Supporting Information for:

Chemical Optogenetic Modulation of Inflammation and Immunity

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Contents

Materials and methods

General materials and methods. All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise noted. Organic extracts were dried over Na_2SO_4, and solvents were removed with a rotary evaporator at reduced pressure (20 torr), unless otherwise noted. Flash chromatography was performed using Silicycle Siliaflash P60 40-63Å 230-400 mesh silica gel. Analytical thin layer chromatography (TLC) was performed on glass-backed TLC 60 Å silica gel plates, and compounds were visualized by staining with ceric ammonium molybdate and the absorbance of UV light (λ = 254 nm or 365 nm). Reverse-phase HPLC was performed using a Shimadzu system equipped with a CBM-20A controller, SPD-20AV UV-Vis detector, LC-20AR liquid chromatograph unit, FRC-10A fraction collector, and an Epic Polar 5μm 120Å C18 analytical column (4.6 x 250 mm) at a flow rate of 1 mL/min or a semipreparative column (10 x 250 mm) at a flow rate of 4 mL/min. HPLC samples were filtered with a Millex-LH syringe filter equipped with a 0.45 μm PTFE membrane prior to injection. The water:acetonitrile gradient varied from 100:0 to 55:45 over 50 min with curve value of -4, after which the gradient increased to 0:100 over 5 min at a linear rate and remained at 0:100 for an additional 10 minutes. Dichloromethane (DCM), methanol (MeOH), dimethylsulfoxide (DMSO), and HPLC-grade water and acetonitrile were used from commercial sources without further purification. Suberoylanilide hydroxamic acid (SAHA) and sodium hydroxide (NaOH) were purchased from Cayman Chemical and Macron Fine Chemicals, respectively. HDAC substrate Boc-Lys(acetyl)-AMC was purchased from Enzo Life Sciences. 4,5-Dimethoxy-2-nitrobenzyl bromide (NV-Br) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Acros Organics. NMR spectra were acquired using a Bruker 500 spectrometer and are referenced to residual solvent peaks. High-resolution mass spectra were obtained at the Cornell University Mass Spectrometry (MS) Laboratory.

Dulbecco's phosphate-buffered saline (PBS) pH 7.4, RPMI-1640 media, and cell culture supplements (L-glutamine, sodium pyruvate, HEPES buffer, and penicillin/streptomycin) were purchased from Corning. Fetal bovine serum, Lipofectamine 2000, and ProLong Diamond mounting media were purchased from Thermo Fisher Scientific. Bovine serum albumin (BSA) was obtained from Amresco. Petri and tissue culture-treated dishes were obtained from BD Falcon, and glass-bottom microwell dishes (35 mm) were obtained from MatTek Corporation. Lipopolysaccharide (LPS) from Escherichia coli 055:B5 was purchased from Sigma Aldrich. Antibodies against CD16/CD32 (clone 93), IL-6 (clone MP5-20F3), TNF-α (clone TN3-19), and rabbit polyclonal biotinylated anti–TNF-α were purchased from eBiosciences. Biotinylated IL-6 (clone MP5-32C11), IL-12p40 (clone C15.6), and biotinylated IL-12p40 (clone C17.8) were purchased from BD Biosciences. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solutions and streptavidin-conjugated horseradish peroxidase for ELISA development were purchased from BD Biosciences. Goat anti-rat Alexa Fluor 647 was purchased from Thermo Fisher Scientific.

UV absorbance readings were measured on a Bio-Tek PowerWave XS microplate spectrophotometer. Fluorescence readings were collected with a Molecular Devices SpectraMax Gemini EM fluorescent plate reader. Imaging was performed on a Zeiss LSM 800 confocal laser scanning microscope equipped with 20X 0.8 NA and 40X 1.4 NA Plan Apochromat objectives, 405, 488, 561, and 640 nm solid-state lasers, and two GaAsP PMT detectors.

Mice. C57Bl/6 mice were purchased from Harlan Laboratories and bred at the animal facility of Cornell University. Mice were used at 8-20 weeks of age in accordance with the guidelines of the Institutional Animal Care and Use Committee and the Cornell Center for Animal Resources and Education (Protocol Number 2015-0069).

In vitro photo-uncaging of 1. Compound **1** was dissolved in DMSO and irradiated with 365 nm UV light for 6 h. The solution was periodically analyzed by HPLC and MS to monitor conversion of **1** to SAHA. Percent conversion was calculated by analyzing standards of **1** and SAHA by HPLC, using integration of the area under the curve for normalization. These data were used to calculate the first-order reaction kinetics.

In vitro stability studies. Compound **1** was dissolved in a mixture of 5:1 DMSO:PBS and incubated at 37 °C for 24 h. The solution was periodically analyzed by HPLC and MS to monitor decomposition of **1**. Percent of **1** remaining was calculated by analyzing a standard of **1** by HPLC, using integration of the area under the curve for normalization.

BMDM cultures. BMDM were cultured as previously described.[1] Briefly, bone marrow progenitors were harvested from C57Bl/6 mice and cultured for 6 d on Petri dishes in RPMI-1640 media supplemented with macrophage colony-stimulating factor (macrophage media). Cells were fed on day 4 and were lifted with 5 mM EDTA and replated on tissue culture-treated dishes or glass-bottom microwell dishes for imaging the day before stimulation. The cells were maintained at 37 °C and 5% CO_2 in a water-saturated incubator and counted using a hemocytometer.

Cell viability assay. BMDM were plated ($1x10^5$ cells/well) in a 96-well plate. After incubation overnight, the media was replaced with 100 μL of 1.2 mM MTT in macrophage media. The cells were incubated for 3 h, after which 75 μL of media was removed and 50 μL of DMSO was added to each well. After incubating for an additional 10 min at 37 °C, the UV absorbance readings were measured at 540 nm using a plate reader.

