Highly selective inhibition of IMPDH2 provides the basis of anti-neuroinflammation therapy

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I. Supplementary methods

1. Chemicals

1.1. Synthesis of sappanone A (SA)

Step 1

3-chloro-1-(2,4-dihydroxy-phenyl)-propan-1-one

To a stirred mixture of resorcinol (30.2 g, 275 mmol) and 3-chloropropionic acid (29.8 g, 275 mmol) trifluoromethanesulfonic acid (75 g, 498 mmol) was added in one portion. The mixture was heated at 80°C for 30 min. The reaction was cooled to room temperature and the mixture was diluted with chloroform (300 mL). The solution was slowly poured into ice water (300 mL) with strong stirring. The bi-layer was separated, and the aqueous phase was extracted with chloroform. The combined organic phase was washed with brine and dried over sodium sulfate. The 3-chloro-1-(2,4-dihydroxy-phenyl)-propan-1-one was obtained as orange oil (25.3 g) after evaporation of solvent. The crude product was used in the next step without further purification.

Step 2

7-hydroxychroman-4-one

To a cooled (0°C, ice/water bath) 2 N aqueous NaOH (750 mL) was added crude 3-chloro-1-(2,4-dihydroxyphenyl)propan-1-one (25.3 g, 126.11 mmol) in one portion. The resultant solution was stirred at room temperature for 2 h then cooled down to 5°C. The solution was acidified with 6M H₂SO₄ to pH = 2. The mixture was extracted with ethyl acetate. The combined organics were washed with brine and dried over Na₂SO₄ and filtered. The solution was concentrated in a vacuum, the solid was washed with 300 mL hexane, dried in a vacuum, and yielded brown solid (12 g, 57.96%).

Step 3

Sappanone A (SA)

7-hydroxychroman-4-one (7.38 g, 45 mmol) and 3,4-dihydroxybenzaldehyde (8.97 g, 65 mmol) were dissolved in 100 mL methanol. To this solution, 3.5 mL of concentrated HCl (37%) was added. The solution was refluxed for 21 h and cooled to room temperature then concentrated to dryness in vacuum. The residue was dissolved in 1.2 L ethyl acetate and filtered with a silica gel
pad. The filtrate was concentrated under vacuum to yield a yellow solid, which was re-crystallized from ethanol to give Sappanone A (6.3 g, 50.95% three steps). $^1$H NMR (DMSO-$d_6$, 500 MHz) $\delta_{H}$ 10.58 (brs, 1H), 9.57 (brs, 1H), 9.21 (brs, 1H), 8.24 (s, 1H), 7.72 (d, $J = 8.7$ Hz, 1H), 7.52 (s, 1H), 6.83 (m, 2H), 6.76 (dd, $J = 8.3$, 1.7 Hz, 1H), 6.54 (dd, $J = 8.7$, 2.2 Hz, 1H), 6.31 (d, $J = 2.2$ Hz, 1H), 5.35 (d, $J = 1.6$ Hz, 2H); and (−)-ESIMS $m/z$ 283.4 [M – H]$^–$.

1.2. Synthesis of alkynyl-sappanone A (Alk-SA)

A stirred suspension of 7-hydroxychroman-4-one (600 mg, 3.66 mmol), 3-bromoprop-1-yn (1.32 mL, 15.31 mmol), $K_2$CO$_3$ (3000 mg, 21.74 mmol) in dry acetone was refluxed for 3 h. The reaction was monitored by TLC. The hot reaction mixture was filtered and washed with acetone. The filtrate was concentrated in a vacuum to give 7-(prop-2-yn-1-yloxy) chroman-4-one.

7-(prop-2-yn-1-yloxy) chroman-4-one (450 mg, 2.23 mmol) and 3,4-dihydroxybenzaldehyde (340 mg, 2.46 mmol) were dissolved in 100 mL methanol. To this solution, 1 mL of concentrated HCl (37%) was added. The solution was refluxed for 20 h and cooled to room temperature then concentrated to dryness under vacuum. The residue was re-crystallized from methanol to yield a yellow-green solid (550 mg, 76.5% two steps). $^1$H NMR (DMSO-$d_6$, 500 MHz) $\delta_{H}$ 9.63 (brs, 1H), 9.25 (brs, 1H), 7.81 (d, $J = 8.8$ Hz, 1H), 7.55 (s, 1H), 6.85 (d, $J = 1.3$ Hz, 1H), 6.84 (d, $J = 8.2$ Hz, 1H), 6.78 (dd, $J = 8.2$, 1.3 Hz, 1H), 6.73 (dd, $J = 8.8$, 2.3 Hz, 1H), 6.62 (d, $J = 2.3$ Hz, 1H), 5.41 (s, 2H), 4.90 (d, $J = 2.1$ Hz, 2H), 3.65 (t, $J = 2.1$ Hz, 1H); (−)-ESIMS $m/z$ 321.2 [M – H]$^–$; and (+)-ESIMS $m/z$ 323.2 [M + H]$^+$.

