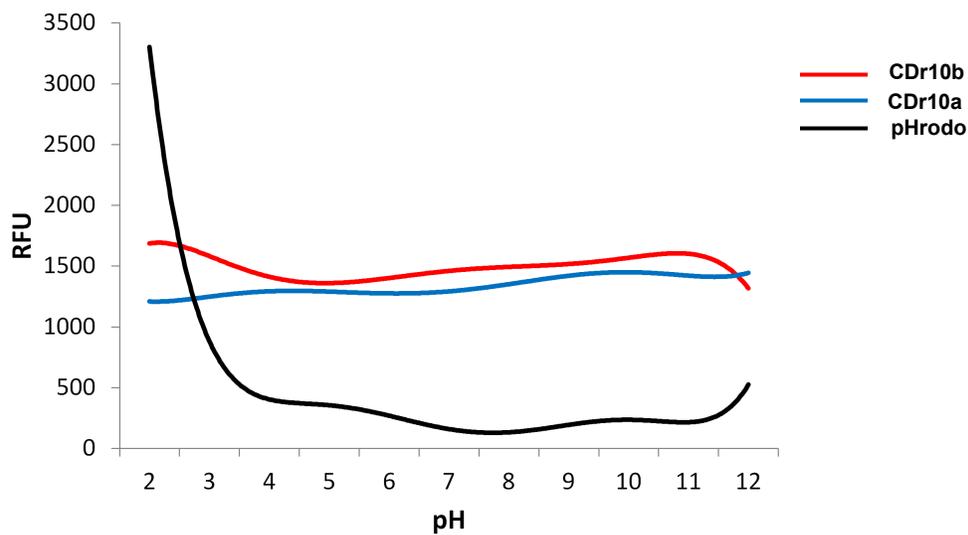
Supplementary Figure 8. Microglia specificity of CDr10a. Primary neural cells from CX3CR1-GFP mice were stained with 500 nM of CDr10a and imaged by fluorescence microscopy (a) and analyzed by flow cytometry (b). Scale bar, 10 μ m.



Supplementary Figure 9. Time-course analysis of CDr10b and CDr10a destaining. A percentage average intensity plot of **CDr10b** and **CDr10a** stained cells over 18 hours in media without compounds. **CDr10a** signal is rapidly washed out but **CDr10b** signal remains relatively constant.



Supplementary Figure 10. SDS-PAGE of CDr10a- and CDr10b-stained microglia cell lysates. (a) 1D SDS-PAGE - **CDr10b**-stained BV2 cell lysate shows prominent dye labeled bands. No signal was detected from control and **CDr10a**-stained cell lysate. **(b)** HPLC detection and fractionation- The major fluorescence peak from HPLC of **CDr10b**-stained BV2 cell lysate was fractionated and run on 1D SDS-PAGE. **(c)** Silver staining confirmed that it corresponded with the major fluorescent band identified from **(a)**.



Supplementary Figure 11. pH response of CDr10b and CDr10a. CDr10b (red) and CDr10a (blue) did not show any pH response compared to pHrodo, a known acid responsive fluorescent dye.