HDAC activity assay. BMDM were lysed in Nonidet P-40 lysis buffer (25 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 1 mM DTT) with protease inhibitor cocktail (Roche cOmplete EDTA-free tablets), and the samples were sonicated briefly. The samples were then centrifuged at 15,000 × g for 15 min, and the supernatants were collected as whole cell lysates. The lysates (10 μg) were treated with SAHA (50 μM) or **1** (50 μM) with or without UV irradiation (365 nm, 10 min). The samples were diluted with reaction buffer (25 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT) up to 55 μL in the presence of 100 μM substrate Boc-Lys(acetyl)-AMC and incubated at 37 °C for 1 h. To quench, an equal volume of trypsin (5 mg/mL) was added, and the reactions were incubated at 37 °C for an additional 20 min. The samples (100 μL) were transferred to a black-bottom 96-well plate, and fluorescence was recorded using a plate reader at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

Treatment of BMDM with SAHA. BMDM were plated ($1x10^6$ cells/well) in a 24-well plate. After incubation overnight, the cells were stimulated with LPS (100 ng/mL) with and without various concentrations of SAHA (i.e., 0.01 nM – 1 μM). Cell supernatants were analyzed by ELISA and the Griess assay.

Alternatively, for imaging by confocal microscopy, BMDM were stimulated with LPS and SAHA in the presence of Brefeldin A (5 μg/mL) and incubated for 6 h. Afterwards, the cells were

washed with PBS (1 x 2 mL) and fixed with paraformaldehyde (4% in PBS) for 10 min at RT. The samples were then washed with PBS (3 x 2 mL) and permeabilized with 0.1% Triton X-100 for 15 min at RT. The samples were washed with PBS (3 x 2 mL), and the Fc receptors were blocked with anti-mouse CD16/CD32 in 5% BSA/PBS for 40 min at RT. The samples were subsequently stained with anti-IL-12 antibody in 5% BSA/PBS for 1 h at RT. The samples were washed with PBS (4 x 2 mL), after which goat anti-rat Alexa Fluor 647 was added in 5% BSA/PBS for 30 min at RT. The samples were washed with PBS (3 x 2 mL), and the coverslips were mounted with ProLong Diamond with DAPI and allowed to dry overnight in the dark before imaging. Image analysis was performed using the Zeiss Zen Blue 2.1 and FIJI software packages. Images shown are maximum-intensity z-projection images, and quantification was performed using FIJI and analyzed in a blinded manner.

Cytokine quantification by sandwich ELISA. After stimulation, BMDM were incubated for an additional 24 h after which supernatants were collected. Cytokines were detected with paired antibodies as previously described, with the following modifications.[2] Antibody binding was detected by streptavidin-conjugated horseradish peroxidase, and ELISA samples were developed with TMB substrate. UV absorbance readings of the samples were measured at 450 nm using a plate reader.

Nitric oxide (NO) quantification by the Griess assay. After stimulation, BMDM were incubated for an additional 24 h, after which supernatants were collected. NO was detected by reacting equal volumes of culture supernatants with 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine dihydrochloride in 2.5% (v/v) phosphoric acid. UV absorbance readings of the samples were measured at 550 nm using a plate reader.

Photo-uncaging of 1 in BMDM. BMDM were plated in 24-well plates ($1x10^6$ cells/well). After incubation overnight, the cells were treated with vehicle (DMSO), LPS (100 ng/mL), LPS (100 ng/mL) and SAHA (500 nM), or LPS (100 ng/mL) and **1** (3 μM). The cells were either irradiated with UV light (365 nm) for 10 min using a UVP Transilluminator 2UV or shielded from light. The cells were incubated for an additional 24 h prior to analysis.

Photo-uncaging of 1 in subpopulation of BMDM and imaging by confocal microscopy. The photoswitchable fluorescent protein mEos3.2 was cloned into pMSCV, a retroviral vector expressing a puromycin resistance gene, using XhoI and EcoRI. This construct was transfected into HEK 293T cells together with the pCl-ECO packaging plasmid. Retroviral supernatants were mixed with Lipofectamine 2000, and viruses were introduced into bone marrow progenitors by centrifugation at 1,172 × g for 90 min at 32 °C. This step was repeated again after 24 h. Puromycin was added to the growth media 48 h after the second spin infection.

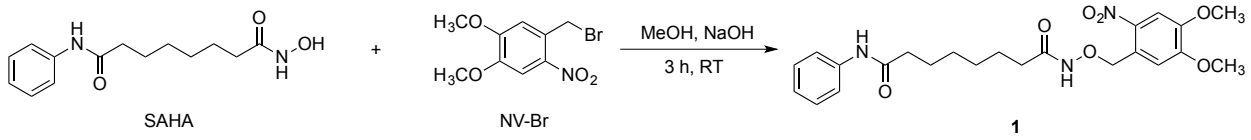
BMDM were plated in 35 mm glass-bottom microwell dishes. After incubation overnight, the cells were pretreated with **1** (3 μM) for 18 h. The samples treated with **1** were washed with PBS (1 x 2 mL). The cells were irradiated on the microscope stage with UV light using epifluorescence illumination (HXP 120 V light source) using a 20X objective at 100% intensity through a DAPI filter set (Zeiss #49) for 30 s. The cells were then treated with LPS (100 ng/mL) or LPS (100 ng/mL) and SAHA (500 nM) in the presence of Brefeldin A (5 μg/mL) and incubated for 6 h. Afterwards, the cells were washed with PBS (1 x 2 mL) and fixed with paraformaldehyde (4% in