1.3. Synthesis of Cy3-sappanone A (Cy3-SA)

Cy3-azide and Alk-SA (1:1 molar) were dissolved with Click buffer (TBTA, 160 μM; ascorbic acid, 2.5 mM; TCEP, 2 mM; t-BuOH, 10%) in a round-bottomed flask. To this solution CuSO$_4$ solution (final concentration 2 mM) was added. The reaction was kept in room temperature for 5 h. The solution was concentrated to dryness under vacuum. The residue was re-crystallized from methanol to yield a red solid (1.25 mg, 89.2%). Purplish red, amorphous powder; $^1$H NMR (DMSO-$d_6$, 500 MHz) $\delta_{H}$ 8.31 (m, 1H), 7.79 (m, 2H), 7.68 (m, 2H), 7.54 (d, $J = 5.4$ Hz, 1H), 6.85 (m, 2H), 6.75 (m, 3H), 6.65 (m, 1H), 5.40 (d, $J = 4.0$ Hz, 2H), 5.23 (m, 2H), 4.10-4.48 (m, 5H), 2.09 (m, 3H), 1.89 (m, 1H), 1.68-1.71 (m, 10H); and (+)-ESIMS $m/z$ 1129.8 [M + H]$^+$.

1.4. Synthesis of biotin-sappanone A (bio-SA)

Biotin-PEG3-SA and Alk-SA (1:1 molar) were dissolved with Click buffer (TBTA, 160 μM;
ascorbic acid, 2.5 mM; TCEP, 2 mM; t-BuOH, 10%) in a round-bottomed flask. To this solution, a
CuSO₄ solution (final concentration 2 mM) was added. The reaction was kept in room
temperature for 5 h. The solution was concentrated to dryness in a vacuum. The residue was
re-crystallized from methanol to yield a red solid (16.5 mg, 95.7%). ¹H NMR (DMSO-d₆, 500
MHz) δH 9.64 (brs, 1H), 9.26 (brs, 1H), 8.24 (s, 1H), 7.80 (s, 1H), 7.79 (m, 1H), 7.55 (s, 1H),
6.84-8.73 (m, 5H), 6.41 (brs, 1H), 6.35 (brs, 1H), 5.40 (brs, 2H), 5.23 (brs, 2H), 4.54 (brs, 2H),
4.29 (brs, 1H), 4.10 (brs, 6H), 3.82 (brs, 2H), 3.51 (brs, 2H), 3.45 (brs, 7H), 3.17 (brs, 16H), 3.07
(brs, 1H), 2.79 (m, 1H), 2.56 (d, J = 12.3, 1H), 2.06 (m, 2H), 1.59-1.28 (m, 6H);
(−)-ESIMS m/z 765.2 [M − H]−; and (+)-ESIMS m/z 789.2 [M + Na]+.

1.5. Compound 1

Compound 1 was purchased from WuXi AppTec (Shanghai, China). ¹H NMR (DMSO-d₆, 500
MHz) δH 7.93 (d, J = 8.5 Hz, 1H), 7.74 (s, 1H), 7.74 (m, 3H), 6.92 (m, 2H), 5.45 (s, 2H), 2.31 (s,
6H), 2.29 (s, 3H); (+)-ESIMS m/z 411.2 [M + H]+, 433.0 [M + Na]+.

1.6 Compounds 2 and 3

Compound 2 was purchased from Topscience Co., Ltd (Shanghai, China). ¹H NMR (DMSO-d₆,
500 MHz) δH 10.54 (s, 1H), 8.78 (s, 1H), 8.70 (s, 1H), 7.64 (d, J = 8.7 Hz, 1H), 6.64 (d, J = 7.9 Hz,
1H), 6.59 (d, J = 1.7 Hz, 1H), 6.50 (dd, J = 8.7, 2.1 Hz, 1H), 6.46 (dd, J = 7.9, 1.7 Hz), 6.30 (d, J =
2.1 Hz, 1H), 4.27 (dd, J = 11.3, 4.5 Hz, 1H), 4.08 (dd, J = 11.3, 9.0, 1H), 2.91 (dd, J = 13.8, 4.6
Hz, 1H), 2.78 (m, 1H), 2.46 (dd, J = 13.8, 10.0 Hz, 1H); (−)-ESIMS m/z 285.2 [M − H]−; and
(+)-ESIMS m/z 287.1 [M + H]+.

Compound 3 was isolated from the plant Caesalpinia sappan L. as previously described by our
group (Michio et al., 1987). Yellow, amorphous powder; ¹H NMR (DMSO-d₆, 500 MHz) δH 7.08
(d, J = 8.4 Hz, 1H), 6.81 (d, J = 1.8 Hz, 1H), 6.71 (d, J = 8.0 Hz, 1H), 6.65 (dd, J = 8.9, 1.8 Hz,
1H), 6.37 (dd, J = 8.4, 2.4 Hz, 1H), 6.24 (d, J = 2.4 Hz, 1H), 4.20 (s, 1H), 4.01 (d, J = 11.3 Hz,
1H), 3.75 (dd, J = 11.3, 1.2 Hz, 1H), 2.84 (d, J = 13.9 Hz, 1H), 2.67 (d, J = 13.9 Hz, 1H); and
(−)-ESIMS m/z 303.2 [M − H]−.

2. Primary microglia: Primary microglia were isolated from the cortices of 1 to 3 day-old ICR
mice pups. Briefly, the brain was removed and cut into 1 mm³ fragments. After incubation in 0.2%
trypsin for 20 min in 37°C, the tissues were dissociated by mild mechanical trituration. The cell
suspension (in DMEM with 10% FBS) was seeded into 175 cm² culture flasks at a density of 1 ×
10^7 cells and grown in a humidified incubator with 5% CO_2 at 37°C for two weeks. Then, the culture flask was gently shaken to collect microglia. The prepared microglia were more than 95% pure as determined with the microglia-specific marker, Iba-1.

3. Construction of plasmids and Cys mutant: Human IMPDH2 was cloned into a pcDNA3.1 vector containing a His tag sequence at the N-terminal region. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene) using His tag-IMPDH2 as a template. These proteins were expressed in HEK293 T cells and subsequently purified.

4. IMPDH2 protein expression and purification: HEK293 T cells (in a 100 mm culture dish) were transfected with IMPDH2 or IMPDH2 Cys mutation plasmids using Lipofectamine® 2000 Transfection Reagent (Invitrogen, CA, USA) in Opti-MEM I Reduced Serum Medium (Invitrogen) for 72 h. Then, the transfected cells were collected and the total lysates were prepared.

5. Cell survival assay: The cell survival assay was performed using the MTT method. Briefly, culture supernatants were removed and exchanged with medium containing 0.5 mg/mL MTT. Then, the cells were incubated for 4 h at 37°C in darkness, followed by the removal of the medium and adding 100 μL dimethyl sulfoxide. The absorbance at 570 nm was detected and the data were expressed as the mean percentage of absorbance in treated vs. control cells. The value of the control was set at 100%.

6. NF-κB activity assay

6.1. NF-κB reporter gene activation assay: Cells were transiently co-transfected with NF-κB reporter plasmids (12 μg), renilla plasmids (12 μg) and Lipofectamine® 2000 Transfection Reagent (60 μL) for 48 h and then treated with LPS (1 μg/mL) in the absence or the presence of SA (5, 10, and 20 μM). After an additional 12 h incubation, cells were lysed and analyzed for luciferase activity using the Dual Luciferase reporter gene Assay System (Bioassays, CA, USA) on a fluorescence spectrophotometer (PerkinElmer, MA, USA).

6.2. NF-κB/p65 nuclear translocation immunofluorescence: The cell-seeded glass cover slips were fixed with cold 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 30 min. Then, the slips were blocked with 5% BSA for 1 h and incubated with a primary antibody specific to the NF-κB p65 subunit for overnight at 4°C, followed by a secondary antibody labeled with Alexa Fluor 594 (1:500) for 1 h at room temperature. After being stained with DAPI (5 μg/mL in PBS) for 30 min at 37°C, the coverslips were washed and sealed. Images
were obtained by OLYMPUS IX73 fluorescence microscope (Olympus, Tokyo, Japan) with excitation/emission wavelengths of 590 nm/617 nm for Alexa Fluor-594 and 360 nm/450 nm for DAPI.