PBS) for 10 min at RT. The samples were then washed with PBS (3 x 2 mL) and permeabilized with 0.1% Triton X-100 for 15 min at RT. The samples were washed with PBS (3 x 2 mL), and the Fc receptors were blocked with anti-mouse CD16/CD32 in 5% BSA/PBS for 40 min at RT. The samples were subsequently stained with anti-IL-12 antibody in 5% BSA/PBS for 1 h at RT. The samples were washed with PBS (4 x 2 mL), after which goat anti-rat Alexa Fluor 647 was added in 5% BSA/PBS for 30 min at RT. The samples were washed with PBS (3 x 2 mL), and the coverslips were mounted with ProLong Diamond and allowed to dry overnight in the dark before imaging. Image analysis was performed using the Zeiss Zen Blue 2.1 and FIJI software packages. Images shown are maximum-intensity z-projection images, and quantification was performed using FIJI and analyzed in a blinded manner.

Synthetic procedures

Compound 1. SAHA (50 mg, 0.189 mmol) was dissolved in MeOH (2 mL). To this mixture was added NV-Br (80 mg, 0.284 mmol, 1.5 eq.), followed by 40% (w/v) NaOH (40 µL, 2.1 eq.) dropwise. The reaction was stirred for 3 h at RT. The solvent was then removed via rotary evaporation. The resulting residue was dissolved in ddH_2O (3 mL) and acidified to pH 1, dropwise with 1 N HCl. An extraction was then performed with DCM (3×25 mL). The organic layer was collected and concentrated by rotary evaporation. The resulting crude product was purified by silica gel chromatography, eluting with 95:5 DCM:MeOH to yield 70 mg (80%) of a white solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.06 (s, 1H), 9.85 (s, 1H), 7.66 (s, 1H), 7.58 (d, 2H, J = 12 Hz), 7.39 (s, 1H), 7.27 (t, 2H, J = 8 Hz), 7.00 (t, 1H, J = 12 Hz), 5.15 (s, 2H), 3.92 (s, 3H), 3.85 (s, 3H), 2.26 (t, 2H, J = 8 Hz), 1.93 (t, 2H, J = 12 Hz), 1.54 (m, 2H), 1. 47 (m, 2H), 1.22 (m, 4H). ^{13}C NMR (500 MHz, DMSO-d_6): δ 171.65, 170.10, 153.61, 148.26, 140.24, 139.82, 129.08, 127.70, 123.36, 119.48, 111.74, 108.46, 73.68, 56.69, 56.52, 36.82, 28.86, 28.72, 25.43, 25.24. DART-MS: Calcd. for $C_{23}H_{30}N_3O_7^+$ $(M+H)^+$ 459.2084, found 460.2076.

Scheme 1. Synthesis of 1.