7. RNA extraction and real-time PCR analysis: Quantitative real-time PCR was performed using Agilent Technologies Stratagene Mx3005P (USA). The total RNA was isolated using an RNA Purification Kit (TianGen, Beijing, China). The total RNA was reverse transcribed at 42°C for 15 min using Fast Quant RT Super Mix (TianGen, Beijing, China) to obtain the cDNA. The cDNA was then diluted 40 times and amplified using Trans Start® Green qPCR Super Mix (Transgen, Beijing, China). GAPDH was used as the internal control. The program for the PCR reactions was: 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s. The primers for real-time PCR are presented in Table 1. At the end of the real-time PCR, the CT value of each reaction was provided and the changes in the transcriptional level of the target genes normalized to GAPDH were calculated by the following formula:

Relative mRNA level of target gene (folds of control) = $2^{-\Delta\Delta CT}$.

8. Preparation of SA-tagged beads (SA beads): Bio-SA or 0.2% DMSO as a control was incubated with Avidin-agarose (Pierce, Rockford, IL, USA) for 2 h at 4°C. Then, immobilized beads were washed 10 times with washing buffer (50 mM HEPES, 30 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween20, pH7.5) and kept at 4°C before use.

9. Proteomics analysis of SA target proteins. (a) Analysis of SA target proteins based SILAC proteomics (Sharma et al., 2009): BV-2 cells were grown in medium containing either arginine and lysine (Light) or heavier isotopic variants of these amino acids (Heavy). Proteins captured from the cell lysates (Heavy-labeled) by SA beads were eluted and subjected to tryptic digestion. Meanwhile, an excess amount of SA for competitive binding was added into Light-labeled cell lysates and subjected to tryptic digestion. After that, the proteins from both treatments were combined and analysed by quantitative MS. (b) Analysis of SA target proteins based shotgun proteomics: The samples were separated by SDS-PAGE followed by silver staining. The bands were isolated, trypsin-digested and identified by LC/MS/MS analysis. The trypsin-digested samples were first filtered through a 0.22 µm micro-pore membrane and then subjected to liquid chromatography coupled with a LTQ Velos pro mass spectrometer (Thermo Scientific, USA). The
Captrap Peptide column (20 μL/min) was used to load the Peptide solution (10 μL), and separation of the analytes were achieved on a RP-C18AQ column (100 μm id × 15 cm, Michrom Bioresources, USA), with a column oven temperature of 35°C. The electro spray voltage was operated at 1.8 kV.

10. **Fluorescence labeling of IMPDH2 using Cy3-SA:** Recombinant IMPDH2 was incubated with Cy3-SA (5-20 μM) overnight at 4°C, and the reactions were terminated by adding the SDS-loading buffer followed by boiling for 5 min. Then, the samples were resolved by SDS-PAGE and the gels were scanned using Tanon-5200Multi Gel Imaging System (Tanon Science & Technology, Shanghai, China).

11. **Determination of GSH-SA complex by LC-MS:** Glutathione (GSH, 500 μM) was incubated with SA (500 μM) for 2 h at 37°C. Then, the GSH-SA complex was identified by LC-pMRM-MS on a hyphenated platform consisting of a Shimadzu LC-20A UHPLC system (Kyoto, Japan) and a Sciex Qtrap 4500 mass spectrometer (Foster City, CA, USA). The chromatographic separation were conducted on an Acquity UPLC BEH® C18 column (2.1 × 50 mm, 1.7 μm, Waters, Ltd., USA) that was protected by a van guard™ BEH® C18 (5 mm × 2.1 mm i.d., 1.7 μm, Waters, Ltd., USA). The mobile phase consisted of ACN (A) and 0.1% aqueous formic acid (B), and was delivered at 1.0 mL·min⁻¹ with a gradient program: 0-3 min, 5%-25%A; 3-7 min, 25%-80%A; 7-9 min, 80%-100%A; 9-10 min, 100-100%. The column temperature was maintained at 50°C. An aliquot of 1 μL supernatant was subjected for LC-MS analysis. The source temperature was set at 450°C. The sprayer voltages were fixed at 5500 V and -4500 V for positive and negative polarities, respectively. In order to guarantee adequate dwell time (4 ms) for each ion pair in a total acquisition time (also known as cycle time) of less than 1.5 s, two product ion (EPI) scans were triggered by employing MRM as survey experiments to generate complementary MS² spectra for structure characterization. Criteria for the information-dependent acquisition (IDA) of EPI was set for the two most intense ions in each dynamic background subtracted survey scan spectrum with an intensity threshold of 500 counts per second (cps).

12. **Determination of SA-binding site on IMPDH2**

12.1. **Sample preparation:** Recombinant IMPDH2 was incubated with SA for overnight at 4°C, and the reactions were resolved by SDS-PAGE. Bands corresponding to IMPDH2 were excised, and digested in gel with trypsin.
12.2. LC-MS/MS analysis on LTQ-Orbitrap: The nano-LC-MS/MS experiments were performed using a LTQ-Orbitrap velos pro mass spectrometer (Thermo Fisher Scientific, U.S.A.). Extracted peptides were separated using an EASY-nLC II system. The samples were autosampled directly and bound onto a trapping column packed with 5 μm C18 reversed-phase material. The peptide mixtures were separated on an analytical column (75 μm, 10 cm) packed with 3 μm C18 reversed-phase material and eluted with the following gradient: 2% - 40% B for 70 min; 40% - 95% B for 5 min; 95% B for 20 min (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in ACN). The eluent was introduced to the mass spectrometer at a flow rate of 300 nL/min. The LTQ-Orbitrap mass spectrometer equipped with a nano-electrospray ion source was operated in a data dependent mode. Full scan MS spectra (from m/z 350−2000) were acquired in the Orbitrap analyzer with a resolution of 60,000 (FWHM). The top 15 most abundant precursor ions from each MS scan with charge states ≥ 2 were selected for MS/MS scans in the linear ion trap analyzer with a CID of 35% collision energy.

12.3. Database search: Mass spectrometric data were analyzed with Proteome Discoverer (1.4) software with the SEQUEST search engine (Thermo Scientific Scientific) using the following criteria: taxonomy, human; enzyme, trypsin; missed cleavage sites, 2; variable modifications, methionine oxidation (+15.995 Da), cysteine carbamidomethylation (+57.021 Da), cysteine binding with IMPDH2 (+284.069 Da); precursor mass tolerance as 10 ppm, fragment mass tolerance as 0.6 Da; and the false discovery rate (FDR) at 0.01.

13. Determination of Peptide \(^{140}\text{SA}\) complex: Sample preparation, LC-MS/MS analysis and database search was the same as described in 15.

14. Determination of IMPDH2 clustering: To evaluate the effect of SA on IMPDH2 clustering, dual-label fluorescence analysis was employed by confocal microscopy. HEK293T cells were transiently co-transfected GFP- and mCherry-tagged IMPDH2 plasmids using lipofectamine 2000 transfection reagent according to the manufacturer’s instructions, and then, cells were treated with or without SA (20 µM) for 4 h. For examination of IMPDH2 clustering, cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 30 min and stained in DAPI for 20 min at 37 °C. The images were captured with confocal microscopy (Leica TCS SP8 X, Leica Microsystems, Germany). The colocalization ratio of IMPDH2 clusters was calculated using Leica Application Suite X software.
15. **Transient transfection with IMPDH2 siRNA:** Cells were transfected with IMPDH2 or control siRNA (Suzhou GenePharma, Jiangsu, China) using Lipofectamine® 2000 Transfection Reagent (Invitrogen, CA, USA) in Opti-MEM I Reduced Serum Medium (Invitrogen) for 72 h. Then, the transfected cells were used for further research.

16. **Molecular dynamics (MD) simulation** (Capoferri et al., 2015):

16.1. Preparation of Cys140-Ligand covalent bond parameters: The force field parameters for inhibitor covalently bonded to Cys140 residue of IMPDH2 protein were generated by the General AMBER Force Field (GAFF) and Restrained Electrostatic Potential (RESP). Geometry optimization and the electrostatic potential calculations were carried out at the HF/6-31G⁄ level of Gaussian09 suite.