Supplemental data

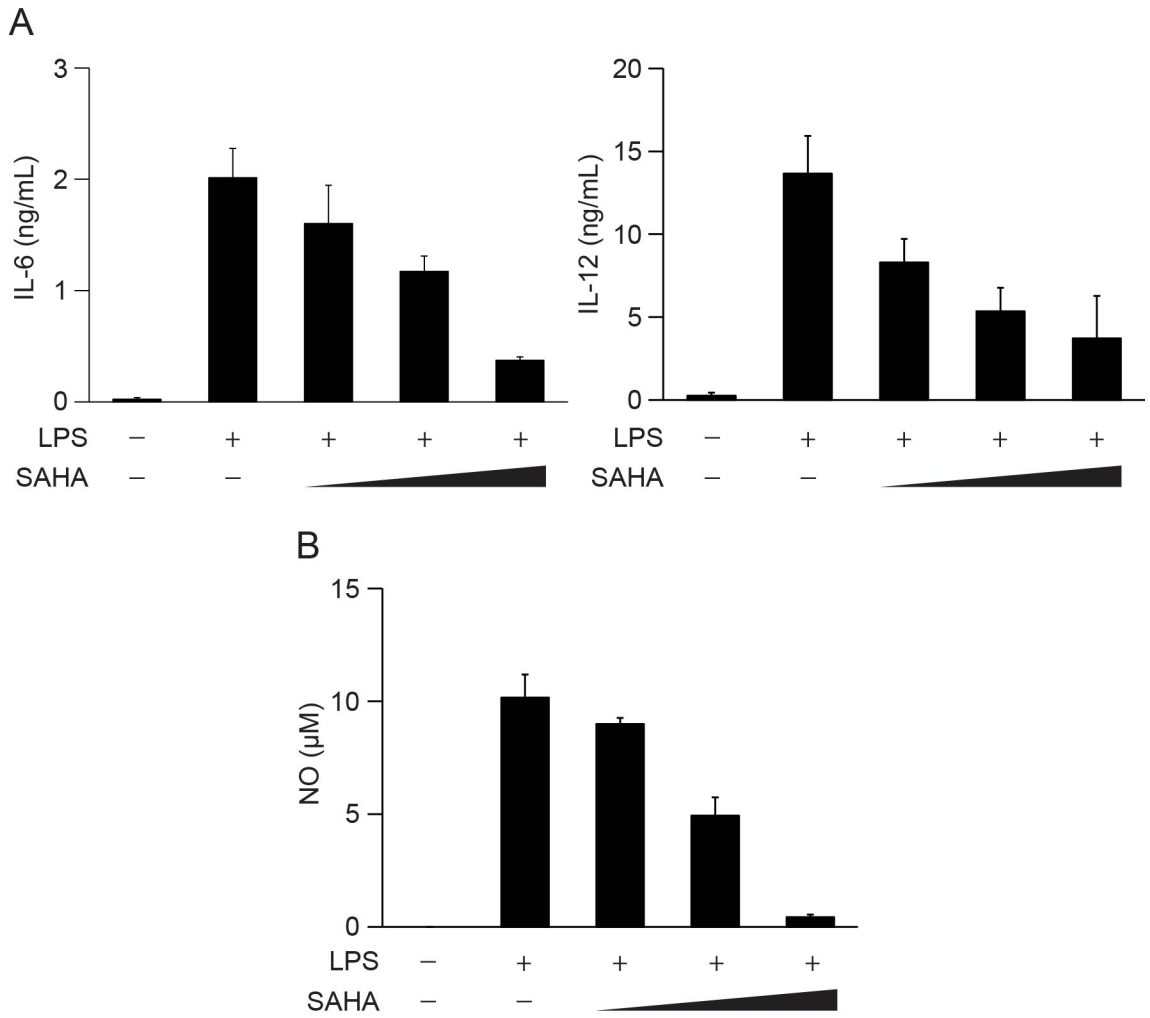


Figure S1. SAHA inhibits pro-inflammatory mediator production by macrophages. BMDM were treated with LPS (100 ng/mL) with or without various concentrations of SAHA (50 nM, 250 nM, and 1 μM). The amount of secreted (A) IL-6 and IL-12 was measured by ELISA and (B) nitric oxide (NO) was measured by the Griess assay. Data are representative of three independent experiments. Error bars represent mean ± SD.

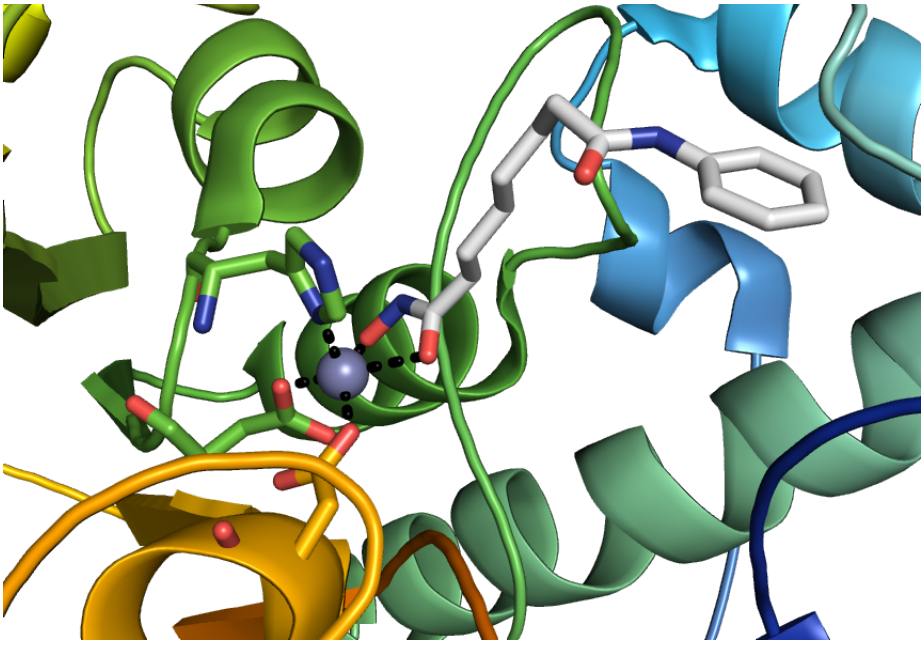


Figure S2. Crystal structure of the active site of human HDAC2 (PDB ID: 4LXZ)[3] with the inhibitor SAHA (gray) bound. Note the importance of the hydroxamic acid moiety in binding to the Zn^{2+} cofactor.

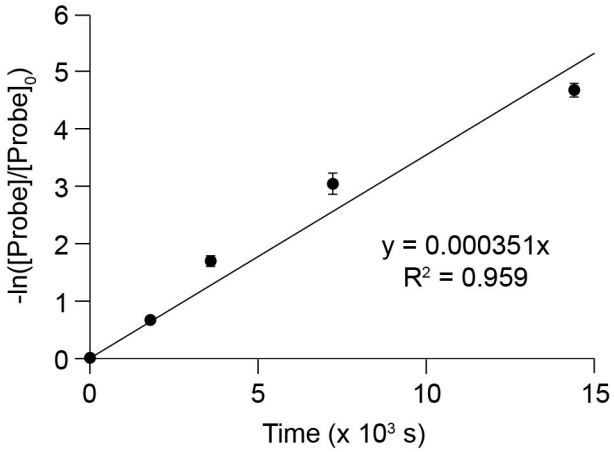


Figure S3. Photo-uncaging of 1 is a first-order reaction. Compound **1** was irradiated with 365 nm UV light for 6 h. The reaction was periodically analyzed by HPLC and MS to monitor conversion of **1** to SAHA. Percent conversion was calculated by analyzing standards of **1** and SAHA by HPLC, using integration of the area under the curve for normalization.

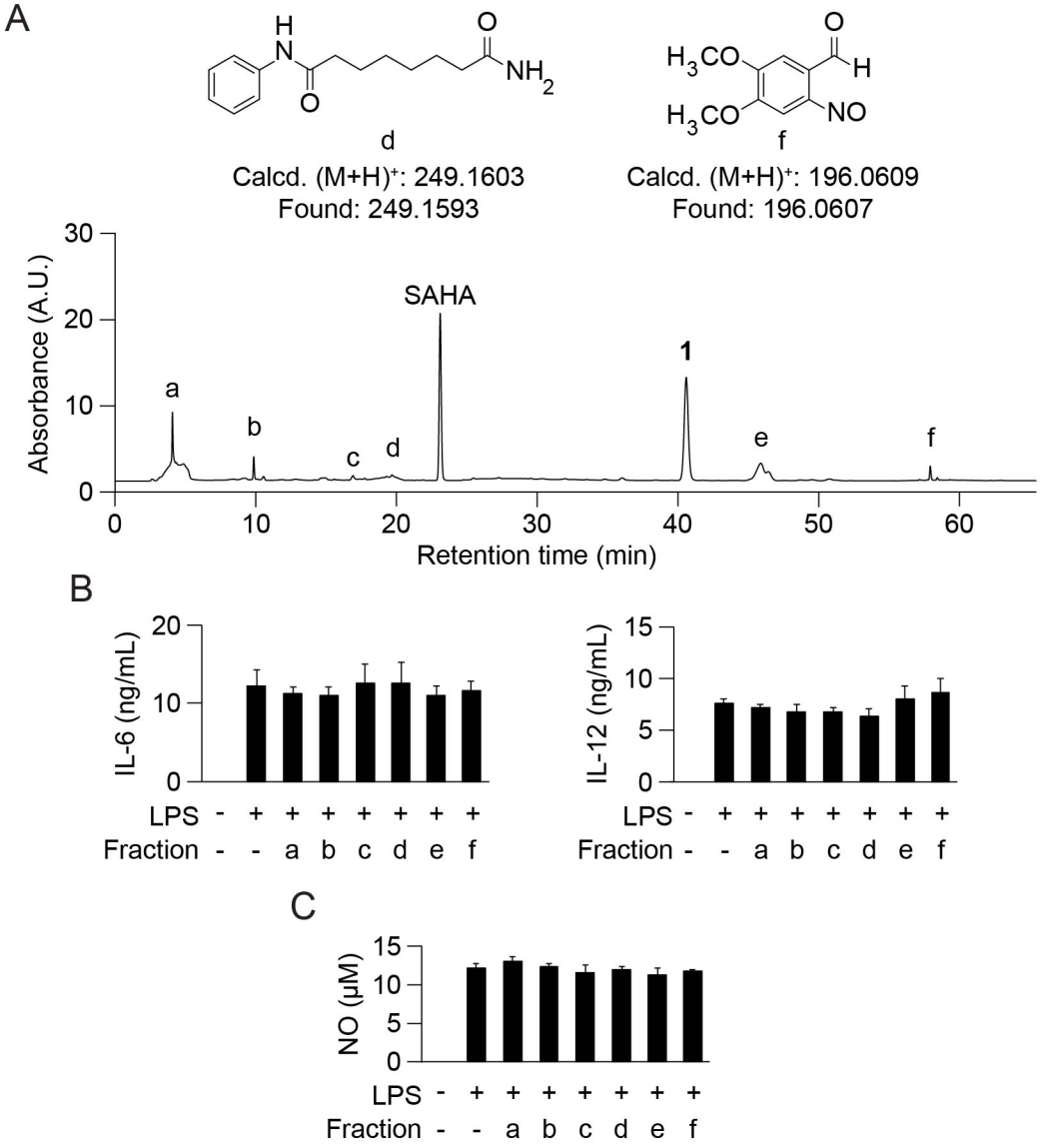


Figure S4. Photo-uncaging of 1 produces minor byproducts that do not affect pro-inflammatory mediator production by macrophages. Compound **1** was photo-uncaged in vitro and the byproducts were purified by HPLC. BMDM were treated with LPS (100 ng/mL) with or without the various byproducts (Fractions a-f) at concentrations based on photo-uncaging of **1** (3 μM). (A) Representative HPLC trace of collected fractions and identification of byproducts by NMR and HRMS. The amount of secreted (B) IL-6 and IL-12 was measured by ELISA and (C) NO was measured by the Griess assay. Data are representative of two independent experiments. Error bars represent mean ± SD.

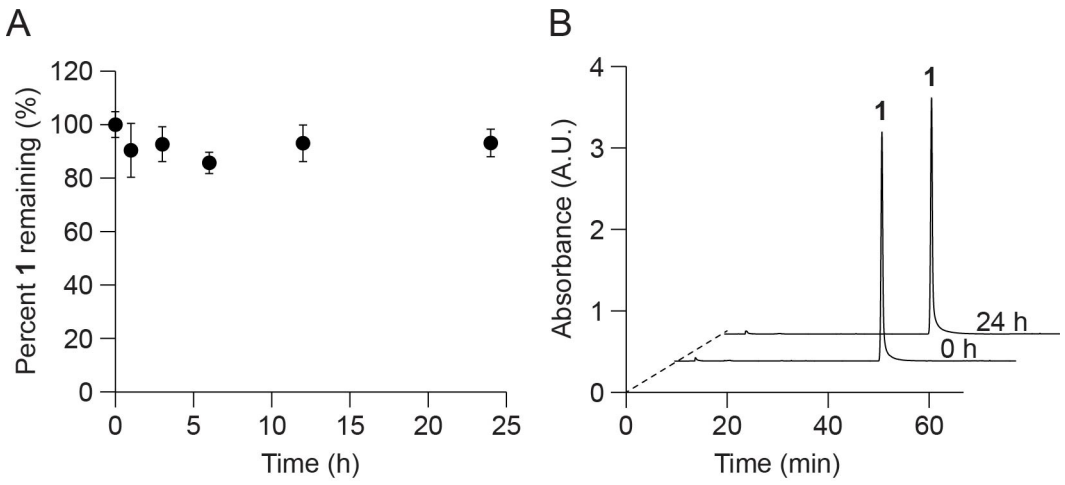


Figure S5. Compound 1 is stable in aqueous solution. Compound **1** was dissolved in a mixture of 5:1 DMSO:PBS and incubated at 37 ºC for 24 h. (A) Percent remaining of **1**. (B) HPLC analyses of samples. Traces shown were monitored by UV absorbance at 254 nm. A.U. = arbitrary units. Data are representative of three independent experiments. Error bars represent mean ± SD.