16.2. Model building and molecular dynamics (MD) simulation: X-ray structures of human IMPDH2 (PDB ID:1B3O, 1NFB, 1NF7) were downloaded from the Protein Data Bank, but the detail structural information of these structures are still unpublished. Next, we searched all the sequences in the Protein Data Bank and compared them with the sequence of human IMPDH2. Then, the IMPDH2 structure (chain A) of Bacillus anthracis (PDB ID:3TSB) possessing full structure and 43.5% sequence identity with human IMPDH2 was found by Swiss-Model web server. Therefore, chain A of 3TSB was selected as the template protein from the PDB database to generate three dimensional models of the human IMPDH2 protein using Swiss-Model. MD simulation (100 ns) were carried out using Amber14 package with the AMBER14SB force field and the explicit TIP3P water model for the apo protein system and the Cys140-Ligand covalent bond system. The initial structures were solvated in a TIP3 water box with a distance of 10 Å between protein surface and the solvation boundary. During the simulations, the periodic boundary conditions were employed and interactions between atoms were truncated with a cutoff radius of 1.0 nm for van der Waals and Coulomb using Particle-mesh Ewald (PME4) for the long-term electrostatic treatment. The net charge of the systems was neutralized with the addition of 1 Na⁺ counter ions. All the MD simulations were performed by 2 fs time step, and all the covalent bonds involving hydrogen atoms were constrained with the SHAKE algorithm.

First, each system was energy minimized for 5000 steps with constraints on protein using steepest descent method, followed by conjugated gradient method for 5000 steps full minimization without any constraints. The systems were sequentially heated up from 0 to 300.0 K over 100 ps.
restrained MD simulation in the NVT ensemble with protein atoms constrained at a force constant of 5 kcal/mol; so that the solvent and ions can be properly positioned around the protein. Temperature was regulated using Langevin dynamics with the collision frequency setting to 2 ps⁻¹. Another 50 ps NPT simulation was run for adjusting the density of the system under 1 atm pressure. At last, MD simulations production continued 100 ns without any constraints in the NPT ensemble at a temperature of 300.0 K and a pressure of 1 atm. Snapshots were conserved once per every 5,000 steps.

17. Analysis of inflammatory mediators

17.1. Nitric oxide (NO) assay: The production of NO were determined by Griess reagent. Cell culture supernatants (100 μL) were mixed with 100 μL Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in a 96-well plate for 10 min at room temperature. Then, the optical density was measured at 540 nm using a microplate reader. Sodium nitrite was used as a standard curve in the assay.

17.2. ELISA assay for TNF-α, IL-6, and PGE₂: Culture medium were collected and centrifuged at 16000 rpm for 20 min. Then, 50 μL of the supernatants were used for detecting TNF-α, IL-6, and PGE₂ levels by ELISA from R&D systems (MI, USA).

18. Cellular thermal shift assay (CETSA) (Martinez et al., 2013): For the cell lysate CETSA experiments, the cells were collected and freeze-thawed three times using liquid nitrogen. The lysates were diluted with PBS and divided into two aliquots, with one aliquot being treated with SA (20 μM) and the other aliquot as control (DMSO). After 10 min at room temperature, the lysates were heated individually at different temperatures (42 to 64°C) for 3 min followed by cooling for 3 min at room temperature. The lysates were then analyzed by SDS-PAGE followed by western blot. For the intact cell experiments, the treated cells were heated as above described followed by lysis using 2 cycles of freeze-thawing with liquid nitrogen. The soluble fractions were isolated and analyzed by western blot.

19. Drug affinity responsive target stability (DARTS) assay (Pai et al., 2015): The cells were collected and total protein was isolated using lysis buffer (0.4% Triton X-100, 400 mM NaCl, 100 mM Tris·Cl, pH 7.5, 20% glycerol). The lysates were 1:10 diluted with TNC buffer (50 mM Tris·Cl, pH 8.0, 50 mM NaCl, 10 mM CaCl₂) and treated with different concentrations of SA or DMSO as control. After incubation for 1 h at room temperature, pronase (25 μg/mL) was
added into the lysates for a further 30 min at 37°C. Reactions were ceased by adding the SDS-PAGE loading buffer and analysed via western blot with a specific anti-IMPDH2 antibody.

20. GTP analysis: BV-2 cells were treated with 20 μM SA for 18 h and then lysed in cold water using a Ultrasonic Disruptor (Ubrina Cell, SONICS, USA). The extract was centrifuged and further purified by HPLC-grade methanol in order to remove insoluble material. The resulting supernatant was concentrated before use. Chromatographic analyses were conducted on a Shimadzu HPLC-DAD system. Sample separation was performed on an Agilent Zorbax Extend-C18 column (4.6 mm × 250 mm, 5 μm) with a constant flow rate of 1 mL/min. Mobile phase consisted of water (A) and acetonitrile (B) using a gradient elution of 2-3% B at 0-8 min, 3-100% B at 8-15 min. The detection wavelength was set at 254 nm. Shimadzu Labsolutions software was used for the chromatographic analysis. GTP peak was identified by comparison with the retention time of reference GTP (Amersco, OH, USA).