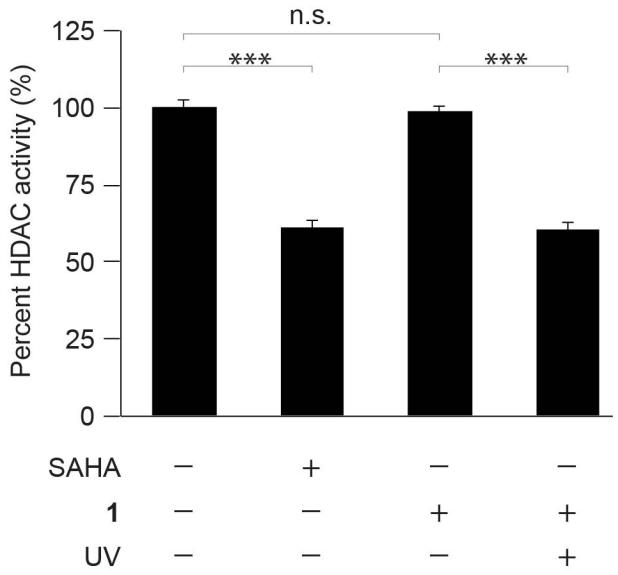


Figure S6. Compound 1 does not inhibit histone deacetylase (HDAC) activity. BMDM whole cell lysates (10 μg) were treated with SAHA (50 μM) or **1** (50 μM) with or without UV irradiation (365 nm, 10 min). HDAC activity was measured using a fluorogenic substrate. Data are representative of three independent experiments. Error bars represent mean ± SD. n.s. = not significant, *** $p<0.001$ (Student's t-test).

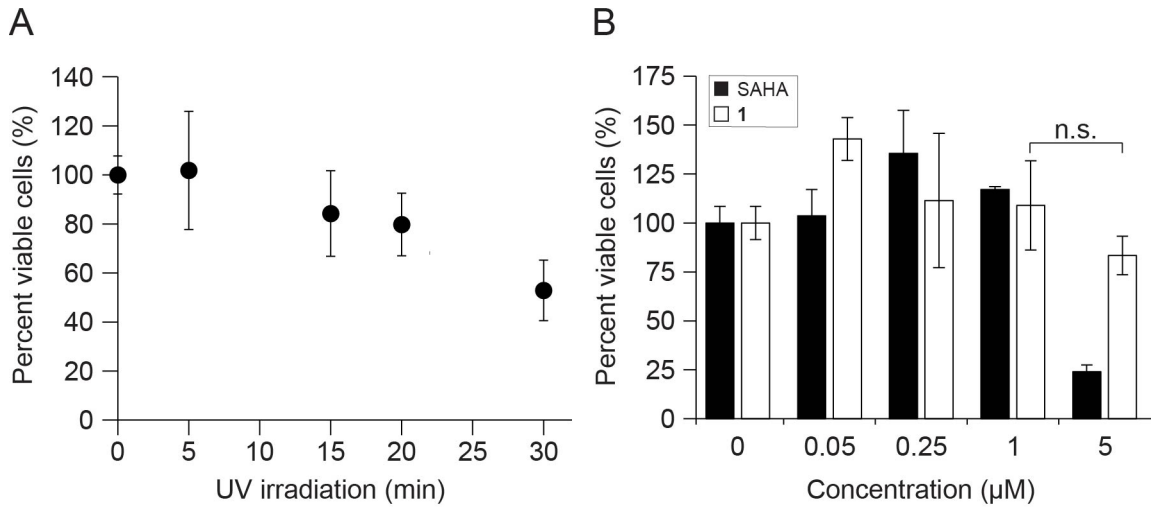


Figure S7. Photo-uncaging conditions are not toxic to BMDM. MTT assay was used to measure cell viability of BMDM exposed to (A) UV light (365 nm) for various amounts of time and (B) different concentrations of SAHA or **1** for 24 h. Data are representative of three independent experiments. Error bars represent mean ± SD. n.s. = not significant (Student's t-test).