21. Chemical cross-linking of IMPDH2 proteins: Cell lysates were cross-linked in 1 mM disuccinimidyl suberate in buffer (20 mM HEPES, 100 mM KCl, 1 mM dithiothreitol, pH 8.0) at room temperature for 30 min. The reaction was quenched by adding 50 mM Tris-HCl for 15 min. Then, the samples were detected using western blot. IMPDH2 dimer band is around 112 kDa, and tetramer band is around 224 kDa.

22. Survival curve analysis: BALB/c mice were cared and administrated as described in above mentioned neuroinflammation models. For the survival curve analysis, the animals were not anesthetized and sacrificed following the LPS injection. Animals were continuously observed and the number of surviving mice was documented daily for 10 days. Then, the survival curve was drawn with software GraphPad Prism 5.
II. Supplementary tables for methods

Supplementary Table 1. Primer pairs for real-time PCR

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<th>Gene</th>
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<td>Tnf-α</td>
<td>F: 5'-AAGCAAGCAGCCAACCAG-3’</td>
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<td></td>
<td>R: 5’-CCACAAGCAGGAATGAGAAGA-3’</td>
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<tr>
<td>Il-1β</td>
<td>F: 5’-TGGAGAAGCTGTGCCAGCTACCT-3’</td>
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<td>R: 5’-GAACGTACACACCAGCAGGT-3’</td>
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<td>Gapdh</td>
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**Supplementary Table 2. siRNA sequences for transfection**

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<th>5’-3’ (antisense)</th>
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<td><strong>siRNA</strong></td>
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<td><strong>siRNA</strong></td>
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</table>
III. Supplementary Results

Supplementary Fig. 1. Inhibition of SA on microglial activation. (a) Inhibition of SA on inflammatory mediator production in BV-2 cells. BV-2 cells were treated with LPS in the absence or presence of SA for 4 h (TNF-α), 8 h (IL-6), and 24 h (NO and PGE₂). (b) Inhibition of SA on inflammatory mediator gene expression in BV-2 cells. BV-2 cells were treated with LPS (1 μg/mL) in the absence or presence of SA (5, 10, and 20 μM) for 6 h. Then, the cells were collected and a real-time PCR assay was performed for various inflammatory gene expression. The values represent the means ± S.D. of triplicates in an independent experiment. # # P < 0.01 compared with control group; * P < 0.05, ** P < 0.01 compared with the LPS group.
Supplementary Fig. 2. Inhibition of SA on the inflammatory mediator release in primary cultured microglia. Primary cultured microglia were treated with LPS (1 μg/mL) in the absence or presence of SA (5, 10, and 20 μM) for 4 h (TNF-α), 8 h (IL-6), and 24 h (NO). Various inflammatory mediators were detected. The values represent the means ± S.D. of triplicates in an independent experiment. ## P < 0.01 compared with the control group; ** P < 0.01 compared with the LPS group.

Supplementary Fig. 3. Anti-inflammation activity assay of Cy3-SA and Biotin-SA. BV-2 cells were treated with LPS (1 μg/mL) in the absence or presence of Cy3-SA or Biotin-SA (5, 10, and 20 μM) for 24 h. The production of NO was detected. ### P < 0.01 compared with the control group; ** P < 0.01 compared with the LPS group.
Supplementary Fig. 4. Identification of IMPDH2 by LC-MS. Top: list of identified specific peptide sequences for IMPDH2 protein; Bottom: the proteins for SA-binding.

Supplementary Fig. 5. Identification of IMPDH2 as SA target protein in primary cultured microglial cells. The primary cultured microglial lysates were incubated with SA beads or control beads at 4°C for 12 h. After washing, the proteins bound to the beads were resolved by SDS-PAGE, followed by a western blot with a specific IMPDH2 antibody.
Supplementary Fig. 6. Identification of IMPDH2 as an SA target protein in Balb/c mouse brain extracts. The brain tissue lysates of Balb/c mice (treated with or without 2 mg/kg of LPS for 24 h) were incubated with SA beads or control beads at 4°C for 12 h. After washing, the proteins bound to the beads were resolved by SDS-PAGE, followed by a western blot with a specific IMPDH2 antibody.