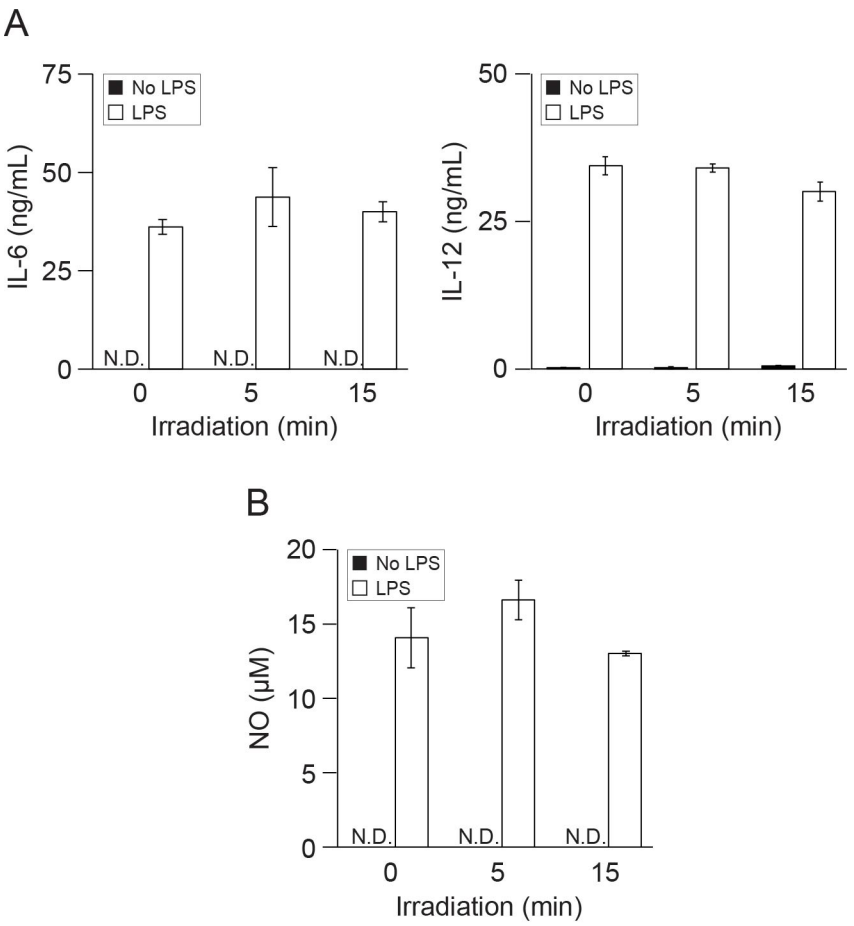


Figure S8. UV irradiation does not affect pro-inflammatory mediator production by BMDM. BMDM were treated with or without LPS (100 ng/mL) and irradiated with UV light (365 nm) for various amounts of time. The amount of secreted (A) IL-6 and IL-12 was measured by ELISA and (B) NO was measured by the Griess assay. Data are representative of three independent experiments. Error bars represent mean ± SD. N.D. = Not detectable.

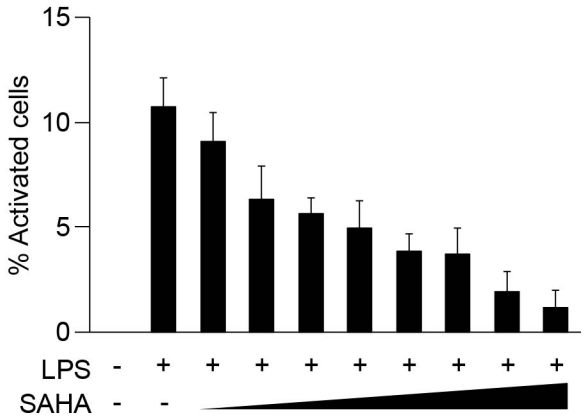


Figure S9. SAHA dose-dependently reduces the percent of activated BMDM. BMDM were treated with LPS (100 ng/mL) +/- SAHA (0.01, 0.1, 0.25, 0.5, 1, 10, 100, and 500 nM). Following immunofluorescence staining for IL-12, cells were imaged by confocal microscopy, and the percentage of activated cells was calculated based on the number of IL-12 positive cells compared to the total number of cells.

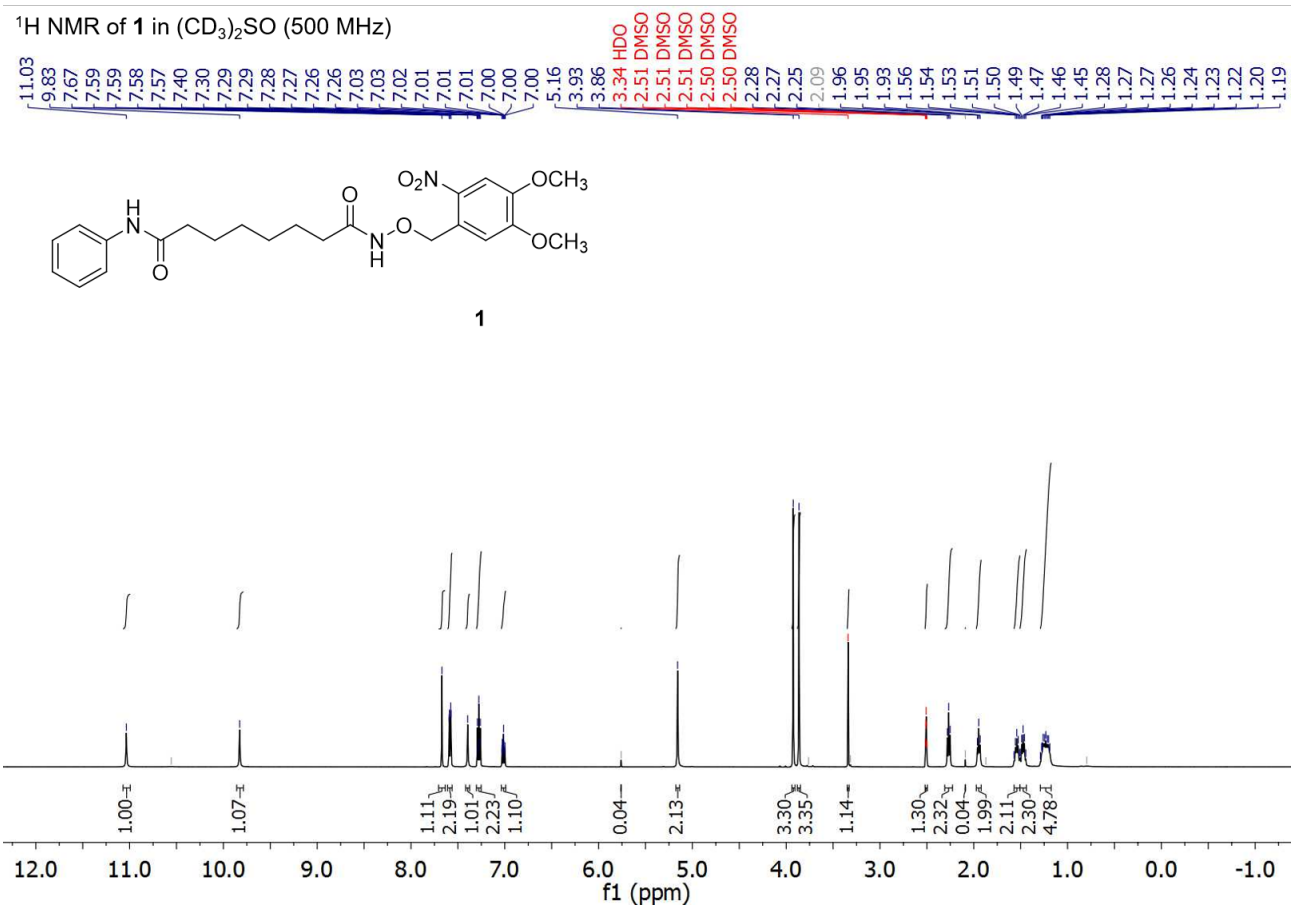


Figure S10. ^{1}H NMR spectrum of compound **1**.

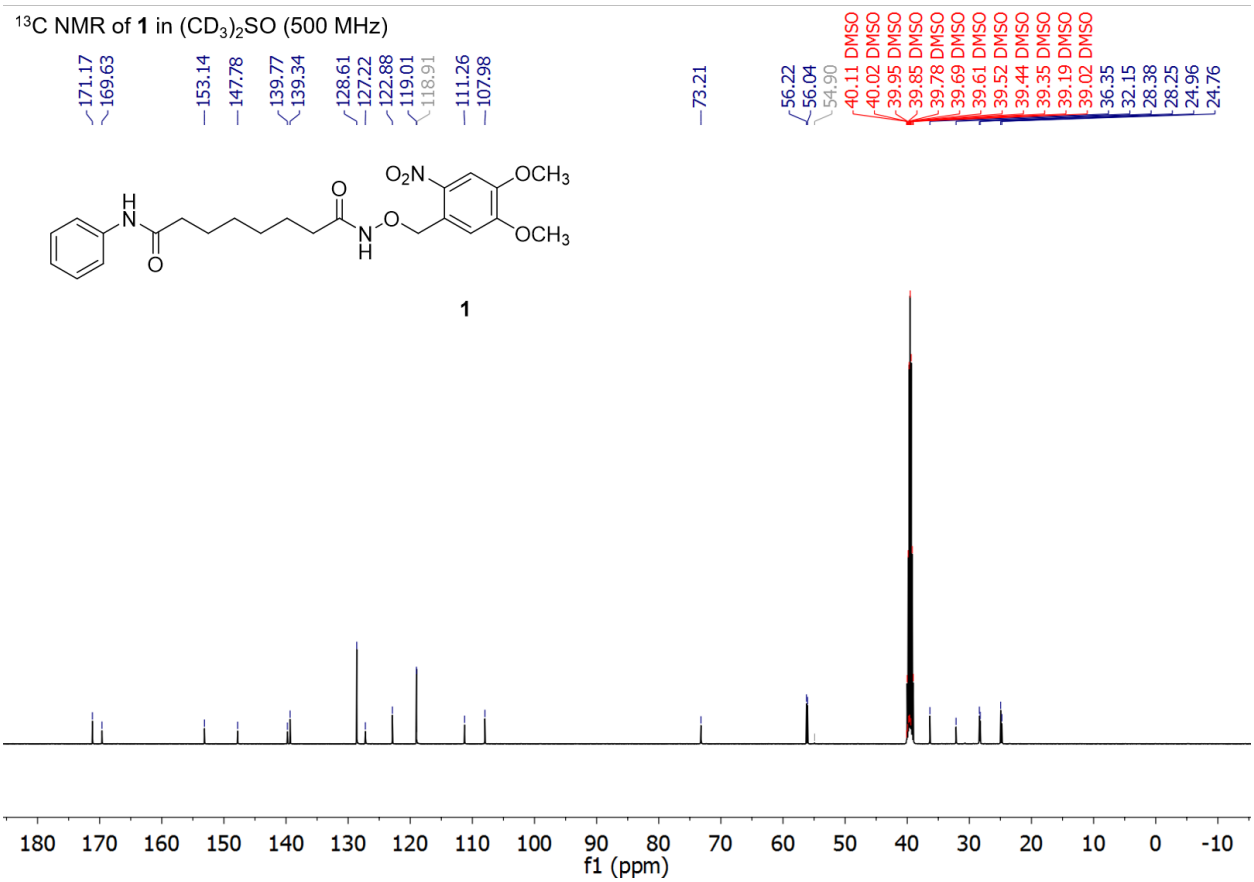


Figure S11. ^{13}C NMR spectrum of compound **1**.

Supplemental references

[1] J. Weischenfeldt, B. Porse, *Cold Spring Harbor Protocols* **2008**, doi:10.1101–pdb.prot5080.

[2] P. V. Hornbeck, *Enzyme-Linked Immunosorbent Assays*, John Wiley & Sons, Inc., Hoboken, NJ, USA, **2001**.

[3] B. E. L. Lauffer, R. Mintzer, R. Fong, S. Mukund, C. Tam, I. Zilberleyb, B. Flicke, A. Ritscher, G. Fedorowicz, R. Vallero, et al., *J. Biol. Chem.* **2013**, *288*, 26926–26943.