Supplementary Fig. 7. SA-GSH complex analysis on the Qtrap-MS. (a) The extracted ion current chromatogram (EIC) by the predicted MRM analysis on the Qtrap-MS. A: positive ion mode, B: negative ion mode; one reaction solution at 2 h and two blank reaction solutions.
(b) The mass profiles obtained by the predicted MRM analysis on the Qtrap-MS (A) and proposed fragmentation pathways (B) in the positive ion mode.
(c) The mass profiles obtained by the predicted MRM analysis on the Qtrap-MS (A) and proposed fragmentation pathways (B) in the negative ion mode.

Supplementary Fig. 8. Conserved cysteine residues in IMPDH2.
Supplementary Fig. 9. Identification of Peptide\textsuperscript{C140}-SA complex by LC-MS. MS/MS analysis of Peptide\textsuperscript{C140} incubated with (bottom) or without (top) SA for 6 h. C* represents the Cys bound by SA.

Supplementary Fig. 10. The variations of hydrogen bond length. PRO (green) indicates IMPDH2 protein alone, PRO-LIG (yellow) indicates SA-IMPDH2 protein complex.

Supplementary Fig. 11. Hydrophobic interaction between catalytic pocket and Loop region. The interaction of Loop region with several hydrophobic residues in IMP-catalytic pocket including Ile304, Ile306, Val310, Leu311 and Ala312. 1 represents the hydrophobic interface diagram (red) of catalytic pocket; 2 represents the hydrophobic interface diagram (red) of Loop region; 1+2 represents the interacting hydrophobic interface diagram of catalytic pocket and Loop region (red in circle).
Supplementary Fig. 12. NF-κB inflammation signal was inhibited by SA. (a) NF-κB activation
detection by nuclear translocation. BV-2 cells were treated with LPS (1 μg/mL) in the absence or presence of SA (5, 10, and 20 μM) for 1 h. NF-κB p65 nuclear translocation was investigated by staining with an anti-p65 subunit antibody (red) and DAPI (blue). Scale bars in the panel indicate 25 μm. (b) SA inhibited IKKβ-NF-κB pathway activation. BV-2 cells were treated with LPS (1 μg/mL) in the absence or presence of SA (5, 10, and 20 μM) for 1 h.

Supplementary Fig. 13. Histopathologic test for the brains of Balb/c mice. Balb/c mice were orally treated with 0.5% sodium carboxyl methyl cellulose (vehicle) and SA (50 or 100 mg/kg body weight). One hour later, microglia were activated by one intraperitoneal injection of LPS (2 mg/kg). Representative sections prepared from the brain tissues collected at 3 h post-LPS challenge and stained with hematoxylin-eosin (HE) staining. Arrows indicate the macrophage infiltration.
Supplementary Fig. 14. Serpentine flow analysis between amino acids. The serpentine flow which SA (LIG) communicates with catalytic domain via Arg224, Asp226 and Cys140. Light
green indicates Batman domain, green indicates IMPDH domain, red indicates catalytic domain, rose indicates Loop region (322-342) which IMP-binding site Cys331 locates.

Supplementary Fig. 15. Synthetic schemes for sappanone A (SA).

Supplementary Fig. 16. $^1$H NMR spectra for SA.
Supplementary Fig. 17. MS spectra for SA.

Supplementary Fig. 18. Synthetic schemes for alkynyl-sappanone A (Alk-SA).
Supplementary Fig. 19. $^1$H NMR spectra for Alk-SA.
Supplementary Fig. 20. MS spectra for Alk-SA.

Supplementary Fig. 21. Synthetic schemes for Cy3-sappanone A (Cy3-SA).
Supplementary Fig. 22. $^1$H NMR spectra for Cy3-SA.

Supplementary Fig. 23. MS spectra for Cy3-SA.
Supplementary Fig. 24. Synthetic schemes for biotin-sappanone A (bio-SA).

Supplementary Fig. 25. $^1$H NMR spectra for bio-SA.
Supplementary Fig. 26. MS spectra for bio-SA.

Supplementary Fig. 27. $^1$H NMR spectra for Compound 1.
Supplementary Fig. 28. MS spectra for Compound 1.

Supplementary Fig. 29. $^1$H NMR spectra for Compound 2.
Supplementary Fig. 30. MS spectra for Compound 2.
Supplementary Fig. 31. $^1$H NMR spectra for Compound 3.

Supplementary Fig. 32. MS spectra for Compound 3.

IV. Supplementary references


